MOLECULAR ASPECTS OF DIFFERENTIATION IN THE HL-60 HUMAN PROMYELOCYTIC LEUKEMIA CELL LINE:

SIGNAL TRANSDUCTION, GENE REGULATION AND CLONING OF THE MAJOR PROTEIN KINASE C SUBSTRATE OF PLATELETS (P47)

By

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ABSTRACT

Biologically active phorbol esters cause HL-60 promyelocytic leukemia cells to differentiate into cells resembling normal macrophages. Elevation of intracellular Ca^{2+} by ionophores and activation of protein kinase C (PKC) by phorbol esters often cooperate in a synergistic manner to elicit a full biological response; hence, a similar effect was sought in HL-60 cell differentiation. Assessment by a number of criteria conclusively demonstrated that treatment of HL-60 cells with subthreshold concentrations of phorbol esters in the presence Ca^{2+} ionophores caused macrophage-like differentiation. Synergistic HL-60 cell differentiation was phenotypicaly similar to the TPA-induced response yet was associated with a distinct pattern of gene regulation. In particular, induction of the proto-oncogene c-fos did not correlate with synergistic differentiation. This precluded a number of possible mechanisms which might underlie the observed cooperativity and suggested that Ca^{2+} may independently trigger essential components of the HL-60 differentiation program.

In an immunological survey of various tissues and cell lines, the major PKC substrate of platelets (P47) was detected in HL-60 cells. P47 abundance in HL-60 cells was increased 4.0 ± 0.5 fold upon differentiation with retinoic acid (RA), apparently because of a parallel induction of P47 mRNA. RA-differentiated cells were therefore

chosen as an mRNA source from which to clone P47. Immunological screening of a lambda gt11 library yielded positive recombinants expressing an open reading frame that matched peptide sequences obtained from purified platelet P47. Expression of P47 sequences in vitro and in E. coli showed the the P47 cDNA encoded a protein of M_r 40,087. The P47 sequence was not similar to any other known sequence but did contain a putative Ca^{2+} binding EF-hand and a strong candidate region for phosphorylation by PKC. The 3.0 kb P47 mRNA had some unusual structural features and was restricted to differentiated white blood cells. Analysis of the P47 gene suggested it has been conserved through vertebrate evolution and that in humans it has a complex 5' end. A Msp I polymorphism detected in HL-60 cells by sequencing of cDNA clones was confirmed in the normal human population. Preliminary characterization of human P47 genomic clones confirmed the gene structure deduced from Southern analysis. The P47 sequence refuted two candidate functions for this protein in the platelet.

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LIST OF ABBREVIATIONS

AA	arachidonic acid
ADP	adenosine diphosphate
ATP	adenosine triphosphate
AMV	avian myeloblastosis virus
ARS	autonomously replicating sequence
ATCC	American Type Culture Collection
bp	base pair
$[Ca^{2+}]_i$	intracellular calcium concentration
cAMP	5',3'-cyclic adenosine monophosphate
CANP	Ca^{2+}-activated neutral protease (calpain)
cDNA	DNA complementary to mRNA
CGD	chronic granulomatous disease
CM	calmodulin
CNBr	cyanogen bromide
CSF-1	colony stimulating factor 1
d	day(s)
dATP	deoxyadenosine triphosphate
dNTP	deoxynucleoside triphosphate
$1,25-(OH)_2D_3$	1,25-dihydroxyvitamin D_3
DAG	1,2-sn-diacylglycerol
dbcAMP	dibutyryl cAMP
DIF	differentiation inducing factor

DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
EDTA	ethylene diamine tetraacetic acid
EGTA	ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid
FAD	flavin adenine dinucleotide
FBS	fetal bovine serum
FITC	fluoresceinisothiocyanate
fMLP	formyl-methionyl-leucyl-phenylalanine
g	unit gravitational force
G-CSF	granulocyte colony stimulating factor
G2/M	gap 2/mitosis phase of cell cycle
GM-CSF	granulocyte/macrophage colony stimulating factor
G_p	G protein coupled to PLC
GRP78	78 kDa glucose regulated protein
GTP	guanosine triphosphate
h	hour(s)
HMBA	hexamethylene bisacetamide
HPLC	high pressure liquid chromatography
IFN	interferon
IgG	immunoglobulin G
IL	interleukin
IP_3	inositol-1,4,5-trisphosphate
1,3,4-IP_3	inositol-1,3,4-trisphosphate
IP_3ase	inositol-1,4,5-trisphosphate 5-phosphomonoesterase

IP_4	inositol-1,3,4,5-tetrakisphosphate
IPTG	isopropylthiogalactoside
kb	kilobase
kbp	kilobase pair
kDa	kilodalton
LCM	lymphocyte-conditioned medium
LTB_4	leukotriene B_4
M-CSF	monocyte/macrophage colony stimulating factor
MBN	mung bean nuclease
MEL	murine erythroid leukemia
µl	microlitre
µM	micromolar
MEM	minimal essential medium
ml	millilitre
M_r	relative molecular mass
mRNA	messenger RNA
MT	metallothionein
NADPH	nicotinamide adenosine diphosphate
NBT	nitroblue tetrazolium
nM	nanomolar
OAG	1-oleoyl-2-acetylglycerol
ORF	open reading frame
PA	phosphatidic acid
2-D PAGE	two-dimensional polyacrylamide gel electrophoresis
PBS	Dulbecco's phosphate buffered saline

PC	phosphatidylcholine
PDBu	phorbol-12,13-dibutyrate
4-α-PDD	4-α-phorbol-12,13-didecanoate
PDD	4-β-phorbol-12,13-didecanoate
PDGF	platelet derived growth factor
α-PDH	pyruvate dehydrogenase α-subunit
PE	phosphatidylethanolamine
PEG	polyethylene glycol
PI	phosphoinositide
PIP_2	phosphatidylinositol-4,5-bisphosphate
PKC	protein kinase C
PKM	protein kinase M
PLA_2	phospholipase A_2
PLC	phospholipase C
PLD	phospholipase D
pM	picomolar
PNP	para-nitrophenol
RA	retinoic acid
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
s	second(s)
SDS	sodium dodecyl sulphate
SEM	standard error of the mean
SL	sphingolipid
SM	simplified medium

SRE	serum response element
SRF	serum response factor
TCA	trichloroacetic acid
TNF	tumour necrosis factor
TPA	12-O-tetradecanoylphorbol-13-acetate
TRE	TPA response element
XGAL	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
UWGCG	University of Wisconsin Genetics Computer Group

INTRODUCTION

1.1 TERMINAL DIFFERENTIATION AS A PARADIGM FOR ORGANISMIC AGING

The global perspective of this work has been the molecular basis for aging. Although the experiments described fall into a much smaller framework than this, a background on cellular and molecular aspects of aging will be presented so that the approaches taken can be placed in this context.

Most organisms which exhibit cellular specialization inevitably experience a predictable decline in tissue function that culminates in organ failure and death. This process is called aging. On a cellular level, impairment of tissue function must derive from inadequate renewal of differentiated cells from a relatively small population of undifferentiated stem cells which themselves have little or no overt phenotype (reviewed in Walton, 1982; Harley, 1988). The process of stem cell differentiation usually involves one or more divisions after which daughter cells acquire a progressively more differentiated phenotype and an increasingly restricted division potential. The eventual transition to a highly differentiated cell type incapable of further division is called terminal differentiation. Two schematic representations of this process are shown in Figure 1.1.1.

Various aging theories based on cell damage and/or accumulated

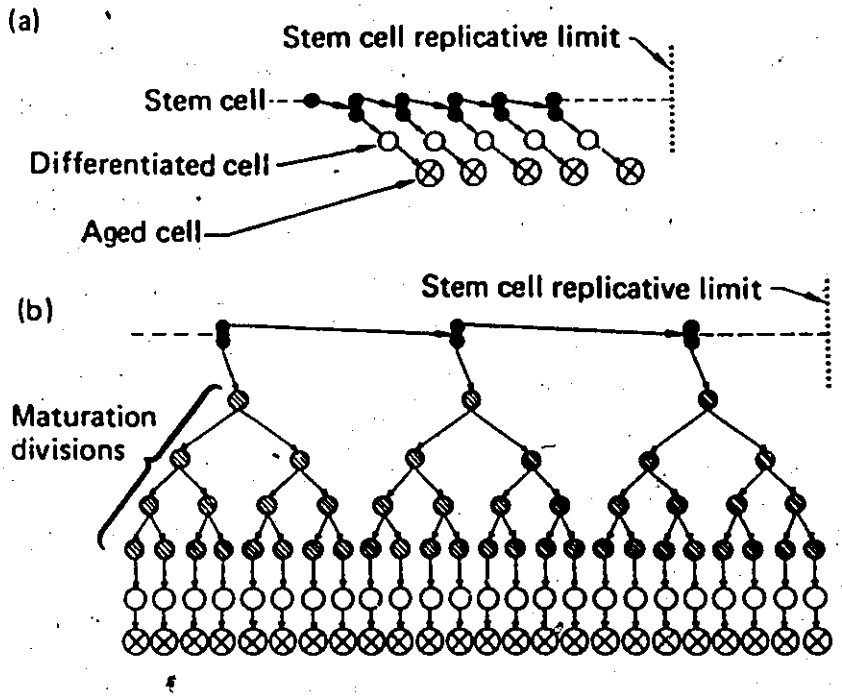


Fig. 1.1.1. Possible heirarchies culminating in terminal differentiation. b, Terminal differentiation preceded by expansion of the cell population. a, Direct descendence of a differentiated cell from a self-renewing precursor. In each case the dashed line represents commitment to a lineage of restricted division potential. Taken directly from Walton (1982).

errors seem unlikely in the absence of abnormal gene regulation and protein synthesis in senescent cells (e.g. Gupta, 1980; Rittling et al, 1986; Harley et al, 1980). The seminal observation of Hayflick and Moore on the correlation between in vitro fibroblast lifespan and species lifespan suggests there is a genetic or programmed element in aging (reviewed in Hayflick, 1984). Fibroblast senescence itself can be defined as a differentiation pathway in which distinct cellular phenotypes are associated with a loss of division potential (Bell et al, 1978; Bayreuther et al, 1988). Despite the intriguing correlations between lifespan and fibroblast proliferation (see Hayflick, 1984), it is unclear whether fibroblast senescence is a good model for organismic aging, since finite division potential in vitro may not be limiting in the organism. In a more extreme example, serial transplantation of mouse bone marrow to lethally irradiated donors can be carried out over several individual lifespans (e.g. Schofield et al, 1986). It is even less certain whether the stem cells which re-populate each tissue are themselves immortal. This question is difficult to resolve and probably depends critically on the cellular microenvironment. For example, mouse fibroblasts can be maintained indefinitely in serum-free medium but undergo a predictable crisis in the presence of serum (Loo et al, 1987). Even a reproducibly finite lifespan in vitro does not necessarily indicate that all cells in a primary culture are mortal since each cell passage results in successive dilution of the culture; this would eliminate the stem cell population once the expected frequency of stem cells fell below one per culture (for review and critique, see Harley and Goldstein 1980). In the context of aging, however, the important

point is that most somatic cells are apparently mortal and hence must be eventually replaced to maintain tissue integrity.

Naively, it is not at all clear why an organism should not be able to constantly renew damaged or aged cells and be immortal. The very existence of cancer cells which can be indefinitely maintained in a continously proliferating state belies any biochemical necessity for cell mortality. However, it is clear that stringent control mechanisms prevent unlimited cell growth. The finite cell growth potential of normal cells overides the transformed phenotype in cell fusions (reviewed in Stanbridge et al, 1982). A molecular basis for this may reside in the function of recessive oncogenes (reviewed in Harris, 1986). More to the point, fibroblast cell fusions indicate that the senescent phenotype is dominant over young cells with high proliferative potential (see Lumpkin et al (1986) for an overview).

Several reasons can be imagined why an organism should limit the division potential of most of its cells. Irreversible commitment to cell cycle exit may be a primary mechanism of reducing neoplastic potential. Thus, cancer is often thought of as blocked differentiation coupled to uncontrolled growth stimulation (reviewed in Tzen et al, 1988). A bias of stem cell populations towards differentiation would reduce the propensity towards uncontrolled self renewal. From an economic standpoint, it is wasteful to maintain cells in a state of division readiness when functionally there is no need, particularly if normal function entails severe cellular damage. The suprastructure of tissues undoubtedly also militates against random division and renewal. Organisms may maintain a negative first derivative of stem cell renewal

in order to avoid the situation of inappropriate tissue hypertrophy in mature individuals. Certainly from an evolutionary perspective, in which successful reproduction is paramount, this is sensible. Only a few species satisfy the evolutionary dictates which permit a long lifespan (Todd, 1978). These postulates still do not precisely answer why the dual requirement of stem cell maintainance and provision of differentiated cells to ensure tissue integrity and function cannot be maintained indefinitely, as they are for most of the adult life of even very long-lived species. Understanding the nature of the stem cell commitment event is necessary to resolve this question.

Nearly all aspects of biochemistry, cellular and molecular biology conceivably impinge on the regulation of stem cell commitment. Mechanisms of intercellular signalling, signal transduction and gene regulation as well as various biochemical alterations may influence this process. Emphasis of any one of these areas leads to a biased theory of aging, many of which have been described. Ultimately though, the fine line between differentiation and stem cell renewal is no longer maintained. In spite of the numerous possibilities, a sensible place to begin elucidation of this problem is the molecular nature of differentiation itself. The influence of aging on this process may then be examined.

Conceptually, the simplest notion of differentiation is a hierarchy of gene regulation in which one or a few master genes control the expression of the many genes whose products confer specialized cellular function (Blau, 1988). The terminal differentiation of myoblasts into myotubules is the most striking example of this process

yet discovered. A single gene called MyoD1 confers an entire program of muscle specific gene expression on mouse embryonic fibroblasts (Davis, R.L. et al, 1987; reviewed in Blau, 1988). Moreover, a hierarchy of gene expression exists since the product of the myd gene acts in unresponsive fibroblast sublines by inducing MyoD1 expression, presumably amongst other changes necessary to confer susceptibility to MyoD1 action (Pinney et al, 1988). Happily, the MyoD1 gene product is a nuclear phosphoprotein that bears strong similarity to the c-myc oncoprotein (Tapscott et al, 1988). This example illustrates perhaps the most fruitful approach available, that is, progressive identification of genes in a differentiation cascade and finally determination of how the master gene for a particular lineage is controlled. Thus far only the muscle cell system has yielded such gratifying results.

Although a cause and effect relationship between a master gene and a given differentiated state has been demonstrated, differentiation is not a rigid, irreversible program of gene expression. Plasticity has been demonstrated by muscle-nonmuscle cell fusion, in which the cytoplasm of muscle cells redirects gene expression in fibroblast, keratinocyte and hepatocyte nuclei (Blau et al, 1985). Other examples include conversion of clonal Eμ-myc B-cell lines to stable macrophage-like cell lines by v-raf (Klinken et al, 1988) and rapid induction of globin transcription in non-erythroid nuclei after fusion to erythroleukemia cells (Baron and Maniatis, 1986). It is possible that a hierarchy of differentiated states corresponding to tissue specificities may exist.

Given the apparent interchangeability of differentiated states, it is probable that they share common subsets of gene expression and associated regulatory mechanisms. One such fundamental unit may encode the program that commits cells to a terminal lineage. A potential candidate in this process has been identified as an mRNA component of senescent fibroblasts which causes cell cycle exit upon microinjection into young fibroblasts (Lumpkin et al, 1986). A similar inhibitory mRNA is present in quiescent T cells (Pepperkok et al, 1988). If fibroblast aging in vitro is considered as a protracted differentiation program, then this mRNA or other growth arrest specific genes (Schneider et al, 1988) may specify the part of the cascade that leads to cell cycle exit. This argues strongly for a genetic component to the process of senescence, as inferred from organism lifespan and in vitro population doubling studies. Naively, the control mechanism of senescent cell mRNA production or higher genes in this heirarchy could provide a molecular basis for commitment to terminal differentiation (reviewed in Levenson and Housman, 1981; Weir and Scott, 1986).

With the outlook that the decision between renewal and differentiation critically influences the aging process, and that common mechanisms underlie this decision in different tissues, the HL-60 cell line will be introduced as a model system for the study of terminal differentiation.

1.2 THE HL-60 CELL LINE AS A MODEL SYSTEM FOR TERMINAL DIFFERENTIATION

1.2.1 Overview of Hemopoiesis

Hemopoiesis entails the generation of all mature blood cell types from aphenotypic stem cell precursors. Much work has confirmed the basic idea that hemopoietic differentiation is accompanied by lineage restriction and a reduction in division potential (reviewed in Whetton and Dexter, 1986; Dexter and Spooncer, 1987; Clark and Kamen, 1987). The hierarchy outlined in Fig. 1.2.1 indicates the various lineages which culminate in fully differentiated blood cells and some of the factors which influence their differentiation. The pluripotent stem cell capable of regenerating all mature blood cell forms has recently been purified to homogeneity and as few as 20 of these cells can completely reconstitute lethally irradiated mice (Spangrude et al, 1988). Stem cells comprise less than 0.05% of the total bone marrow population (Spangrude et al, 1988). Remarkably, all blood cell compartments can be re-populated from only 1 or 2 retrovirally marked stem cells transplanted into lethally irradiated mice (Lemischka et al, 1986). Moreover, clonal fluctuation occurs amongst different stem cells, so that as one stem cell clone declines another rises to take its place (Lemischka et al, 1986). This is consistent with the observation of Spangrude et al (1988) that 97% of the stem cell population is in the G1/G0 phase of the cell cycle.

The hemopoietic system must respond to many varied stresses over the lifetime of the organism. Not only is rapid renewal required (10^{11}

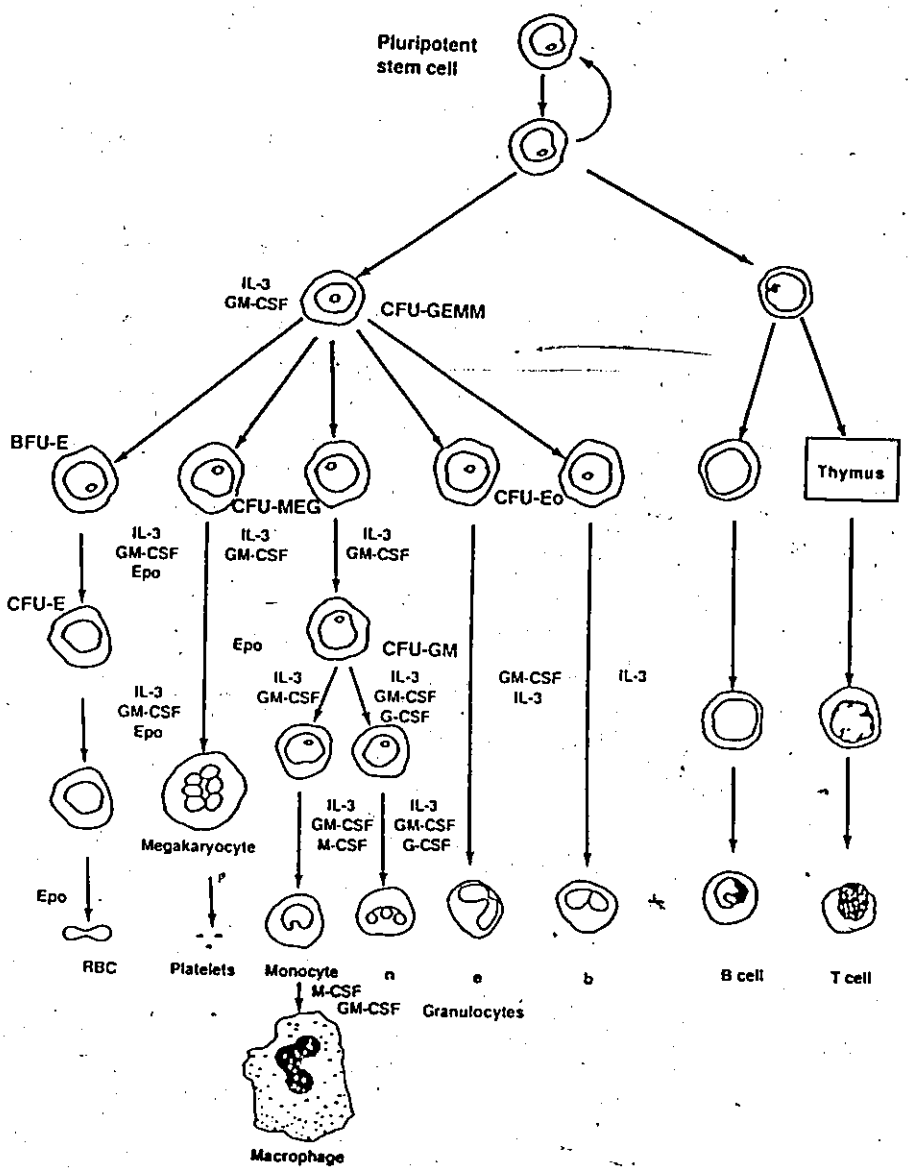


Fig. 1.2.1. Schematic overview of hemopoiesis. The cascade of differentiation in response to various cytokines is shown. Abbreviations for colony forming units (CFUs) are: GEMM, granulocyte-erythrocyte-monocyte-megakaryocyte; Meg, megakaryocyte; Eo, eosinophil; GM, granulocyte-monocyte; E, erythroid; BFU-E, erythroid burst forming unit. Abbreviations for cells are: n, neutrophil; e, eosinophil; b, basophil; m, monocyte/macrophage; E, erythrocyte; M, megakaryocyte. Various colony stimulating factors (CSFs) are described in the text except for erythropoeitin (Epo). Taken directly from Clarke and Kamen (1987).

cells/day in normal individuals (Dexter and Spooncer, 1987)) but enhanced renewal of specific compartments must be achieved in various infected states, depending on the effector cell required. Two basic parameters influence stem cell differentiation. Soluble protein factors profoundly influence blood cell growth, differentiation and viability in vitro; these have been the subject of intense molecular analysis (reviewed in Clark and Kamen, 1987). To date these include four major colony stimulating factors (G-CSF, GM-CSF, M-CSF and IL-3), six other different interleukins, the α, β and τ-interferons, tumour necrosis factor (TNF), and various differentiation-inducing factors and inhibitors. All except the latter have been molecularly cloned and thereby obtained in sufficient quantity to test in vitro and in vivo. As indicated in Fig. 1.2.1., the CSFs are major regulators of hemopoeitic differentiation. Recent evidence suggests the lineage specificity of each factor is multipotent and can ultimately influence stem cells themselves (Sonoda et al, 1988). It appears that interplay between the various factors is critical to the resultant pattern of proliferation and differentiation. Not only are various blood cells targets for these factors, but under appropriate stimulation the same cells may act as a source of cytokines. Administration of specific CSFs in vivo can selectively enhance specific blood cell populations. For instance, recombinant G-CSF induces marked neutropenia whereas GM-CSF causes not only neutrophil proliferation but also increased numbers of macrophages and other granulocytic forms (Clarke and Kamen, 1987).

A second and much less well defined influence on stem cell differentiation is the microenvironment created by bone marrow stromal

cells. This unresolved heterogeneous matrix of cells supports long term growth and survival of stem cells in vitro in the absence of added CSFs (Dexter and Spooncer, 1987). Moreover, effects of the matrix are not mediated by soluble factors released during co-culture as culture supernatants do not support growth; this implies cell-cell contact between stromal cells and hemopoietic stem cells carries growth and differentiation signals (Dexter and Spooncer, 1987).

1.2.2 The HL-60 Promyelocytic Leukemia Cell Line

This brief overview of hemopoiesis sets the stage for a discussion of the HL-60 cell line used as an in vitro model for differentiation in these studies. HL-60 cells were isolated in 1977 from a patient thought to have acute promyelocytic leukemia (Collins et al, 1977). Recently, the HL-60 bone marrow has been reclassified as FAB M2, an acute myeloblastic leukemia, rather than FAB M3, acute promyelocytic leukemia (Dalton et al, 1988). Despite a clear morphological relationship to normal myeloid precursors, the HL-60 line is multipotent in that it can be induced along granulocytic, monocytic/macrophage and eosinophilic lineages (reviewed in Abita, 1984; Harris and Ralph, 1985; Collins, 1987; Leglise et al, 1988). This apparent discrepancy is illustrated in Fig. 1.2.2 in which the morphological lineage assignment is at odds with the ability to acquire characteristics of three distinct terminal cell types. Whether described as "lineage infidelity" (Greaves et al, 1986) or "partially unblocked" differentiation (Ross et al, 1986), the HL-60 cell response is yet another indicator of the plasticity of the differentiated state.

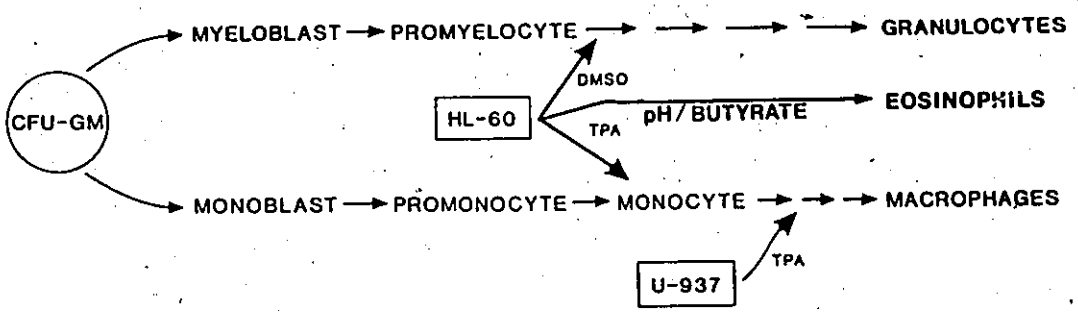


Fig. 1.2.2. HL-60 differentiation in relation to normal myelopoiesis. HL-60 cells morphologically resemble FAB M2 promyelocytes but clearly retain the ability to differentiate along 3 distinct lineages. This may be yet another example of the plasticity of the differentiated state. Modified from Mitchell et al (1985).

HL-60 cells have been used extensively as an *in vitro* model for myeloid differentiation and as a test system for chemotherapeutic agents. The cell line has also been widely used as a tissue source for the isolation and/or cloning of a variety of specific proteins and genes (see Section 1.3.2). Many oncogenes have been characterized in the HL-60 line, and their regulation during differentiation has provided insight into their possible role in transformation (see Section 1.3.1). HL-60 cells comprise a relatively homogenous population of promyelocytes that can be more or less synchronously induced to functional terminal cell types, which express intact signal transduction pathways. Thus differentiated HL-60 cells have been used to study all aspects of receptor coupled signal transduction (see section 1.5).

With regard to terminal differentiation and stem cell exhaustion, the HL-60 line is clearly only a poor substitute for stem cells themselves. Nevertheless, HL-60 is one of the few multipotent lines available (Collins, 1987). If the decision mechanism for commitment to a terminal lineage and eventual cell cycle exit is common amongst different cell types, then information gleaned from HL-60 cells should be applicable to to differentiation in general. Alternatively, the block in HL-60 differentiation may have nothing to do with the normal decision making apparatus, in which case studies on the control of stem cell renewal must await routine isolation of large populations of stem cells for experimental manipulation.

HL-60 cells grow continuously in suspension culture, typically in the presence of 10-20% fetal bovine serum. Proliferation can occur in the absence of serum supplements provided insulin and transferrin are

maintained in the culture medium (Breitman et al, 1980a). An autocrine factor secreted by HL-60 cells apparently contributes to their ability to grow in culture (Brennan et al, 1981; Perkins et al, 1984). HL-60 cells exhibit a pseudodiploid karyotype with a modal chromosome number of 46 (American Type Culture Collection Catalogue, 1988). Several specific genetic defects in the line have been identified. Foremost, is the 10-30 fold amplification of a 95 kb region which encompasses the c-myc locus (Collins and Groudine, 1982). Although present as double minute chromosomes or homogenously staining regions in later passage HL-60 cells (Dalla-Favera et al, 1982), the original amplification event appears to have proceeded via sub-microscopic 250 kb autonomously replicating units (Von Hoff et al, 1988). A second defect also associated with the original tumour is a point mutation in the N-ras locus which activates it to a dominant transforming gene (Murray et al, 1983). Yet a third defect in oncogene loci of HL-60 cells is a deletion of both alleles of the p53 gene by chromosome loss and a micro-deletion event (Wolf and Rotter, 1985). Another chromosomal abberation related to hemopoiesis has also been discovered in the HL-60 genome in that one allele of the GM-CSF gene is deleted from chromosome 5 as part of the 5q$^-$ anomaly (Huebner et al, 1985). It is unknown if this event contributed to the establishment or progression of the HL-60 neoplasm. Finally, unlike most acute promyelocytic leukemias of the M3 subtype, HL-60 cells do not show the t(15:17) translocation (Weil et al, 1988). Amplified c-myc sequences have, however, been localized to this breakpoint region in HL-60 cells (Wolman et al. 1985).

Finally, it should be noted that the phenotype of the HL-60 line

can drift considerably during prolonged culture. Changes which occur early in cell passage include a dramatic loss of granulation, ultrastructural alterations detectable by electron microscopy and a reduction in the number of spontaneously differentiated cells (Leglise et al, 1988; Parmley et al, 1987). Prolonged culture is also accompanied by chromosomal alterations, including double-minute chromosome formation of regions bearing the c-myc locus, alterations in oncogene expression, increased resistance to differentiation inducing agents and increased growth rate (Von Hoff et al, 1988; Leglise et al, 1988; Kaplinsky et al, 1988). Thus, care must be exercised in extrapolating the behavior of any given HL-60 subline to other in vitro systems, let alone normal myelopoiesis.

1.2.3 Modes of HL-60 Cell Differentiation

The list of agents which evoke HL-60 differentiation has become nearly unmanageable. Partial compilations from a recent review are shown in Table 1.2.1. Classification of HL-60 phenotypes have often been disputed in the literature; this undoubtedly reflects the abnormal context of HL-60 differentiation. All forms of HL-60 differentiation are defective in that the phenotypes achieved are at best only a subset of the characteristics embodied by normal cells (Skubitz and August, 1983; Murao et al, 1983; reviewed in Collins et al, 1987). Such effects in part form the basis for descriptions of lineage promiscuity or partial unblocking of differentiated states in leukemia cells (Greaves et al, 1986; Ross et al, 1986). In any event, this caveat must be borne in mind when attempting to identify an HL-60 cell phenotype or use

Table 1.2.1 Agents capable of inducing HL-60 differentiation

A

Type of Inducer	Concentration	Path-way	% Mature
Polar-planar compounds			
DMSO	10^{-1}, 10^{-2} M	G	90
DMF	10^{-1}, 10^{-2} M	G	90
HMBA	$2.5 \cdot 10^{-3}$ M	G	90
Other chemicals			
BA	10^{-6} M	M	90
Purine and pyrimidine analogs			
3 deazauridine	$2.5 \cdot 10^{-5}$ M	G	85
hypoxanthine	$5 \cdot 10^{-3}$ M	G	85
thymidine	10^{-3} M	G	30
neoplacin A	$3 \cdot 10^{-6}$ M	G	40
cyclopenthenyl-Cyt	10^{-7} M	G	95
5 azacytidine	$3 \cdot 10^{-6}$ M	G	30
Chemotherapeutic agents			
actinomycin D	$8 \cdot 10^{-9}$ M	G	85
methotrexate	$1.4 \cdot 10^{-8}$ M	G	35
brdUridine	$3 \cdot 10^{-5}$ M	G	45
hydroxyurea	8 μg/ml	G	15
daunomycin	0.01 μg/ml	G	35
ara-C	$3.6 \cdot 10^{-8}$ M	M	30
vincristine	$1.2 \cdot 10^{-9}$ M	–	–
mitomycin C	$6 \cdot 10^{-8}$ M	–	–
adriamycine	$7 \cdot 10^{-8}$ M	–	–
x-irradiation	100 rads	–	–
Agents that increase cAMP (dcbAMP, theophilline/PGE)	500 μM	M	90
Inhibitors of PADPR-polymerase			
3 aminobenzamide	10 μM	M	50
theophylline	$5 \cdot 10^{-3}$ M		52
phosphodiesterase inhibitor: papaverine	$113 \cdot 10^{-6}$ M	M	10
Tumor promoters			
TPA	10^{-7}, 10^{-8} M	M	90
teleocidins	$5 \cdot 10^{-8}$ M	M	
Proteolytic enzymes			
chymotrypsin, elastase	$40 \cdot 10^{-3}$ M	G	40
Antithymocyte globulin	10 μg/ml	G	35

B

Type of inducer	Concentration	Pathway	% Mature
DIF		M and G	20G, 30M
PHA-LCM	10%	M	65
G-IFN	10·3 U/ml	M	20
G-IFN + 1.25 diOHD3	10·3 U/ml + 10^{-9} M	M	60
vitD_3	10^{-9} M	M	50
3	10^{-7} M	M	30
Ara-C + 1.25 diOHD3	$4 \cdot 10^{-7} + 10^{-7}$ M	M	60
RA and metabolites	10^{-7} M	G	40
RA	$2 \cdot 10^{-7}$ M	G	50
Ara-C + RA	$4 \cdot 10^{-7} + 10^{-7}$ M	G	60
Aphidicolin + RA	$4 \cdot 10^{-7} + 2 \cdot 10^{-7}$ M	G	50
Trypsin + RA	$4 \cdot 10^{-3} + 10^{-8}$ M	G	90

Non-physiological inducers are listed in A and apparent physiological inducers are listed in B. Both parts were taken directly from Leglise et al (1988).

differentiated HL-60 cells as a model for their normal hemopoeitic counterparts. Some of the many criteria used to classify differentiated HL-60 cells are listed in Table 1.2.2. By no means exhaustive, this list is merely intended to place the assays used in this work in the context of other work on this cell line. Finally the structures of some of the compounds used in this work are shown in Fig. 1.2.3. The activity of each inducing agent will be considered according to the lineage along which it induces differentiation.

i) Granulocytic Differentiation

Many structurally unrelated agents have been identified which induce HL-60 cells along this lineage. DMSO was the first compound discovered to induce HL-60 differentiation (Collins et al, 1978) and is representative of several other planar polar solvents which cause granulocytic maturation (reviewed in Langdon and Hickman, 1987). Differentiation is accompanied by one or two cell divisions before permanent arrest in G1 occurs (Collins et al, 1978) while commitment, defined as the minimum period of exposure required to cause differentiation after withdrawal of inducer, requires at least 12 h exposure to DMSO (Tarella et al, 1982). Proliferation is not obligatory for differentiation as cells arrested in G1 by thymidine treatment for 48 h undergo rapid maturation upon exposure to DMSO (Tarella et al, 1982). Although DMSO-differentiated HL-60 cells share many features with normal granulocytes, differentiation appears defective in several regards and may thus represent incomplete maturation (Collins, 1987). Despite extensive use of these compounds as differentiation inducing agents their mode of action is unknown (see Leglise et al, 1988). One

Table 1.2.2 Various HL-60 phenotypes

Characteristic	Uninduced	Granulocyte	Monocyte	Macrophagelike	Eosinophil
Myeloperoxidase	+[3,4]	↓[22,147]	↓[22,42]	↓[61]	+[86,87]
Eosinophil peroxidase	−[87]				+[86,87]
ASD chloroacetate esterase	+[4]	+[4]	±[42,61]	↓[61]	+[87]
Nonspecific esterase	−[4,61]	−[61]	+[61]	+[61]	−[87]
Acid phosphatase	+[148]	↑[148]		↑↑[148]	+[87]
Biebrich scarlet	[96]	−[96]		±[96]	+[90]
Plastic adherence	−[61,155]	−[61,155]	−[43,155]	+[61]	
Chemotaxis	−[74,150]	+[74,150]	+[149]	−[152]	
Chemotactic receptors	+[150,151]	↑[150,151]	↑[50,149]	−[26]	
Complement receptors	+[47,74]	↑[47,74]	↑[47]	↑[47]	
F_c receptors	+[47]	↑[47]	↑[56]	↑[56,152]	
Lysozyme	+[4,61]	↑[61]		↑↑[61]	
NBT reduction	−[74]	+[74]	+[62]	−[25,86]	
Phagocytosis	−[17,47,74]	+[17,47,74]	+[47]	+[47,61]	
Microbicidal	−[21]	+[21]		+[152]	
Monocyte/macrophage surface antigens	±[42,154]	±[44,86,154]	+[42-44,154]	+[42,65]	
Granulocyte surface antigens	±[66,153]	+[65,153]	−[65]	−[65]	
Insulin receptors	+[10]	↓[10]	↑[10]		
Transferrin receptors	+[7,8]	↓[7,8]		↓[87]	

Arrows indicate a quantitative increase (↑) or decrease (↓) from uninduced HL-60 levels. Blank spaces correspond to characteristics absent from the literature. (+) indicates present, (−) indicates absent, ± indicates discrepant or equivocal literature findings.

A partial summary of cellular markers is listed. Characteristics may vary within a given lineage depending on the inducing agent used and on the HL-60 subline. Taken directly from Collins (1987).

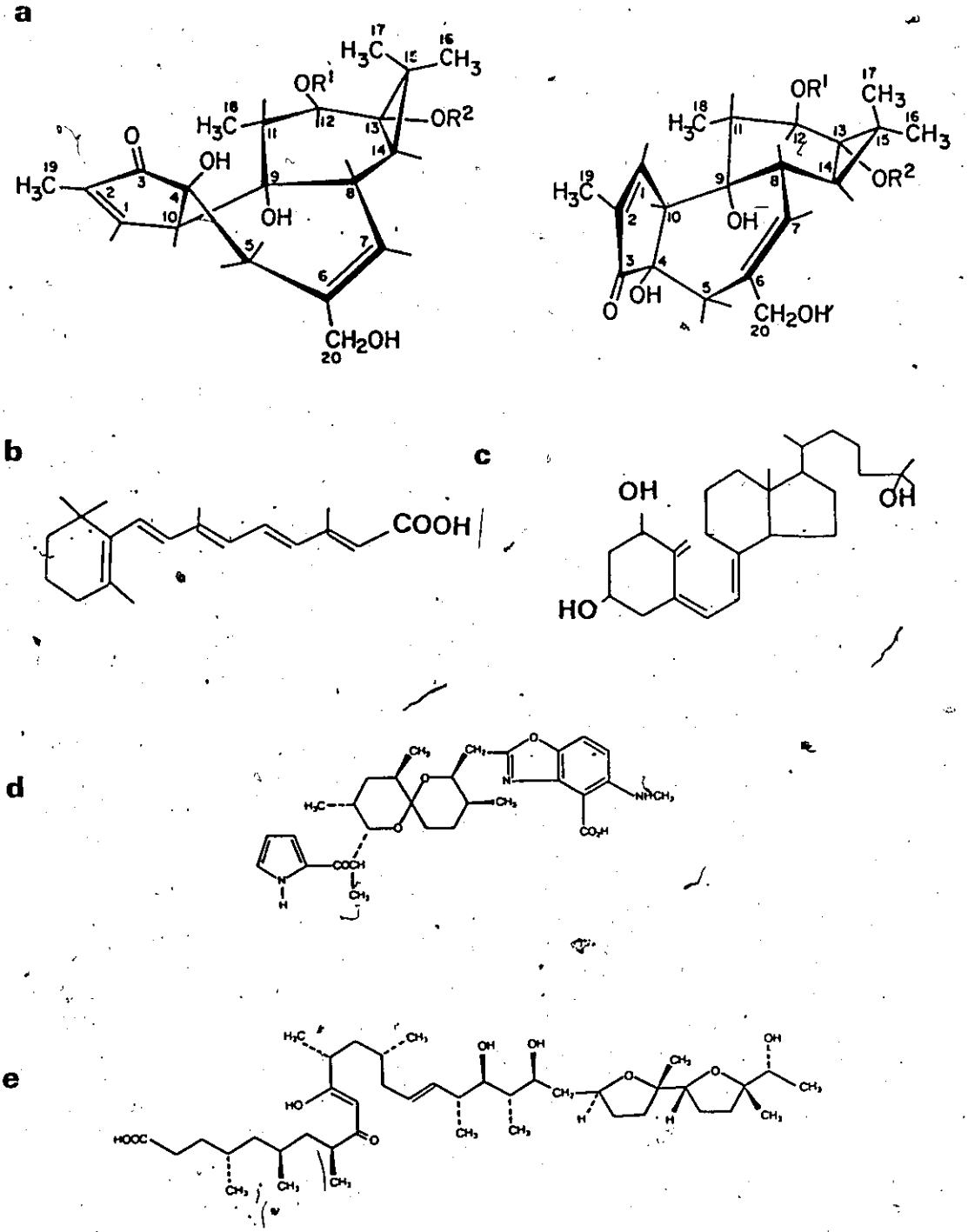


Fig. 1.2.3. Structures of HL-60 inducing agents. a, phorbol ester parent ring system (4-β conformation on left, 4-α conformation on right); b, retinoic acid; c, 1,25-dihydroxyvitamin D_3; d, Ca^{2+} ionophore A23187; e, Ca^{2+} ionophore ionomycin. Taken from Diamond et al (1980), Green and Chambon (1988) and 1988 Calbiochem catalog.

of the earliest effects of DMSO is rapid conversion of phosphatidylethanolamine (PE) to phosphatidylcholine (PC) and release of arachidonic acid from the new PC pool (Ziboh et al, 1986) but the significance of this is unclear. The wide variety of other chemicals which induce granulocytic HL-60 differentiation (see Table 1.2.1) provide little clue as to a common event which could initiate differentiation. Interestingly, it has been suggested that agents which disturb purine metabolism (see Table 1.2.1) may act by decreasing cellular GTP pools and inhibiting G protein function (Wright, 1987).

Several biological mediators also induce HL-60 cells towards a granulocytic phenotype. Physiological concentrations of retinoic acid (RA) cause maturation similar but not identical to that observed with DMSO (Breitman et al, 1980b; Matzner et al, 1987). One difference of note is the apparent absence of chemotactic peptide receptors in RA-treated cells (Skubitz et al, 1982). Despite this the normally coupled oxidative burst system is induced and can be activated by phorbol esters (Imaizumi and Breitman, 1986). Both sensitivity to differentiation and uptake of RA are most pronounced in S phase cells (Yen and Albright, 1984); RA itself appears to prolong division time by increasing the length of G1 and S phase (Gezer et al, 1988). Commitment to differentiation induced by RA occurs after 48 h of exposure and approximately two cell divisions (Yen et al, 1984a). This is accompanied by distinct alterations in nuclear structure which are retained through several cell divisions in the absence of inducer (Yen et al, 1984a). Elucidation of this event at a molecular level may provide insight to the switch which governs renewal versus

differentiation. Towards this end, nuclear proteins that are either induced during differentiation (Chou et al, 1984; Barque et al, 1987), specific to HL-60 and other myeloid lines (Goldberger et al, 1986) or altered in post translational modification upon differentiation (Barque et al, 1987; Briggs and Casey, 1988) have been identified.

The presumed mode of action of RA on HL-60 cells occurs via a RA-receptor which acts in much the same manner as steroid hormone receptors (reviewed in Green and Chambon, 1988). Two isoforms of receptor with different tissue specificities have been discovered thus far (Petkovich et al, 1987; Giguere et al, 1987; Benbrook et al, 1988). Initially, there was no evidence for specific binding of RA to HL-60 cells (summarized in Collins, 1987) but high affinity sites have been recently detected (Wathne et al, 1988). This is consistent with the low level of RA-receptor transcripts found in rat spleen tissues (Benbrook et al, 1988). Despite the accepted mode of action of steroid hormone like-receptors and apparently direct effects on transcription in mouse myeloid cells (Chiocca et al, 1988), intracellular RA-receptors may not be necessary for HL-60 differentiation since immobilized RA still induces differentiation (Yen et al, 1984b; Wahtne et al, 1988). There is evidence for post transcriptional control of mRNA abundance by RA in F9 teratocarcinoma cells (Wang and Gudas, 1988). It is possible that some form of membrane perturbation by the alkyl moeity of RA may trigger differentiation (Yen et al, 1984b). Hydrophobicity is one characteristic shared by many inducing agents including polar solvents, phorbol esters and other lipophilic drugs.

Perhaps the most physiologically relevant inducer of

granulocytic HL-60 differentiation is G-CSF. However, the effects of this cytokine as either conditioned medium or purified or recombinant protein are less marked than with other inducers (Metcalf, 1983; Begley et al, 1987). Thus, granulocytic cell surface antigens are evoked but in the absence of overt morphological alterations. G-CSF transiently stimulates HL-60 proliferation, however this is followed by clonal extinction as expected in a terminal lineage (Begley et al, 1987). Additional cytokines or other physiological signals may augment the response to G-CSF. In contrast to G-CSF, GM-CSF elicits HL-60 surface antigens representative of both monocytes and granulocytes (Begley et al, 1987) in accord with its ability to stimulate the progression of normal precursors along several lineages in vitro. Lastly, pluripotent CSF (IL-3) also apparently induces HL-60 cells along an as yet undefined lineage (Welte et al, 1985).

Other protein factors which influence HL-60 differentiation have been described. Serine proteases such as trypsin, chymotrypsin and elastase cause granulocytic maturation within 3-6 days (Fibach et al, 1985). This process depends absolutely on proteolytic activity directed towards the cell surface. Curiously, a long incubation period (up to 12 days) results in replacement of granulocytic characteristics with a monocytic phenotype (Fibach et al, 1985). Another unusual protein inducer is anti-thymocyte globulin which evokes granulocytic characteristics apparently through tight binding to HL-60 cells (Hunter et al, 1985). This equine IgG preparation also induces differentiation of normal myeloid precursors and is used clincally as an anti-leukemic agent (Hunter et al, 1985).

The spectrum of known physiological granulocytic inducers is completed by a class of sphingolipids, the neo-lacto series gangliosides (Nojiri et al, 1988). These lipids are synthesized early in DMSO-induced differentiation, raising the possibility that perturbation of lipid metabolism leads to the production of agents which induce differentiation. Significantly, the ganglio-series ganglioside GM_3 is increased by phorbol ester treatment and itself elicits monocytic HL-60 differentiation (Nojiri et al, 1986). Such effects may represent a differentiation counterpart to autocrine growth stimulation of undifferentiated HL-60 cells (Brennan et al, 1981; Perkins et al, 1984).

ii) Monocytic/Macrophage-Like Differentiation

Following the discovery of granulocytic HL-60 differentiation, HL-60 cells were found to be capable of differentiation towards cells resembling normal monocytes and their activated form, macrophages. Tumour promoting phorbol esters such as 12-O-tetradecanoylphorbol-13-acetate (TPA) have pleiotropic effects on virtually every cell type examined (reviewed in Diamond et al, 1980); the tetracyclic diterpene phorbol ring structure is shown in Fig. 1.2.3. Initially, TPA was reported to cause granulocytic maturation (Huberman and Callaham, 1979), but this was soon re-classified as a macrophage-like phenotype (Rovera et al, 1979). Even short exposure to TPA results in strong adherence to plastic and rapid cessation of cell division (Rovera et al, 1980) as well as a host of cellular and biochemical changes (see Table 1.2.1). Cell cycle arrest occurs nearly uniformly in late G1 so that cells beyond this point proceed through a final division before accumulating in G1/G0 (Rovera et al, 1980; Yun and Sugihara, 1986; Yen et al, 1987a).

Undifferentiated HL-60 cells express approximately 10^5 phorbol diester receptors per cell with a K_d on the order of 10 nM (Vandenbark et al, 1984). The phorbol ester receptor co-purifies with protein kinase C (PKC) and it is widely accepted that most if not all effects of phorbol esters and similar tumour promoters are mediated by PKC (Castanaga et al, 1982; reviewed in Nishizuka 1984a,b). Most agents which activate PKC induce HL-60 differentiation at the expected concentration for biological activity (Huberman et al, 1982; Vandenbark, 1984). Not unexpectedly, marked alterations in the HL-60 phosphoprotein profile occur upon TPA treatment. For instance, phosphorylation of the transferrin receptor by PKC leads to its subsequent downregulation (May et al, 1984) while the Na^+/H^+ antiporter is phosphorylated and activated thereby increasing intracellular pH (Besterman et al, 1985). Many other unidentified phosphoproteins incorporate ^{32}P upon phorbol ester stimulation (e.g. Feuerstein and Cooper, 1983; Mita et al, 1984; Feuerstein et al, 1985; Anderson et al, 1985), however the role of these PKC substrates is unknown. Detailed aspects of PKC activation and HL-60 differentiation are discussed in Section 1.4 and 1.5.

Perturbation of the cAMP-based second messenger system also causes HL-60 cell maturation. Originally, dibutyryl cAMP and PGE (which elevates intracellular cAMP levels) were characterized as granulocytic inducers (Chaplinski et al, 1982). However, a more detailed analysis has shown that in addition to markers common to both granulocytic and monocytic differentiation, monocyte-specific esterase activity is induced by these agents (Chaplinski et al, 1985). As with many other inducers, differentiation in response to dbcAMP occurs without any overt

morphological changes in the cells (see Leglise et al, 1988). Other chemicals which evoke a monocytic phenotype are listed in Table 1.2.1.

Physiological inducers of monocytoid HL-60 differentiation have been recognized more recently. Among these 1,25-dihydroxyvitamin D3 ($1,25\text{-}(OH)_2D_3$) elicits a phenotype most like that of normal macrophages (Murao et al, 1983). Although originally suggested to evoke granulocytic maturation, $1,25\text{-}(OH)_2D_3$ has since been found to cause a "myelomonocytic" phenotype (Rigby et al, 1985). This active metabolite of vitamin D_3 apparently acts in a steroid hormone-like fashion through a specific cellular receptor, recently cloned by Baker et al (1988). HL-60 cells express approximately 4,000 $1,25\text{-}(OH)_2D_3$ receptors that have a K_d of 5 nM (Mangelsdorf et al, 1984). The ability of vitamin D_3 analogs to induce HL-60 differentiation parallels both their affinity for the receptor and their activity in other biological systems (Ostrem et al, 1987). As for RA, differentiation by $1,25\text{-}(OH)_2D_3$ can be uncoupled from proliferation although sensitivity to $1,25\text{-}(OH)_2D_3$ is greatest in G1/S to S phase (Studzinski et al, 1985). Continuous treatment with $1,25\text{-}(OH)_2D_3$ culminates in G1/G0 arrest after 2-3 cell divisions (Yen et al, 1987a). One of the earliest events detected after exposure to $1,25\text{-}(OH)_2D_3$ is an increase in the ratio of PC to PE (Levy et al, 1987), much as observed in DMSO treated cells (Ziboh et al, 1986). Another candidate for a differentiation mediator accumulates in $1,25\text{-}(OH)_2D_3$ treated cells and is able to induce differentiation when delivered to undifferentiated cells either directly or in cytoplasts (Okazaki et al, 1988). It has been suggested that this proteinaceous factor is associated with commitment (Okazaki et al, 1988). Finally it

is probably noteworthy that 1,25-$(OH)_2D_3$ causes normal macrophages to become osteoclasts, the principal mediators of bone resorption, and that this capability is also acquired by HL-60 cells upon exposure to 1,25-$(OH)_2D_3$ (Bar-Shavitt et al, 1983).

Two other cytokines aside from GM-CSF (see above), namely γ-IFN and TNF, have been shown to induce a monocytic HL-60 phenotype (Takei et al, 1984; Trinchieri et al, 1986). Although the latter has only weak activity by itself, it synergistically augments the action of subthreshold doses of γ-IFN (Trinchieri et al, 1986). The mechanism for each of these inducers is not well defined. γ-IFN may in part regulate transcription via activation of PKC (Fan et al, 1988). One effect of TNF on HL-60 is the activation of a pertussin toxin-sensitive GTP binding protein (Imamura et al, 1988). Other proteinaceous inducers of monocytic differentiation include a M_r 40,000 protein known as differentiation-inducing factor (DIF) present in lymphocyte conditioned medium (Olsson et al, 1984). Differentiation in this case requires only a single division before G1 arrest occurs (Yen and Chiao, 1983). In contrast to DIF, a peptide factor secreted by an HL-60 subline resistant to monocytic differentiation is apparently able to block monocytic differentiation (Mendelsohn et al, 1983). Lastly, the effect of the bone marrow environment on HL-60 differentiation should be mentioned. Co-culture with a human bone marrow cell line (KM-102) predisposes HL-60 cells towards differentiation by 1,25-$(OH)_2D_3$ (Ohkawa and Harigaya, 1987). This effect depends on cell-cell contact rather than secreted factors, although an interaction with extracellular matrix components from bone marrow enhances the cellular response to TPA (Luikart et al,

1987).

iii) Eosinophilic/Basophilic Differentiation

Finally, to illustrate the multipotent nature of the HL-60 cell line, induction along a third myeloid lineage will be mentioned. Prolonged culture of HL-60 cells in alkaline media (pH > 7.8) followed by sodium butyrate treatment causes the expresssion of eosinophil-specific histochemical reactivity and increased histamine levels, characteristic of basophilic granulocytes (Fischkoff et al, 1984; Fischkoff and Condon, 1985). Maturation is incomplete both in function and loss of proliferative capacity, although strongly eosinophilic sublines have been developed (Tomonaga et al, 1986). Thus, HL-60 cells retain enough plasticity to allow gene expression representative of 3 distinct terminal blood cell lineages.

iv) Interactions Between Various Inducers

It is clear from the above discussion that many programs of HL-60 differentiation share 2 features: maximal sensitivity to inducers in late G1 phase to early S phase of the cell cycle and G1/G0 arrest subsequent to a variable but low number of cell divisions in the presence of inducer. Many combinations of inducing agents have been tested in an attempt to define at least at a cellular level events important for differentiation. Often this has occured in the search for effective chemotherapeutic regimes against myeloid leukemias. Two themes have emerged from this work. First, monocytic inducers predominate over agents which induce granulocytic differentiation. Phorbol ester-induced maturation is unaffected by the presence of

hexamethylene-bisacetamide (HMBA), a strong granulocytic inducer, whereas TPA completely overides HMBA even after 3 days prior exposure to the latter (Fontana et al, 1981). Similarly, 1,25-$(OH)_2D_3$ at a concentration of 1-120 nM induces monocytic markers even in the presence of 1 μM RA (Miyaura et al, 1985). TPA also dominates RA-induced differentiation (Rovera et al, 1979) even if cells are exposed to 10 nM TPA for only 1 h followed by 48 h exposure to 1 μM RA (Zylber-Katz et al, 1986). Perhaps the most dramatic example of monocytic hierarchy is the HL-60/MRI subline which differentiates into monocytes when treated with RA (Imaizumi et al, 1987). Not only are these cells extremely sensitive to RA (ED_{50} = 0.41 nM) but they undergo normal granulocytic differentiation in response to DMSO or HMBA (Imaizumi et al, 1987). Although there are considerable karyotypic differences between parental and HL-60/MRI cells, it is likely that only relatively few events in the RA program have been altered to subvert it to monocytic differentiation. Thus most key events necessary for the commitment decision should be common to both modes of differentiation.

This leads to the second point, in that commitment to differentiation is not lineage specific. Pre-treatment of HL-60 cells with either RA or 1,25-$(OH)_2D_3$ for 24 h enhances the rate of differentiation in response to the second drug without altering its lineage specificity (Yen et al, 1987b). In addition, a lower concentration of the final drug is required for full differentiation, implying that commitment has a higher threshold than lineage determination. DMSO similarly enhances the effects of 1,25-$(OH)_2D_3$ but in this case the converse cannot be demonstrated (Momoi et al, 1986).

This phenomenon has also been demonstrated with TPA in that pre-treatment with DMSO, HMBA, butyric acid or RA markedly accelerates differentiation in response to 16 nM TPA (Fibach et al, 1982). A monocytic differentiation inducing activity (DIA = DIF, see above) as well as dbcAMP also exhibit enhanced potency if HL-60 cells are pre-treated with RA (Olsson et al, 1982). These results all support the notion that early events in terminal differentiation are common to all lineages. To some extent differentiation and growth arrest are separable in HL-60 cells and other cell types (Yen, 1985; Yen et al, 1987b), again suggesting that differentiation may be composed of discrete interchangeable blocks of gene expression. The programs encoding cell cycle exit and commitment to a terminal lineage may be universal to all differentiating systems within an organism. Elements common to many programs of differentiation are logical candidate components of such genetic programs. The next section is devoted to genes regulated during various modes of HL-60 differentiation.

1.3 GENE REGULATION DURING HL-60 CELL DIFFERENTIATION

The HL-60 system is ideal for investigating differential gene control since several differentiated states can be attained in a homogeneous and reproducible manner. All differentiation-regulated genes provide a means with which the controlling elements of the renewal/differentiation decision can be sought. In part, this can be done by comparing the various inducers of HL-60 differentiation with regard to expression of the gene of interest. Patterns of gene

expression common to all differentiation pathways may indicate a crucial role for the genes involved. Genes characterized in the HL-60 system thus far will be reviewed with emphasis on those most pertinent to this work. For the sake of clarity, genes that are regulated during HL-60 differentiation will be divided into proto-oncogenes and other genes either with a known function or as differentiation-specific products of undetermined function.

1.3.1 Proto-oncogenes

The normal cellular counterparts to transforming oncogenes appear to form an integral part of the control network for growth and differentiation (Bishop, 1985). Given the links between oncogenesis and differentiation it is not surprising that these transforming genes, often first identified in various leukemias, impinge on terminal hemopoetic differentiation. A heirarchy of oncogene effects places nuclear genes such as c-myc, c-fos and c-jun at the centre of gene regulation (Zarbl et al, 1987; Schonthal et al, 1988). Understanding the function and associations of oncogene products in other systems bears strongly on their possible roles in HL-60 differentiation. Discussion of each oncogene is necessarily incomplete but will be roughly in proportion to the significance to the work reported in this thesis.

i) c-myc

This highly conserved gene was first recognized as the transforming gene of the MC29 acute avian leukemia virus (Bister et al, 1977) and is the archetypal nuclear oncogene (Donner et al, 1982). Increased

expression of c-myc is associated with cell cycle competence, although neither c-myc mRNA or protein (Myc) are cell cycle regulated (reviewed in Piechaczyk et al, 1987). Expression of c-myc stimulates the transcription of early genes in the G0 to G1 transition (Schweinfest et al, 1988) as well as some heat shock genes (Kingston et al, 1984). Induction of c-myc is not, however, sufficient to confer proliferative competence (Smeland et al, 1985), nor is it necessary for the mitogenic effect of platelet-derived growth factor (Coughlin et al, 1985). Rather, c-myc appears to correlate with maintenance of a non-terminal or pre-differentiated state, which usually but not always includes proliferation. Transgenic mice bearing an Eμ-myc fusion gene have increased pre-B cell populations at the expense of more differentiated forms (Langdon et al, 1986). Differentiation of most cell types in vitro is accompanied by a decline in c-myc mRNA levels (summarized in Freytag, 1988). Conversely, constitutive expression of c-myc from a heterologous promoter in transfected murine erythroleukemia (MEL) cells abrogates differentiation (Coppola and Cole, 1986; Prowchownik and Kukowska, 1986). De-regulated c-myc similarly blocks 3T3-L1 cell differentiation by preventing the transition to a pre-differentiated state (Freytag, 1988). From another viewpoint, expression of antisense c-myc mRNA in several cell lines predisposes them towards differentiation (Prowchownik et al, 1988; Griep and Westphal, 1988). In spite of these results and in contrast to downregulation of the mRNA, it has been found that Myc abundance in MEL cells does not decline dramatically upon differentiation (Wingrove et al, 1988).

Regulation of c-myc expression is extremely complex, having

multiple translational and transcriptional components (reviewed in Piechaczyk et al, 1987). The human c-myc gene is expressed in virtually all cell types and comprises 3 exons (Colby et al, 1983; Battey et al, 1983). The latter two contain the major open reading frames that encode the 64/67 kDa Myc nuclear phosphoproteins (Hann and Eisenmann, 1984), while the untranslated first exon apparently has multiple regulatory roles (see Piechaczyk et al, 1987). Various negative and positive upstream regulatory elements interact to control c-myc transcription (Chung et al, 1986; Remmers et al, 1986) including a phorbol ester/cAMP responsive element which binds transcription factor AP-2 (Imagawa et al, 1987). Both the c-myc mRNA and protein are extremely unstable (reviewed in Piechaczyk et al, 1987).

Expression of c-myc has been investigated thoroughly in the HL-60 cell line (reviewed in Collins, 1987). HL-60 cells overexpress c-myc as a result of c-myc gene amplification (see Section 1.2.2), although it has been argued that the normal counterparts to HL-60 cells express equally high c-myc mRNA levels (Gowda et al, 1986). Downregulation of c-myc mRNA is often but not always an early event after exposure to an inducing agent. For instance, DMSO (Wantanabe et al, 1985), TPA (Westin et al, 1982a), dbcAMP (Trepel et al, 1987) and TNF (M^{c}Cachren et al, 1988) all reduce c-myc mRNA abundance to nearly undetectable levels within 6 h. On the other hand, 1,25-$(OH)_2D_3$ and RA cause a gradual reduction on the order of days (Wantanabe et al, 1985; Yen and Guernsey, 1986) while cytosine arabinoside and τ-IFN have no effect at all on c-myc transcript abundance (Leglise et al, 1988; M^{c}Cachren et al, 1988). In each case tested mRNA levels are closely paralleled by Myc abundance

(see above references). Despite a few exceptions, downregulation of c-myc mRNA is now widely regarded as a marker of HL-60 cell differentiation (e.g. Salehi et al, 1988). Repression of c-myc is closely linked to G1/G0 arrest (Einat et al, 1985) and may even directly trigger cell maturation; a partial monocytic phenotype is exhibited by HL-60 transfectants which express high levels of antisense c-myc mRNA (Yokoyama and Imamoto, 1987). Anti-sense c-myc oligonucleotides also evoke weak differentiation and growth arrest of HL-60 cultures (Wickstrom et al, 1988). The similar kinetics of c-myc repression and the appearance of differentiated cells, as opposed to remaining proliferating cells, has been used to argue that c-myc is associated with a predifferentiated state (Filmus and Buick, 1985). Conversely, non-specific arrest of HL-60 proliferation by hydroxyurea is not accompanied by a decrease in c-myc (Grosso and Pitot, 1984). Thus c-myc is a candidate for a controlling element in the renewal decision, albeit as a repressor of differentiation.

Unlike some other differentiating cell systems in which c-myc mRNA is decreased by a post-transcriptional mechanism (e.g. Dony et al, 1985), c-myc transcription is downregulated in all modes of HL-60 cell differentiation tested thus far (Grosso and Pitot, 1985a; Simpson et al, 1987; Trepel et al, 1987). Altered S_1 nuclease sensitivity at one site upstream of exon 1 correlates with this event (Grosso and Pitot, 1985b). Interestingly, RA does not lower the rate of c-myc transcription initiation but rather causes an elongation block at the end of exon 1 (Bentley and Groudine, 1986). Both TPA and TNF reduce c-myc expression by an identical mechanism (McCachren et al, 1988). At first it was

suggested this arose from a bifunctional P_2 c-myc promoter in which RNA polymerase III replaced RNA polymerase II-mediated transcription during differentiation (Chung et al, 1987). However, it seems more likely that specific sequences at the end of exon I cause a specific block in transcription elongation in differentiated cells (Bentley and Groudine, 1988). Other oncogene loci including c-fos are regulated in a similar manner (reviewed in Bentley and Groudine, 1988).

Finally, recent evidence gleaned in part from HL-60 cells suggests a possible function for c-myc in DNA replication. Although by no means unequivocal, antiserum to human Myc can inhibit DNA replication in isolated HL-60 nuclei implying that c-myc expression is required for replicative competence (Studzinski et al, 1986). However, the specificity of this effect has been seriously questioned (Gutierrez et al, 1988). More convincingly, a plasmid containing human autonomously replicating sequences (ARS) will not replicate in HL-60 cells if it is co-transfected with Myc antiserum (Iguchi-Ariga et al, 1987). In vitro replication of ARS constructs is inhibited in a similar manner (Iguchi-Ariga et al, 1987). SV40 replication is also enhanced by high level c-myc expression (Classon et al, 1987). Accordingly, an antisense c-myc oligonucleotide specifically arrests T cells in S phase (Heikkila et al, 1988). Very recently, Iguchi-Ariga et al (1988) have reported that in addition to its role in DNA replication, Myc is necessary for an enhancer activity that resides 2 kbp upstream of the c-myc coding region and that Myc may positively regulate its own expression. These findings may provide a basis for the correlation between c-myc expression and DNA synthesis in HL-60 cells (Brevli et al, 1986).

ii) c-fos

Rapid induction of the cellular cognate of the FBJ sarcomavirus transforming gene (Curran and Teich, 1982) is often associated with the onset of new programs of gene expression (reviewed in Verma and Sassone-Corsi, 1987; Curran and Franza, 1988). As in other cell types, treatment of HL-60 cells with phorbol esters causes an immediate increase in c-fos transcription that is maximal within 1 h and nearly completely subsides within 4 h (Mitchell et al, 1985; Muller et al, 1985). As expected, the c-fos protein (Fos) closely parallels increases in mRNA levels (Mitchell et al, 1985). Induction of c-fos transcription by TPA and serum is mediated through the serum response element (SRE) and its associated serum response factor (SRF); this multifunctional transcription element also confers PKC independent activation of c-fos (Gilman, 1988). The c-fos promoter contains several additional transcriptional elements. A sequence adjacent to the SRE is the binding site for the AP-1 family of transcription factors typified by $p39^{c-jun}$ (Rauscher et al, 1988a,b; Chui et al, 1988; reviewed in Curran and Franza, 1988). However, this AP-1 site does not appear to contribute to the TPA responsiveness of c-fos (Gilman, 1988). Two other upstream elements responsive to intracellular messengers have been identified in the c-fos gene. One of these confers Ca^{2+}-inducibility while another is required for cAMP-induced transcription (Sheng et al, 1988; Gilman, 1988). In HL-60 cells, cAMP treatment elevates c-fos in a kinetically similar fashion to TPA (Tsuda et al, 1987).

Fos apparently has no sequence specific DNA binding capacity but upon complex formation with $p39^{c-jun}$ is able to specifically activate

transcription from promoters containing the AP-1 recognition sequence (Rauscher et al, 1988b; Sassone-Corsi et al, 1988a). Examples of genes activated by c-fos via AP-1 sites include collagenase (Schonthal et al, 1988), human metallothioein II_A (Angel et al, 1987) and c-Ha-ras (Imler et al, 1988). Surprisingly, Fos apparently negatively regulates its own synthesis via the SRE and a heat shock promoter-like element; Fos may compete with $p39^{c-jun}$ in transcription complex formation with SRF (Sassone-Corsi et al, 1988b; Schonthal et al, 1988). In conjunction with destabilizing sequences in the c-fos mRNA (Wilson and Treisman, 1988), this accounts for the characteristic "spiked" induction of c-fos mRNA. Other genes repressed by Fos include the adipocyte aP2 gene (reviewed in Speigelman et al, 1988) and HSP70 (Sassone-Corsi et al, 1988b). Subtlties in recognition sequences and their associated binding factors may underlie the diverse responses of genes under the control of Fos (see Curran and Franza, 1988). It is certain that post-translational modification of Fos, such as phosphorylation in response to TPA (e.g. Barber and Verma, 1987), also modulates the response of any given set of genes. This is discussed further in Section 1.4.1.

Unlike repression of c-myc, c-fos induction is not a general feature of HL-60 differentiation as it occurs only upon treatment with TPA or other activators of PKC (Mitchell et al, 1985). One report has suggested that $1,25\text{-}(OH)_2D_3$ induces c-fos (Mitchell et al, 1986) but this has not been reproduced (Calabretta, 1987). HL-60 sublines resistant to TPA but susceptible to $1,25\text{-}(OH)_2D_3$ differentiate in the absence of c-fos induction (Mitchell et al, 1986). Further, 1-oleoyl-2-acetylglycerol cannot induce HL-60 differentiation but nonetheless

causes rapid accumulation of c-fos (Calabretta, 1987). Serum also induces c-fos in serum starved HL-60 cells but without any evidence of differentiation (Mitchell et al, 1986). Finally, the c-fos response has been dissociated from TPA-induced differentiation by cotreating cells with very high concentrations of retinal (an inhibitor of PKC) and TPA for a 30 min period, then resuspending cells in fresh medium (Calabretta, 1987). This procedure apparently removes retinal from the culture but not TPA. Thus on several grounds it has been argued that c-fos is neither necessary nor sufficient for the induction of macrophage-like cells by TPA. Based on the continued growth of HL-60 cell cultures after macrophage-like differentiation by 1,25-$(OH)_2D_3$, it has been suggested that c-fos expression may be necessary (but not sufficient) for cessation of HL-60 cell proliferation rather than the acquisition of a differentiated phenotype (Mitchell et al, 1986). Other systems suggest c-fos plays a role in growth (Holt et al, 1986), differentiation (Ruther et al, 1985) and development (Ruther et al, 1987).

Finally, monocytic differentiation of HL-60 cells is accompanied by an approximate 2 fold rise in c-fos transcripts in the final differentiated state, regardless of inducing agent (Muller et al, 1985; Mitchell et al, 1985), possibly because of high levels of CSF-1 in serum (see Sherr, 1988). Normal macrophages contain detectable c-fos mRNA that is inducible to high levels, implying a functional role for c-fos in these cells (Bravo et al, 1987; Muller et al, 1985). However, c-fos is particularly abundant in normal granulocytes so that regulation of c-fos in this form of HL-60 differentiation appears defective (Kreipe et al, 1986).

iii) c-fms

This proto-oncogene, isolated by virtue of its similarity to the transforming gene of the Susan McDonough feline sarcoma virus, encodes the CSF-1 (or M-CSF) receptor which is expressed in normal monocytes (Sherr et al, 1985). The effects of M-CSF are largely mediated through ligand-dependent tyrosine kinase activity of the receptor but may also depend on receptor stimulated phospholipid turnover (reviewed in Sherr, 1988). Truncated forms of c-fms which encode non-regulated tyrosine kinase activity are oncogenic in cell types other than monocyte/macrophages (Sherr, 1988). Although the c-fms transcript is upregulated late in monocytic HL-60 differentiation and thus probably plays no causal role in cell maturation (Sariban et al, 1985), it does serve as a useful endpoint marker indicative of complete differentiation (e.g. Mitchell et al, 1986; Sariban et al, 1987).

iv) Miscellaneous oncogenes

A variety of other oncogenes are regulated during various modes of HL-60 differentiation. The cellular homolog of the AMV transforming gene, c-myb, (reviewed in Thompson et al, 1986) shares many properties with c-myc including downregulation during HL-60 differentiation (Westin et al, 1982b). In contrast to c-myc, it is expressed mainly in hemopoietic cells and thus may have a more limited role in growth and/or differentiation (Thompson et al, 1986). Although c-myb expression is strongly associated with hemopoietic cell proliferation, its expression can be dissociated from c-myc in HL-60 cells induced to differentiate with tiazofurin (Kharbanda et al, 1988). Nonetheless, given its numerous other similarities to c-myc (reviewed in Brevli and Studzinski,

1987), c-myb may contribute to maintenance of the undifferentiated state. Other evidence suggests that c-myb may play a more crucial role than c-myc in erythroleukemia differentiation (Ramsay et al, 1986). In any event, c-myb was not investigated in these studies.

The β-chain of platelet derived growth factor (PDGF) encoded by c-sis is normally expressed in several cell types including platelets, endothelial cells and macrophages (reviewed in Pantazis et al, 1986). Treatment of HL-60 cells with monocytic inducers such as TPA or TNF but not granulocytic inducers causes the appearance of both PDGF transcripts and PDGF activity after 18 h exposure (Pantazis et al, 1986; Alitalo et al, 1987). Activity of the c-src tyrosine kinase increases substantially during both monocytic and granulocytic HL-60 differentiation although the evidence for increased c-src mRNA is equivocal (Barkenow and Gessler, 1986). Similarly, c-fes tyrosine kinase activity is increased several fold in all forms of differentiated HL-60 cells even though protein abundance is reduced by a similar factor (Smithgall et al, 1988). The c-fes mRNA is weakly induced after several days exposure to RA (Ferrari et al, 1985). Treatment of HL-60 cells with TPA but not RA evokes expression of the c-ets nuclear proteins, again as a late event in differentation (Ghysdael et al, 1986; Fujiwara et al, 1988). Induction of c-Ha-ras transcripts may occur during granulocytic HL-60 maturation (Studzinski and Brevli, 1987) whereas the c-N-ras mRNA is unaltered in all forms of HL-60 differentiation (Wantanabe et al, 1985). Finally, the c-raf mRNA, which encodes a serine/threonine kinase that itself is a substrate for the c-fms tyrosine kinase (Sherr, 1988), is expressed in HL-60 cells but not

regulated during differentiation (Sariban et al, 1987).

1.3.2 Other Genes Regulated During HL-60 Differentiation

The HL-60 line has proved an invaluable tissue source for the cloning of several genes regulated during myelopoiesis; however, all of these relate to differentiated cell function rather than control of the process. A recent survey of the literature includes the following genomic or cDNA clones: myeloperoxidase which is strongly depressed upon differentiation (Weil et al, 1987); the NADPH oxidase system cytochrome b heavy chain that is induced during granulocytic maturation (Royer-Pokora et al, 1986; Barker et al, 1988); ferritin heavy and light subunits that undergo complex regulation during various modes of HL-60 differentiation (Chou et al, 1986); unusual mutant actin and tubulin species also regulated in a complex manner (Salser et al, 1985; Chou et al, 1987); the protein core component of chondroitin sulfate proteoglycan found in secretory granules (Stevens et al, 1988); an ADP/ATP carrier which markedly decreases upon differentiation (Battini et al, 1987); the antimicrobial defensins of neutrophil granules which are upregulated after DMSO treatment (Daher et al, 1988).

The HL-60 system has also been used by several groups to identify genes whose expression is altered during differentiation. This approach has not yet identified any "master" genes comparable to those of the myoblast system (Blau, 1988). Differential hybridization of cDNA libraries has been used to detect clones which are either increased or decreased upon HL-60 differentiation (Davis et al, 1987). This approach also yielded novel τ-actin and β-tubulin genes that are variously

regulated during differentiation (Chou et al, 1987). Moreover, re-introduction of corresponding genomic clones back into HL-60 cells showed that they were regulated in a normal manner (Concannon et al, 1985). A similar approach has also identified clones which are controlled by nucleo-cytoplasmic transport during HL-60 differentiation (Graham and Birnie, 1988). Other genes of unknown function investigated in the HL-60 system include 2 zinc-finger proteins that are downregulated by DMSO and TPA (Pannuti et al, 1988) and a pair of cDNA clones which encode Ca^{2+}-binding proteins that are strongly upregulated by DMSO but repressed by TPA (Lagasse and Clerc, 1988).

Finally with respect to this work, 3 genes not well characterized in HL-60 cells were investigated, based on what is known about their function and regulation in other cell types. These were IL-1β, metallothionein (MT), and the 78 kDa glucose regulated protein (GRP78). IL-1β is a predominantly monocyte derived cytokine with an enormous range of biological activities including stimulation of thymocyte proliferation and fever induction in vivo (reviewed in Oppenheim et al, 1986). IL1-β is abundantly expressed in monocyte/macrophages and has in fact been cloned from HL-60 cells (Furutani et al, 1985), although its regulation during differentiation has not been characterized. The MT family of small cysteine-rich proteins is presumed to play a protective role against heavy metal toxicity and possibly against damage caused by oxygen radicals (reviewed in Hamer, 1986). Since mature granulocytes and monocytes localize to lesions and upon appropriate stimulation undergo a bacteriocidal oxidative burst (reviewed in Babior, 1984), increased basal and/or

inducible MT expression may be associated with HL-60 maturation. In addition, the human $MTII_A$ gene contains upstream elements which are phorbol ester responsive (Imbra and Karin, 1987; Angel et al, 1987) and which may thus activate MT transcription during TPA-induced HL-60 differentiation. Altered basal and/or Cd^{2+}-induced MT transcripts may also be expected during various forms of HL-60 maturation since PKC levels are decreased by TPA but elevated 2-4 fold by RA, DMSO or 1,25-$(OH)_2D_3$ (Zylber-Katz and Glazer, 1985; Martell et al, 1987). Interestingly, IL-1β moderately increases MT expression in undifferentiated HL-60 cells (Karin et al, 1985).

The major classes of stress response proteins (reviewed in Pelham, 1986) may also play a role in differentiation. Members of the heat shock protein gene family are regulated during differentiation of various cell lines in vitro (Singh and Yu, 1984; Henshold and Housman, 1988) and accumulate in the cells of aged Drosophila melanogaster (Fleming et al, 1988). The related GRPs are also induced in response to a variety of stimuli, particularly Ca^{2+} ionophore treatment (reviewed in Lee, 1987). In this work, GRP78 was used as a reporter gene for the effect of A23187 on HL-60 cells.

Having detailed some of the known alterations in gene expression during HL-60 differentiation, one of the major signal transduction routes impinging on these targets will be discussed.

1.4 PHOSPHOINOSITIDE-BASED SIGNAL TRANSDUCTION

1.4.1 Overview

Alteration of cellular function can ultimately be traced to regulatory signals which impinge on the cell surface. Transduction of signals detected by receptors to intracellular events is mediated by one or, more commonly, a combination of second messenger systems. Considering the diversity of biological signalling, relatively few intracellular mediators have been identified. Ca^{2+} and cAMP have long been assigned this function whereas more recently the role of lipid-derived mediators has become apparent (reviewed in Nishizuka, 1984a,b; Berridge and Irvine, 1984; Nishizuka, 1986; Berridge, 1987).

Turnover of phospholipids in the plasma membrane was first recognized as an early event in cell stimulation by Hokin and Hokin (1953). Much later, receptor-mediated phosphoinositide (PI) turnover was found to derive from the initial hydrolysis of phosphatidylinositol-4,5-bisphosphate (PIP_2), which constitutes less than 5% of the total PI pool (reviewed in Berridge and Irvine, 1984). Receptor activation is coupled via a G protein (G_p) to a PIP_2-specific phospholipase C (reviewed in Cockcroft, 1987). This generates two divergent messenger molecules, the soluble head group inositol-1,4,5-trisphosphate (IP_3) and the hydrophobic 1,2-sn-diacylglycerol (DAG). The latter activates a phospholipid-Ca^{2+}-dependent protein kinase (PKC) by causing its association with (or "translocation" to) the plasma membrane. At the same time IP_3 causes release of Ca^{2+} from a component of the endoplasmic reticulum by binding to a specific receptor (reviewed in Berridge,

1987). Both signals are transient. DAG is metabolized either by DAG kinase to phosphatidic acid which then re-enters the PI pool or by DAG lipase to monoacyl glycerol and arachidonic acid (reviewed in Nishizuka, 1986). The latter is further metabolized to the prostaglandin and leukotriene mediators of inflammation, while monoacylglycerol is completely degraded to glycerol and a second free fatty acid. IP_3 also has two metabolic fates (reviewed in Berridge, 1987). A specific phosphomonoesterase (IP_3ase) hydrolyzes the 5 phosphate on the inositol ring to generate 1,4-inositol bisphosphate, which is inactive for Ca^{2+} release. This is then converted to inositol, which also re-enters the PI pool, by the sequential action of two more phosphatases (Majerus et al, 1988). Alternatively, IP_3 is converted to 1,3,4,5-IP_4 by the action of a specific kinase (Irvine et al, 1986). IP_4 cannot release intracellular Ca^{2+} but, at least in some cell types (e.g. sea urchin oocyte), can open membrane Ca^{2+} channels and thereby further increase intracellular Ca^{2+} concentration (reviewed in Houslay, 1987; Downes, 1988). It has recently been proposed that a more general role for IP_4 may be to re-sequester Ca^{2+} into intracellular stores after IP_3-induced release (Hill et al, 1988). IP_4 is metabolized through as yet unclear phosphorylation and dephosphorylation pathways (see Majerus et al, 1988). Higher phosphorylated forms of inositol may also have biological roles (see Downes, 1988; Fink and Kaczmarek, 1988). The basic scheme of PI-based signal transduction is shown in Figure 1.4.1.

Identification of the major components of this signalling network was accompanied by the discovery that PKC is the major cellular receptor for the tumour promoting phorbol diesters (reviewed in

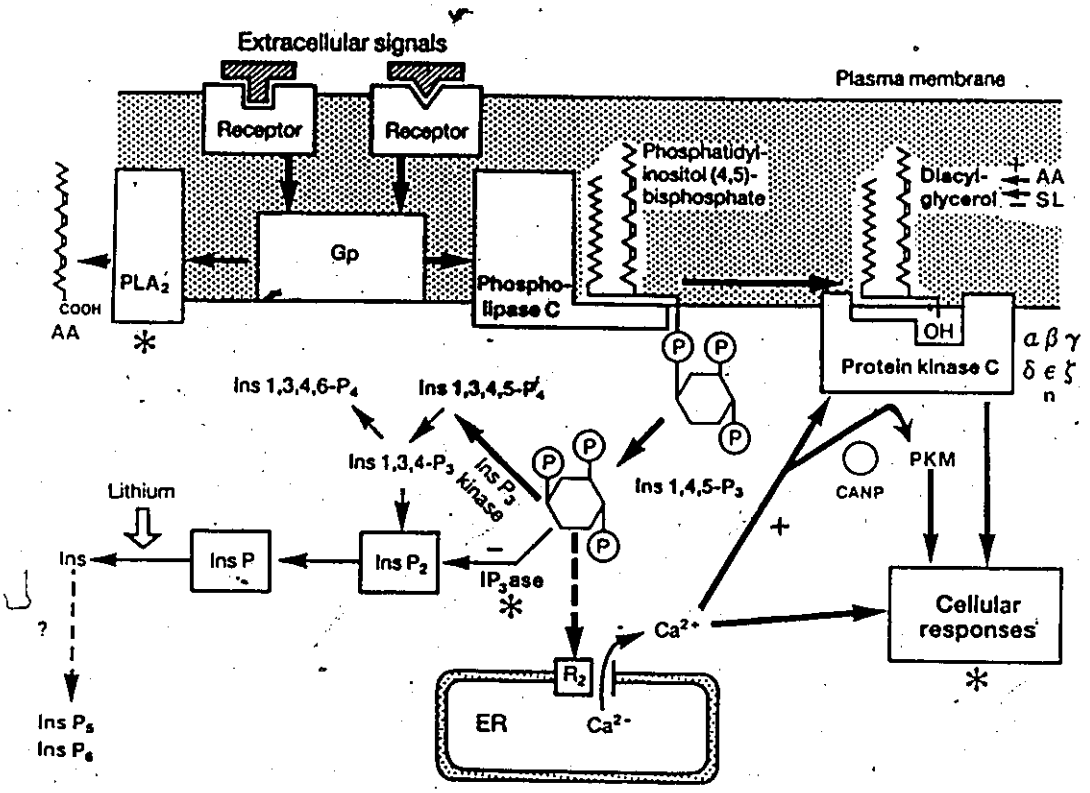


Fig. 1.4.1: Schematic of phosphoinositide-based signal transduction. Possible interactions between the various components are indicated by (+) and (-) for cooperative and antagonistic responses, respectively. The known subspecies of PKC are indicated by Greek letters. Possible points of action of P47, the major PKC substrate of platelets are indicated by * (see Section 1.6). Modified from Altman (1987).

Nishizuka, 1984a; Blumberg, 1988). These compounds (see Fig. 1.2.3) bind to PKC with high affinity and activate the kinase in the presence of resting intracellular Ca^{2+} concentrations. Aside from higher affinity binding than DAG, the major difference between the two activators is the metabolic stability of phorbol esters (e.g. Rodrigez-Pena and Rozengurt, 1984). This property causes prolonged, inappropriate activation of PKC. A number of structurally unrelated lipophilic agents also cause non-physiological activation of PKC; stereochemical alignment of these agents suggests that the oxygens at C3, C4, C9 and C20 and the lipophilic moiety at C12 of the parent phorbol ring system are the centers required for interaction with the kinase (Wender et al, 1986; Jeffrey and Liskamp, 1986). Computer matching of the various conformers has refined this model in terms of bonding interactions between ligand and receptor (Itai et al, 1988). Although more potent PKC activators and inhibitors may eventually be designed from such considerations (Wender et al, 1986), the phorbol esters have proved invaluable probes for investigating the role of PKC in all forms of signal transduction.

1.4.2 Interactions Between PKC and Ca^{2+}

The two intracellular signals generated by PIP_2 hydrolysis act in large part through phosphorylation/dephosphorylation cascades. This is obviously the case for PKC, which phosphorylates a number of cellular proteins, though many of these are of unknown function (Nishizuka, 1986). Ca^{2+} has long been established as a second messenger (reviewed in Veigel et al, 1984). Many of its effects are mediated by calmodulin

(CM), which upon binding Ca^{2+} serves to activate CM-dependent kinases and phosphatases; other target proteins such as cytoskeletal components and phospholipase A_2 (PLA_2) are affected through a direct interaction with Ca^{2+} (see Veigl et al, 1984). With the advent of high quantum yield fluorescent Ca^{2+} chelators such as fura-2 (Grynkiewicz et al, 1985) it has become apparent that Ca^{2+} signals do not merely occur as a continuous elevation of basal Ca^{2+} levels, but rather in any single cell are comprised of oscillations on a timescale of seconds (Woods et al, 1986; Jaconi et al, 1988; Jacob et al, 1988). A model to account for this effect incorporates the known parmeters of Ca^{2+} regulation to predict a bistable state of intracellular Ca^{2+} concentration (Meyer and Stryer, 1988). The role of Ca^{2+} periodic fluctuations is apparently to convert the the strength of agonist stimulation into a frequency modulated signal; various advantages to this scheme have been hypothesized (e.g. Jacob et al, 1988; Meyer and Stryer, 1988).

Not only do Ca^{2+} and PKC generate independent signals, but they are often able to cooperate with each other in a synergistic fashion (Nishizuka 1984a; 1986). This implies that subthreshold levels of either signal alone interact in a greater than additive fashion to elicit a full biological response. This was first noted for platelet granule release (Kaibuchi et al, 1983) and has subsequently been shown in many biological systems for many other short term and a few long term responses (Nishizuka, 1986). The basis for synergism resides at least in part at the level of PKC itself since the kinase is Ca^{2+}-dependent. Ca^{2+} reduces the requirement for DAG or phorbol ester by increasing the affinity of PKC for the lipid cofactor (Dougherty and Neidel, 1986).

Mechanistically, Ca^{2+} appears to "prime" PKC by causing its loose association with the plasma membrane in readiness to interact with DAG (Wolf et al, 1985a; b). This may in part occur via a specific PKC receptor in some cell types (Gopalakrishna et al, 1986). It is more generally accepted, however, that the kinase interacts directly with lipid bilayers. Activation of PKC in Triton X-100 mixed micelles suggests that a single PKC molecule interacts at the membrane surface with 4 phosphatidylserine molecules, one diacylglyceraol molecule and one Ca^{2+} ion in a five point membrane attachment model (Hannun, et al, 1986). It appears that this interaction underlies synergism in many short term responses such as transferrin receptor phosphorylation (May et al, 1985a).

A second more indirect mechanism of Ca^{2+}/DAG cooperativity involves the proteolytic cleavage of activated PKC by Ca^{2+}-activated neutral protease (CANP or calpain; reviewed in Suzuki, 1987). There are two forms of CANP, μ and m (or calpain I and calpain II), which have a micromolar and millimolar dependency on Ca^{2+}, respectively. Both forms are inactive until Ca^{2+}-dependent autolysis in the presence of membranes and phosphatidylinositol generates a fragment that is catalytically active towards membrane as well as cytosolic substrates (Suzuki, 1987). In particular, calpain I cleaves PKC into a Ca^{2+} and phospholipid-independent kinase of 50 kDa and a residual membrane bound regulatory fragment of 30 kDa (Kishimoto et al, 1983). The catalytic fragment was in fact originally isolated as the "active" form of PKC called protein kinase M (PKM) by Inoue et al (1977). Although the biological role of PKM has received little attention, neutrophil calpain I converts PKC to

PKM during stimulation (Melloni et al, 1985). This apparently has functional significance in that prior treatment with either leupeptin or calpain antiserum prevents cleavage of PKC and subsequent degranulation (Melloni et al, 1986; Pontremoli et al, 1988). Moreover, the respiratory burst is prolonged in the presence of intact PKC; this correlates with increased phosphorylation of membrane proteins at the expense of cytoskeletal protein phosphorylation (Pontremoli et al, 1987). Proteolytic regulation of the neutrophil activation response thus suggests a physiological role for PKM. The similar regulation of the two enzymes and their biochemical copurification (Savant et al, 1987) also suggests a physiological role for their interaction. Phorbol esters appear to generate PKM indirectly by continuously maintaining PKC in an activated state which is susceptible to proteolysis. This almost certainly accounts for down-regulation of PKC upon prolonged treatment with phorbol esters (Rodriguez-Pena and Rozengurt, 1984; Chida et al, 1986; Young et al, 1987).

Interactions between Ca^{2+} and PKC can also occur further downstream of the initial signal in that each branch of the PI pathway can have an independent target(s) that converge only in the response generated. In the platelet, Ca^{2+} causes the phosphorylation of myosin light chains via myosin light chain kinase and PKC phosphorylates a 40-47 kDa protein of unknown function (Sano et al, 1983). When subthreshold levels of each agent are employed together, the phosphorylation of each target protein is enhanced to near maximal levels and a biological response (i.e. secretion) ensues (Kaibuchi et al, 1983; Rink et al, 1983). It should be noted that sufficient

stimulation of PKC can evoke a full platelet response in the absence of elevated intracellular Ca^{2+} (Rink et al, 1983). Numerous other instances of synergism in short term responses have been noted (e.g. Vicenti and Villereal, 1985; Park and Rasmussen, 1985; Weissmann et al, 1986) although the mechanistic basis for most of these is less certain than in the platelet.

Ca^{2+} and PKC signalling also interact in long term responses. Synergism between TPA and Ca^{2+} ionophores has been demonstrated for mouse T and B lymphocyte mitogenesis (Truneh et al, 1985; Monroe and Kass, 1985; Clevers et al, 1985). Both agents independently induce c-myc and c-fos transcription in mouse lymphocytes (Moore et al, 1986) and fibroblasts (Kaibuchi et al, 1986). In fibroblasts the effects on transcription are additive whereas synergistic induction of c-fos by TPA and ionomycin occurs in rat thymocytes (Grinstein et al, 1988). Likewise, mezerein and A23187 synergistically induce the γ-IFN and IL-2 mRNAs in human leukocytes (Croll et al, 1987).

In contrast to these observations of cooperativity between Ca^{2+} and PKC, it has also become apparent that PKC often antagonizes the actions of Ca^{2+} (reviewed in Berridge, 1987). For example, the serum induced elevation of Ca^{2+} in 3T3 fibroblasts is abrogated by phorbol esters (McNeil et al, 1985). On a more subtle level, TPA decreases the frequency of α_1-adrenergic-induced Ca^{2+} oscillations in rat hepatocytes (Woods et al, 1987). These effects could occur through negative feedback to phospholipase C (e.g. Kikuchi et al, 1987). Finally, with regard to gene regulation, phorbol esters and Ca^{2+} ionophores can directly antagonize each other; for example, induction of human T cell

receptor τ-subunit mRNA by ionomycin is blocked by TPA (Martinez-Valdez, 1988).

1.4.3 Variations on the Basic PI Theme

Perhaps not unexpectedly for a system which transduces signals for many different stimuli in many cell types, variations on the original scheme of PI turnover have emerged. The original tenet that DAG is derived exclusively from PI hydrolysis no longer holds. Substantial evidence has accumulated that DAG can originate in a receptor-linked fashion from a non-PI source (reviewed in Exton, 1988). Either PC-specific PLC (Besterman et al, 1986; Slivka et al, 1988) or receptor-activated phospholipase D (PLD) in conjunction with phosphatidic acid phosphatase (Cabot et al, 1988; Rosoff et al, 1988) may generate DAG signals in the absence of IP_3. A PI-specific PLD may also generate DAG without concomitant elevation of Ca^{2+} by IP_3 (Balsinde et al, 1988).

Altered regulation of PKC in these instances may also arise due to the different fatty acid components of PC-derived DAG compared to that originating from PI (Mori et al, 1982). Modulation of PKC can be achieved with a variety of lipid cofactors in addition to DAG. Arachidonic acid, liberated either by DAG metabolism or via Ca^{2+} or receptor mediated activation of phospholipase A_2, activates PKC in vitro (Sekiguchi et al, 1988). Other long chain free fatty acids can also activate PKC to various degrees (Sekiguchi et al, 1987).

In contrast, sphingoid lipids and other long chain bases inhibit PKC activation by DAG in a roughly equimolar ratio (reviewed in Wilson

et al, 1988). Inhibition of PKC by receptor linked hydrolysis of sphingolipids may occur in rat pituitary cells (Kolesnick and Clegg, 1988). It has been sugested that the sphingolipidosis syndromes may result from inhibition of PKC by sphigolipids or gangliosides (Hannun and Bell, 1987; Kreutter et al, 1987).

Finally with respect to second messenger generation, two radical variations on the PI scheme have recently emerged. It has been reported that activation of PKC in vitro is 50 fold more efficient with PIP_2 than with DAG (Chauhan and Brockeroff, 1988). If true, this throws doubt on the role of DAG and requires complete re-evaluation of the PI response. A second, less disruptive, finding has been the discovery of PIP_3 generated as part of the early neutrophil response (Traynor-Kaplan et al, 1988). These points underscore a cautionary note that mere association of PI turnover with a specific biological response does not imply causality. The response of T cell lymphomas to IL-2 is a case in point (Sussman et al, 1988; Mills et al, 1988).

Further diversity in transmembrane signalling is generated by the existence of both multiple PI-specific phospholipase C (e.g. Suh et al, 1988) and PKC subspecies (reviewed in Ono and Kikkawa, 1987; Nishizuka, 1988). Currently, 7 distinct PKC subtypes have been discovered. The three major subspecies are α, $\beta 1/\beta 2$ (generated by alternate splicing of the same gene, Ono et al, 1987) and τ (Coussens et al, 1986a; Knopf et al, 1986). These correspond to the type III, II and I forms separable from brain extract by hydroxylapatite chromatography (Yoshida et al, 1988). The different PKCs have a non-identical tissue distribution which may help account for tissue specificity in response to

a given stimulus (e.g. Brandt et al, 1987; reviewed in Nishizuka, 1988). Preliminary studies in vitro suggest that the Ca^{2+} and lipid requirements are somewhat different for each PKC (Sekiguchi et al, 1987; Huang et al, 1988). In addition, the β1/β2 subspecies is more rapidly activated and downregulated by TPA than the α form (Ase et al, 1988).

The presence of more than one PKC subspecies within a given cell type may also explain the discrete concentration requirements of different cellular responses. Adherence of U937 cells to substratum occurs at a 10-fold lower concentration of TPA than membrane translocation and stimulation of choline incorporation (Skoglund et al, 1988). A similar discrepancy exists between adherence and increased intracellular pH in both U937 and HL-60 cells (Ladoux et al, 1988). The more recently discovered delta, epsilon, and zeta PKC subtypes are less similar to the original PKC sequences but still exhibit dependence on Ca^{2+} and phospholipid, again with slight variation amongst different forms (Ono et al, 1988). A related kinase, nPKC, discovered through its homology to conserved regions in other PKCs, is unusual in that it exhibits no dependence on Ca^{2+} (Malviya et al, 1986; Ohno et al, 1988). In contrast, Ca^{2+} alone is sufficient to activate α-PKC in some cell types (Ho et al, 1988). Thus the Ca^{2+} and DAG-based pathways may be separable even at the level of PKC itself. Despite indications of subtle functional differences, the possibility that many PKC subspecies arose through gene duplication and neutral drift should not be discounted. Ultimately, it may be quite difficult to prove a physiological role for each apparent characteristic observed in vitro.

The primary structure of PKC has revealed features which may

underlie behaviour of the kinase under certain conditions. Based on comparisons between the α, β and τ-subspecies, the amino acid sequence can be divided into 3 constant regions and 5 variable regions (reviewed in Nishizuka, 1988). The $V_{1-2}C_{1-2}$ N terminal portion corresponds to the hydrophobic regulatory domain; this is joined to the remaining catalytic domain by the V_3 hinge region. CANP apparently cleaves at this point to liberate the unregulated catalytic fragment (Nishizuka, 1988). Other conserved regions of PKC include a weak putative EF-hand domain and a Zn finger motif characteristic of DNA binding proteins (Parker et al, 1986; reviewed in Nishizuka, 1988). The former could account for a second low affinity Ca^{2+}-binding site detected in the kinase (Murakami et al, 1987). Putative Zn finger domains in the regulatory domain may be responsible for dependence on low concentrations of Zn^{2+} or possibly inhibition at higher Zn^{2+} concentrations (Murakami et al, 1987; Csermely et al, 1988). It has also been suggested that this region may allow association of PKC with the nucleus under some conditions or even bind to DNA (Testori et al, 1988; see Section 1.5.1). Although not identical, the more recently discovered PKC subspecies exhibit a similar overall structure to α, β1/β2 and τ-PKC (Ono et al, 1988).

1.4.4 Gene Regulation by PKC.

Whether associated with cell proliferation or differentiation, phorbol esters dramatically alter gene expression in most cell types (reviewed in Nishizuka, 1986). At least some of the many responses to serum may actually be due to stimulation of PKC by serum lipoproteins (Ways et al, 1986). More directly, overexpression of PKC in fibroblasts

is sufficient to confer a partially transformed phenotype (Housey et al, 1988; Persons et al, 1988). Many individual genes which play a role in differentiation and development respond to phorbol esters; a number of these have been cloned and sequenced (reviewed in Johnson et al, 1987; Tippets et al, 1988).

At least one mechanism of phorbol ester-mediated transcriptional control has recently been clarified by the resolution of several phorbol ester responsive promoter elements and the factors which bind them. This was described in detail as relevant to the induction and function of c-fos (see Section 1.3.1). It is interesting that TPA induces transcription of c-jun as well as c-fos (Lamph et al, 1988). Thus, PKC appears not only to modify existing transcription factors, but also to induce the nuclear mediators of its own actions. Activation of c-jun transcription by Jun/AP-1 has recently been demonstrated (Angel et al, 1988). In addition to the well-characterized Jun/AP-1 components, at least four other TPA responsive motifs and their respective binding factors have been identified (reviewed in Jones et al, 1988). The SV40 enhancer contains 3 more TPA responsive sequences which are distinct from the AP-1 site; the binding of AP-2 and AP-3 to the C element has been demonstrated by DNAse I protection (Chui et al, 1987). Like AP-1, some of these activating elements are sufficient to confer TPA inducibility to heterologous promoters in a cell type specific manner (Chui et al, 1987; Imagawa et al, 1987). Yet another unique element and its lymphoid derived factor, NF-KB, confer TPA inducibility in non-lymphoid cells (Nelsen et al, 1988; Wirth and Baltimore, 1988). All these elements are distinct from the SRE (also known as dyad symmetry

element or DSE) and its associated binding protein (SRF) that mediate both induction and repression of c-fos transcription by either serum or phorbol ester (Sassone-Corsi et al, 1988b). As illustrated by c-fos (Schonthal et al, 1988) and aP2 (Distel et al, 1987) promoters, these various phorbol ester responsive elements can confer positive or negative regulation. Although the known TPA responsive elements account for part of the transcriptional response to serum, it is clear that other less well characterized elements must also contribute to serum inducibility (Sassone-Corsi et al, 1988b).

A plethora of mechanisms for transcription factor activation have been uncovered. SRF binding activity is dependent on the phosphorylation of serine and threonine residues, which occurs only in serum treated cells (Prywes et al, 1988). In like manner, the DNA binding ability of AP-1 depends on phosphorylation. AP-1 purified from TPA-stimulated cells binds the AP-1 site more tightly (Angel et al, 1987) and activates transcription in vitro (Lee et al, 1987); this may actually occur via post-transcriptional modification of Fos, however (Angel et al, 1988). AP-2 behaves in a similar manner (Imagawa et al, 1987). Other transcription factors, such as the heat shock factor, appear to be constitutively phosphorylated and bind DNA equally well in stimulated and unstimulated cells (Larson et al, 1988). Phosphorylation of transcription factors may also confer stability, as in the case of c-ets phosphorylation by PKC (Fujiwara et al, 1988). Yet another variation is the cytosolic interaction of NF-KB with a specific inhibitor, IKB. TPA induced phosphorylation of IKB dissociates the complex and allows NF-KB to associate with the nucleus where it

activates transcription (Baeuerle and Baltimore, 1988). Specific transcriptional control arises not only from various combinations of different elements (e.g. Comb et al, 1986) and modification of their respective binding factors, but also from cross-talk between different elements via multiple interactions with various binding factors (reviewed in Robertson, 1988). For example, AP-2 directs both TPA and cAMP signalling to the same element (Imagawa et al, 1987). In some circumstances AP-1 and a cAMP responsive-factor bind identical consensus sites but in a context dependent fashion (Deutsch et al, 1988). Likewise, the SRE targets activation by PKC dependent and independent pathways (Gilman, 1988). These responses may reflect entire families of closely related binding proteins with only small differences in sequence specificity (e.g. Hai et al, 1988). Several Jun-related transcription factors have recently been cloned and characterized (Nakabeppu et al, 1988). Regulatory loops based on homologous and heterologous transcription factor interactions will also undoubtedly contribute to the complexity of nuclear signal transduction; dimerization of AP-1 with itself and with Fos has recently been demonstrated (Halazonetis et al, 1988). Obviously, the varied elements of the PI-based signal transduction system itself could also contribute heavily to the specificity of PKC mediated transcription.

Post-translational modification of transcription factors is not necessarily the only mechanism whereby PKC transduces information to the nucleus. Translocation of PKC to the nuclear compartment occurs in B cells stimulated with Ia binding ligands or dbcAMP and may thus be part of the cascade which stimulates B cell differentiation (Cambier et al,

1987). This is discussed more thoroughly with respect to HL-60 differentiation in Section 1.5.1. Other pleiotropic effects of phorbol esters such as phospholipid remodeling (e.g. Hoffman and Huberman, 1982) and increased ADP-ribosylation of chromatin (reviewed in Singh et al, 1985) could also influence gene expression.

1.5 PI-BASED SIGNAL TRANSDUCTION AND HL-60 CELL DIFFERENTIATION

Signal transduction may be viewed from two perspectives in a differentiating cell system. The flow of signals that culminates in altered gene expression is the crucial part of differentiation process itself. In the case of HL-60 cell differentiation, phorbol esters clearly act by impinging on the PI signalling pathway. This will be considered in detail. A second aspect, not always distinct from the first, is the acquisition of new transduction routes as a part of the differentiated phenotype. HL-60 cells have been used as a model system to investigate the coupling of chemotactic peptide receptor occupancy to PI turnover and the ultimate biological response. Since this is possibly relevant to the role of P47 (see Section 1.6) it will also be discussed, but not in great detail.

1.5.1 PKC and Induction of HL-60 Cell Differentiation

The basis for the disparate effects of phorbol esters in different cell types is not clear. Although these compounds often initiate cell proliferation (Nishizuka, 1984), many cell types are induced to differentiate instead (reviewed in Gescher, 1985; Abrahm and

Rovera, 1980). It is interesting that whereas TPA is a stimulus for proliferation in early G1, it inhibits mitogenesis if applied to cells in late G1 or S phase (Huang and Ives, 1987; Takada et al, 1988). Importantly for the HL-60 model system, TPA causes monocytic differentiation of normal myeloid precursors (Abrahm and Smiley, 1981; Griffin et al, 1985).

Prior to identification of PKC as the phorbol ester receptor, it was established that these compounds exert their effects on HL-60 cells at the plasma membrane and remain there throughout the differentiation time course (Cooper et al, 1982). One of the very early responses to TPA is phospholipid remodeling in which PE is converted to PC via a methyltransferase reaction (Hoffman and Huberman, 1982). Despite this, the bulk membrane fluidity of HL-60 cells is not altered by TPA (Ip and Cooper, 1980). It was also noted that, as for other cell types, specific phorbol ester binding was lost if HL-60 cells were incubated with TPA for prolonged periods (Solanki et al, 1981). The efficacy of a series of phorbol ester analogues to cause monocytic differentiation paralleled their effects in other biological systems as well as activation of PKC in vitro (Vandenbark et al, 1984). A variety of synthetic DAGs also evoke a monocytic HL-60 phenotype (Ebling et al, 1985; McNamara et al, 1984). Further, inhibitors of PKC such as palmitoyl carnitine (Nakaki et al, 1984), H7 (Matsui et al, 1986) and various long chain bases (Merrill et al, 1986) block the effects of TPA on HL-60 cells. Much work has been carried out on protein phosphorylation in HL-60 cells treated with phorbol esters. TPA evokes rapid phosphorylation of a least 14 proteins as detected by 2-D PAGE

(Kreutter et al, 1985; Homma et al, 1988). Proteins of known identity include the transferrin receptor (May et al, 1985), the Na^+/H^+ exchanger (Besterman et al, 1985) and vinculin (Aquino et al, 1988). Heavy phosphorylation of 2 low M_r species correlates strongly with differentiation (Feuerstein et al, 1984). A causal role in differentiation has not been demonstrated for any of these events.

Properties of differentiation-resistant HL-60 cell sublines confirm the above results. Decreased fluidity of the cytosolic leaflet of the plasma membrane in one TPA-resistant HL-60 subline presumably affects the ability of TPA to interact with PKC (Fisher et al, 1984). A reduction in the number of phorbol ester receptors in another subline correlates with differentiation resistance (Perrella et al, 1986). Decreased protein phosphorylation in response to TPA treatment also correlates with some resistant phenotypes (Anderson et al, 1985; Homma et al, 1986). In one instance, defective PKC translocation was paralleled by a greater dependence on Ca^{2+} and phospholipid for phosphorylation in vitro (Homma et al, 1988). However, other defects can also apparently confer resistance to TPA. Various chromosomal abnormalities including a reduction in double minute chromosome number have been correlated with several differentiation resistant HL-60 sublines (Au et al, 1983). Another subline (HL-60-1E3) is only slightly altered in phorbol ester binding and exhibits some of the morphological changes indicative of macrophage-like differentiation (Leftwich et al, 1987). These cells are apparently unable to commit to differentiation as they resume growth upon removal of phorbol ester (Ely et al, 1987). Obviously this defect is less trivial than mere interruption of PKC

signalling. Despite this, most TPA resistant HL-60 sublines clearly indicate a role for PKC in differentiation to macrophage-like cells.

Despite the wealth of data implicating PKC in TPA induced HL-60 differentiation, two PKC activators which elicit similar patterns of protein phosphorylation as TPA do not cause HL-60 maturation or cell cycle exit. 1-Oleoyl-2-acetylglycerol (OAG) causes phosphorylation of 8 of the 14 proteins identified by TPA treatment but without concomitant differentiation (Kreutter et al, 1985; Yamamoto et al, 1985; Morin et al, 1987). Although suggested to disprove the role of PKC in HL-60 differentiation, OAG is rapidly metabolized and thus may not constitute a strong enough differentiation signal (Kreutter et al, 1985). TPA and OAG also have disparate biochemical effects on HL-60 cells in that only the phorbol ester causes a rapid decrease in endogenous DAG and IP_3 levels (Geny et al, 1988). In contrast to the weak effects of OAG, the bryostatin family of macrocyclic lactones not only have little or no differentiation-inducing activity themselves, but actually block HL-60 maturation caused by TPA (Kraft et al, 1986). Bryostatin 1 activates PKC in HL-60 cells and platelets as judged by initial membrane translocation and protein phosphorylation (Kraft et al, 1987a; Warren et al, 1988; Tallant et al, 1988), but importantly, does not repress c-myc mRNA levels in HL-60 cells (Kraft et al, 1987).

Bryostatins and phorbol esters also have disparate effects in other biological systems. Bryostatin is an ineffective tumour promoter in the mouse skin system (Blumberg, 1988) and only partially mimics the effects of phorbol esters on cultured mouse fibroblasts (Dell'Aquila et al, 1988) and epidermal cells (Sako et al, 1987; Kraft et al, 1988).

Moreover, bryostatin 1 acts as non-competitive inhibitor of the effects of phorbol esters on MEL cell differentiation (Dell'Aquila et al, 1987). These differences may be attributable to the nature of the bryostatin:PKC complex. Phorbol esters bind tightly but reversibly to PKC whereas bryostatin forms an essentially irreversible complex with PKC with a K_d in the pM concentration range (Warren et al, 1988); this suggests it represents a further refinement of the phorbol ester pharmacore (Wender et al, 1988). Tight binding of PKC to bryostatin could cause partitioning of the kinase to incorrect membrane compartments in the cell, making it inaccessible to the substrates necessary for biological response. In HL-60 cells, bryostatin stimulates rapid phosphorylation of the nuclear protein lamin B (Fields et al, 1988) and several 70 kDa proteins which are not phosphorylated in response to PDBu, except at very high concentrations. The latter treatment still causes differentiation, however (Warren et al, 1983). Additionally, TPA causes extensive phospholipid remodeling of the plasma membrane whereas bryostatin and OAG do not (Tetterhorn and Mueller, 1987; Kiss et al, 1987). These observations are compelling in light of the fact that phorbol esters cause translocation of PKC to the plasma membrane of HL-60 cells, as compared to the nuclear translocation which occurs in cell types that do not differentiate (Girard et al, 1987; also see below). In some instances cotreatment of cells with bryostatin and a Ca^{2+} ionophore restores the expected response to PKC activation (Dell'Aquila et al, 1988). Elevated Ca^{2+} causes a transient association of PKC with the plasma membrane (Ito et al, 1988); this may allow bryostatin to activate the kinase in the appropriate context for biological activity.

Finally, different PKC subspecies may be more or less susceptible to each agent. Bryostatin binds less strongly to mouse epidermal cell β-PKC than phorbol esters (Kraft et al, 1988). This may account for the high rate of PKC degradation observed in HL-60 cells treated with bryostatin (Kraft et al, 1987). It is noteworthy that HL-60 cells contain type II (β1/β2) and type III (α) PKC (Makowske et al, 1988) and that normal leukocytes have predominantly type II PKC (Yoshida et al, 1988).

The distribution of PKC immunoreactivity in HL-60 cells has been studied before and after treatment with TPA. Initially, the enzyme redistributes from the cytosol of unstimulated cells to the plasma membrane (Girard et al, 1987). By 24 h, however, much of the immunoreactivity is localized to the nuclear membrane where it remains for several days thereafter (Kiss et al, 1988). It appears that this is not accompanied by phorbol ester repartitioning (Cooper et al, 1982). In contrast, bryostatin evokes only a transient association with the plasma membrane followed by rapid nuclear relocation (Kraft et al, 1987a, 1987b). As for other biological responses (Dell-Aquila et al, 1988), this implies that initial localization of PKC must be to the plasma membrane for HL-60 differentiation to occur. However, a potential role for nuclear PKC in monocytic HL-60 differentiation is also suggested by the effect of cAMP on the TPA response. Low concentrations of TPA which have only marginal effects alone are able to cooperate with dbcAMP to induce differentiation typical of higher TPA concentrations (Deli et al, 1988). This is accompanied by eventual association of PKC with the nuclear membrane that is not observed with

low concentrations of phorbol ester alone (Deli et al, 1988).

Although the physiological significance of nuclear PKC is unclear, PKC phosphorylates and activates topoisomerase II in vitro while novobiocin (a topoisomerase II inhibitor) partially inhibits phorbol ester-induced HL-60 differentiation (Sahyoun et al, 1986). Limited evidence also suggests that PKC may regulate nucleo-cytoplasmic transport of mRNA by phosphorylation of the nuclear membrane NTPase-transporter (Schroder et al, 1988). Regulation of at least one mRNA during HL-60 differentiation occurs at this level (Graham and Birnie, 1988). Several other nuclear substrates for PKC have been noted (Warren et al, 1988). Increased poly(ADP)ribosylation of nuclear proteins in response to TPA (Singh et al, 1985) could also be mediated by PKC in the nucleus. All these events may help initiate the altered pattern of gene expression that culminates in the differentiated phenotype.

The proper physiological counterpart to prolonged PKC activation in myeloid differentiation is unclear. In HL-60 cells, TPA causes a 50% decrease in endogenous IP_3 and DAG levels (Geny et al, 1988); this could contribute to differentiation as a similar reduction occurs early in HMBA-induced MEL differentiation (Faletto et al, 1985). Moreover, phorbol esters directly suppress DAG formation in vitro (Chabbott and Cabot, 1986). These effects could be an important component of the extensive phospholipid remodeling in the plasma membrane induced by TPA (Hoffman and Huberman, 1982). Although PKC stimulation is often accompanied by increases in intracellular Ca^{2+}, many examples of independent DAG signalling have been discovered (reviewed in Exton, 1988). With respect to myeloid differentiation, both G-CSF and GM-CSF

apparently stimulate the production of DAG in the absence of elevated Ca^{2+} (Dexter and Spooncer, 1987), as does IL-3 (Whetton et al, 1988). Another physiological inducer of monocytic HL-60 differentiation, γ-IFN, also mediates transcriptional changes solely through activation of PKC in both HL-60 cells and other cell types (Fan et al, 1988). Finally, agonist-induced production of DAG from PC has been demonstrated in a variety of cell types, including HL-60 cells (Besterman et al, 1986). TPA may thus directly mimic an endogenous signalling pathway in hemopoietic differentiation. In this regard, it is noteworthy that phorbol esters rapidly induce maturation of normal human myeloblasts to macrophage-like cells (Griffin et al, 1985).

1.5.2 Ca^{2+} and HL-60 Cell Differentiation

The role of the Ca^{2+} branch of PI-based signalling in HL-60 differentiation is not clear. Ca^{2+} is probably important for the differentiation of some cell types (reviewed in Veigl et al, 1984); for instance, Ca^{2+} uptake is an early but possibly not essential event in MEL differentiation (Levenson et al, 1982; Faletto and Macara, 1985). Since 1,25-$(OH)_2D_3$ is a physiological regulator of Ca^{2+} metabolism, the effect of this inducer on Ca^{2+} in HL-60 cells has been well studied. Elevation of $[Ca^{2+}]_i$ occurs 6-12 h after exposure to 1,25-$(OH)_2D_3$, but this appears related to a build up of intracellular Ca^{2+} stores in anticipation of differentiated cell function (Hruska et al, 1988). Thus, monocytic HL-60 differentiation induced by 1,25-$(OH)_2D_3$ is not inhibited by CM antagonists (Matsui et al, 1985) and is only marginally supressed if at all by removal of extracellular Ca^{2+} (Levy et al, 1988;

Hruska et al, 1988). Further, CM antagonists are purported to increase differentiation in respose to RA, DMSO and dbcAMP (Veigl et al, 1986) while other Ca^{2+} antagonists and Ca^{2+}-deficient medium apparently enhance the effects of 1,25-$(OH)_2D_3$, RA and DMSO (Okazaki et al, 1986). The CM content of HL-60 cells also decreases as cells are exposed to 1,25-$(OH)_2D_3$ (Brevli et al, 1986). Counter to these observations, in one isolated report Ca^{2+} ionophore A23187 was found to enhance RA-induced HL-60 differentiation (Chapekar et al, 1987). Given the pleiotropic role of Ca^{2+} in cell regulation it is obviously difficult to ascribe a specific effect in differentiation to this second messenger.

1.5.3 PI Responses in Differentiated HL-60 Cells

Much work in signal transduction has been carried out with isolated platelets and neutrophils. In both cell types there are clearly multiple interactions between various signalling networks, particularly those controlled by PLA_2 and PLC. This is manifest in the complex neutrophil response which includes chemotaxis, release of granule contents and generation of a bacteriocidal superoxide anion burst as well as the generation of other secondary inflammatory mediators (reviewed in Lackie, 1988). Since HL-60 cells differentiated with agents such as DMSO provide reproducibly homogeneous cell populations which closely mirror their normal counterparts, they have been extensively used to investigate agonist-induced PI turnover and subsequent events.

Receptor coupling to both PLA_2 and PLC occurs via G proteins (reviewed in Burgoyne et al, 1987). The PI-specific PLC that responds

to fMLP in HL-60 membranes (Kikuchi et al, 1986a; Athnes et al, 1987) and intact cells (Brandt et al, 1985) is activated by non-hydrolyzable GTP analogues and inhibited by pertussis toxin. Stimulation of neutrophils or DMSO differentiated HL-60 cells with fMLP causes an immediate decrease in PIP_2 and a concomitant increase in IP_3 (Dougherty et al, 1984; Burgess et al, 1985). This coincides with or slightly precedes Ca^{2+} release from intracellular stores (Burgess et al, 1984), recently localized to an endoplasmic reticulum-associated organelle called the "calciosome" in HL-60 cells (Volpe et al, 1988). The 1,4,5-IP_3 response peaks within 5 s and then rapidly declines, whereas 1,3,4-IP_3 slowly rises throughout the period of stimulation (Burgess et al, 1985). Production of the former occurs at basal Ca^{2+} levels whereas the latter is not generated unless Ca^{2+} is elevated (Lew et al, 1986). In human neutrophils, the initial Ca^{2+} response due to the action of IP_3 is followed by a prolonged elevation that depends on extracellular Ca^{2+}, possibly through the action of a Ca^{2+} channel (Truett et al, 1988), although an ionophoretic effect of phosphatidic acid cannot be ruled out (Korchak et al, 1988). Activation of the Ca^{2+} influx is ligand specific in that LTB_4 stimulates PI turnover yet causes only a transient Ca^{2+} peak; cytochalasin B, on the other hand, causes a delayed increase in Ca^{2+} without the IP_3-mediated initial peak (Truett et al, 1988). Likewise, concanavalin A evokes only the delayed influx of extracellular Ca^{2+} (Korchak et al, 1988b).

The kinetics of DAG accumulation also follow a complex pattern. In DMSO-differentiated HL-60 cells, fMLP causes DAG levels to peak after 4 min (Priess et al, 1987). A more precise analysis in human

neutrophils suggests that, as for Ca^{2+}, a response occurs with an early maximum at 5-30 s followed by a sustained rise for several minutes (Truett et al, 1988; Reibman et al, 1988). Differential labeling of lipid pools indicates that the initial release of DAG is from PI whereas sustained mobilization occurs from a non-PI source, possibly PC (Reibman et al, 1988). The former is independent of elevated Ca^{2+} while the latter depends absolutely on high levels of Ca^{2+} (Reibman et al, 1988). To complicate matters further, the fMLP receptor is apparently also coupled to a PC-specific PLD in DMSO-differentiated HL-60 cells; this activity completely accounts for the early formation of phosphatidic acid and PE in stimulated cells (Pai et al, 1988). The contribution of these events to DAG formation is unclear. The simultaneous activation of several different phospholipases obviously leads to the complex regulation of second messengers in the neutrophil response.

The order of cellular events which follow stimulation correlate with some of the observed alterations in second messenger abundance (Truett et al, 1988). Agonists such as LTB_4, which weakly activate PI turnover, induce chemotaxis and granule release but not the superoxide burst. Stronger stimulation of cells with fMLP causes a sustained elevation of Ca^{2+} and DAG, thereby activating the NADPH oxidase system. The temporal order of cellular responses coincides with the temporal order of second messenger release, that is migration and granule secretion are early events associated with PI turnover whereas the oxidative burst metabolism is stimulated by the late phase of DAG production (Reibman et al, 1988). There is also a concordance between fMLP concentration and the heirarchy of neutrophil activation; low

concentrations (10^{-8} M) cause chemotaxis, whereas a 10-fold higher concentration evokes bacteriocidal responses (Omann and Sklar, 1988).

Despite confusion in much of the earlier literature (summarized in Tyagi et al, 1988), it is now clear that PKC activation by DAG is largely responsible for activation of NADPH oxidase. TPA has long been known to cause prolonged activation of the respiratory burst (DeChatelet et al, 1976), in spite of its ability to inhibit PLC activity and fMLP-induced PI turnover (Kikuchi et al, 1986b; 1987). Evidence which suggests a central role for PKC in activating the NADPH oxidase system includes "priming" of the neutrophil response to fMLP by low concentrations of TPA (McPhail et al, 1984). Compared to fMLP alone, TPA priming increases DAG levels and concomitant O_2^- production by a factor of 2 and reduces fMLP-induced Ca^{2+} transients by 50% (Tyagi et al, 1988). Blunting of the Ca^{2+} response is due to inhibition of PLC via PKC whereas the increased DAG release under this condition indicates a non-PI lipid source (Tyagi et al, 1988). Accordingly, sphingosine inhibits O_2^- generation without altering the Ca^{2+} response (Tyagi et al, 1988). In contrast, partial activation of the respiratory burst by agents which increase intracellular Ca^{2+} probably arises from mobilization of DAG from a non-PI source, much as in the latter phase of the physiological response (Rider and Niedel et al, 1987). In DMSO-differentiated HL-60 cells, A23187 increases DAG levels by 4-5 fold for at least 30 min (Preiss et al, 1987). Conversely, suppression of Ca^{2+} transients by intracellular buffering also severely depresses DAG formation (Korchak, 1988a). As indicated above, although mobilization of DAG correlates strongly with the respiratory burst, there may be an

additional requirement for phosphatidic acid in this process (Korchak et al, 1988a).

Molecular analysis of the chronic granulomatous disease (CGD) state has corroborated the role of PKC in the oxidative burst. Neutrophils from CGD patients are unable to generate O_2^- upon agonist stimulation (reviewed in Curnutte and Babior, 1987). Defective phosphorylation of several M_r 44-48,000 proteins by PKC correlates with the non-functional NADPH oxidase system (Okamura et al, 1988; see below for details). In normal cells, prolonged phosphorylation of a M_r 47,000 protein is associated with a strong respiratory burst (Reibman et al, 1988). Coincidentally, a similar M_r protein was investigated in HL-60 cells as part of this work. The background for this is covered in the next section.

1.6 PROPERTIES AND PUTATIVE FUNCTIONS OF THE MAJOR PROTEIN KINASE C SUBSTRATE OF PLATELETS (P47)

The platelet is one of the most extensively studied cell types with regard to short term mechanisms of signal transduction (reviewed in Majerus, 1987). In response to agonists such as thrombin, platelets undergo rapid shape change, aggregate, secrete granule contents and release arachidonic acid metabolites. Elucidation of the basic PI scheme of signal transduction was carried out in this cell (reviewed in Nishizuka, 1984). Despite this, the mechanism of PKC action in platelet stimulus-response coupling remains uncertain. Platelets can be fully stimulated by either TPA or OAG in the absence of increased Ca^{2+},

confirming the central role of PKC in this process (Rink et al, 1983). Further, inhibition of PKC by sphingosine abolishes all but the most preliminary platelet responses to agonist (Hannun et al, 1987). Activation of the Na^+/H^+ exchanger by PKC may in fact be a co-requisite for Ca^{2+} release from intracellular stores, implying that PKC may lie at the apex of platelet signal transduction (Siffert and Akkerman, 1987). Other known targets of PKC in the platelet include vinculin (Werth et al, 1983), myosin light chains (Naka et al, 1983) and possibly talin (Litchfield and Ball, 1986). However, quantitatively the most important PKC substrate in platelets is an apparent M_r 40-47,000 protein (Lyons et al, 1975; Haslam and Lynham, 1977), referred to here as P47 (Haslam et al 1979) but also known as the "40K" protein (Sano et al, 1983). P47 phosphorylation occurs within 5 s of thrombin exposure (Lyons et al, 1975) and closely parallels the earliest cellular response to platelet activating factor, that is, a shape change (Kapetina and Siegel, 1983). Weak agonists such as ADP can induce aggregation without P47 phosphorylation, whereas degranulation is always preceded by P47 phosphorylation (Haslam and Lynham, 1977). P47 accounts for about 1% of platelet protein and actually consists of up to 11 variously phosphorylated isoforms distinguishible by 2D-PAGE (Imaoka et al, 1983). Three of these may arise from primary structure variation since they appear as unphosphorylated forms in unstimulated platelets; in contrast P47 isolated from thrombin treated platelets is found almost exclusively as phosphorylated species (Imaoka et al, 1983). Beyond the detailed biophysical characterization reported by Imaoka et al (1983) little is known of P47 structure or function.

At least 5 candidate functions, all based on rather tenous evidence, have been postulated for P47. In one way or another, each concerns some immediate aspect of signal transduction or is part of the early response to stimulation. Where possible, putative P47 functions have been indicated on Fig. 1.4.1. The suggested functions of P47 will be outlined in order of historical occurance.

i) A member of the lipocortin family

The lipocortins inhibit several types of PLA_2 in vitro and may mediate the anti-inflammatory effects of glucocorticoids (reviewed in Hollenberg et al, 1988; Pepinsky et al, 1988; Crompton et al, 1988). This class of at least 6 structurally related proteins has also been given the generic name annexins (Geisow et al, 1987). In addition to PLA_2 inhibition, the functional diversity of the annexins extends to anti-coagulant activity, substrates for various kinases (including the epidermal growth factor receptor, $pp60^{src}$ and PKC (Kaplan et al, 1988)), association with secretory vesicles and Ca^{2+}-dependent binding to membranes and actin filaments (reviewed in Giesow et al, 1987). Limited evidence suggests that calpactin I may induce fusion of secretory vesicles at physiological Ca^{2+} concentrations (Drust and Creutz, 1988). Marked conservation of a 70 amino acid tetrameric repeat amongst the annexins correlates with their ability to bind 4 Ca^{2+} ions in the presence of phospholipid (Crompton et al, 1988). This repeated motif may also contain the site of interaction with actin (Kaplan et al, 1988).

Given the similar size of P47 and the lipocortins and that TPA represses the PLA_2 inhibitory activity of a similar protein in

neutrophils, it was postulated that the proteins may be related (Touqui et al, 1986). This notion was substantiated by partial immuno-crossreactivity with renocortin (lipocortin V) and inhibition of PLA_2 by partially purified P47 (Touqui et al, 1986). In addition, phosphorylated P47 from thrombin treated platelets apparently had less inhibitory activity. There are, however, several weak points in these arguments. Non-specific protein-lipid interaction (e.g. by BSA) also causes apparent inhibition of PLA_2 so that the specificity of inhibition by 40 kDa containing platelet fractions is questionable. In fact, the mechanism of lipocortin action may involve perturbation or sequestration of substrate lipid rather than direct inhibition of PLA_2 (Haigler et al, 1987). Abrogation of platelet 40 kDa anti-PLA_2 activity by the renocortin antibody is incomplete and not validated by pre-immune serum controls. Moreover, only a very small fraction of the phosphorylated 40 kDa species is precipitated by renocortin antiserum (Touqui et al, 1986), again suggestive of a non-specific effect. Finally, this role has been tested indirectly by stimulation of platelets with α and τ thrombin; despite equivalent phosphorylation of P47 by each stimulus α thrombin was able to cause arachidonic acid release (Crouch and Lapetina, 1986). Thus, it seems unlikely that P47 is a member of the lipocortin family.

ii) An inositol-1,4,5-trisphosphate 5-phosphomonoesterase (IP_3ase)

Attenuation of Ca^{2+} release from intracellular stores occurs in part by hydrolysis of 1,4,5-IP_3 to 1,4-IP_2 (see Section 1.4.1). In the platelet and in vitro, PKC appears to activate the IP_3ase by increasing its V_{max}, thereby forming an elegant negative feedback loop on PI-based

Ca^{2+} release (Molina et al, 1986; Connolly et al, 1986). The platelet IP_3ase is a cytosolic enzyme which apparently copurifies with P47 (Connolly et al, 1985; 1986). However, published IP_3ase preparations are not homogeneous as several other bands are visible in gels stained for total protein (Connolly et al, 1986). The low specific activity of the platelet IP_3ase is also suggestive of incomplete purification (compare to Hansen et al, 1987). Given the high abundance of P47 in platelets, it is unlikely on teleological grounds that it is an enzyme which catabolizes minute quantities of a transient messenger molecule. Rather, these results are more reasonably explained by fortuitous copurification of P47 with the true IP_3ase.

iii) A regulator of actin polymerization

Activation of platelets and white blood cells entails rapid reorganization of the cytoskeleton, including a marked increase in polymerized actin. It is estimated that resting platelets contain 50% F actin and that this increases upto 75% upon thrombin stimulation (Jennings et al, 1981; Majerus, 1987). Partially purified P47 from unstimulated platelets supposedly inhibits actin polymerization in vitro at a molar ratio of 1:200, whereas P47 isolated from thrombin-treated platelets apparently does not block the G to F actin transition (Hashimoto et al, 1987). These results obviously coincide with observed changes in actin polymerization upon agonist stimulation of platelets. Many actin regulatory proteins have been characterized but of those identified in the platelet none are in the 40-47,000 M_r range (reviewed in Pollard, 1986).

iv) The α-subunit of pyruvate dehydrogenase (α-PDH)

Very weak evidence based on partial immunoreactivity and similar M_r has been used to suggest that P47 is identical to α-PDH (Chiang et al, 1987). To account for the cytosolic location of P47 it was postulated that α-PDH, a mitochondrial matrix enzyme, is released to the cytosol during cell preparation. Although rapid glucose metabolism occurs upon platelet stimulation (Majerus, 1987), it was reported Chiang et al (1987) that phosphorylation of α-PDH decreases its activity, an unlikely juxtaposition of events. How P47 could be phosphorylated by PKC inside the mitochondria was not addressed.

v) A soluble component of the NADPH oxidase system

Phagocytic cells, mainly neutrophils and eosinophils, contain a specialized system of membrane associated electron carriers which catalyze the one-electron reduction of O_2 by NADPH:

$$\text{NADPH} + O_2 \rightarrow \text{NADP} + O_2^-$$

This reaction and the cellular events surrouding it have been reviewed in detail by Babior (1984) and Rossi (1986). Although not entirely clear, it appears that the NADPH oxidase is a 45-66 kDa protein which transfers electrons via one or all of FAD, ubiquinone-50 and an iron-sulfur centre to a penultimate cytochrome (b_{558}) and finally to O_2 (reviewed in Babior, 1987; Prince and Gunson, 1987). Activation of this chain may occur through the phosphorylation of a group of M_r 44-48,000 proteins (possibly the oxidase itself) by PKC (Okamura et al, 1988). These proteins may comprise a "cytosolic factor" required for activation of the purified oxidase in vitro (Curnutte et al, 1987).

The CGD state in humans is characterized by the inability of phagocytes to generate a respiratory burst (reviewed in Curnutte and

Babior, 1987) and apparently arises from absence of the cytochrome b_{558} heavy chain and/or defective phosphorylation of the 44-48 kDa protein(s) (Okamura et al, 1988; Royera-Parker et al, 1986). The former has been molecularly cloned from HL-60 cells and shown to be absent in the X-linked form of CGD (Royera-Parker et al, 1986; Dinauer et al, 1987). In contrast, the 44-48 kDa proteins are present in all patients, but at least in the autosomal recessive disease state, are not phosphorylated by PKC either in vivo or in vitro (Segal et al, 1985; Kramer et al, 1988). Some evidence suggests that all forms of CGD may be phosphorylation defective (Hayakawa et al, 1986; Okamura et al, 1988). Given its M_r and cytosolic location, P47 may be the PKC substrate involved in activation of the respiratory burst, presuming it is even present in neutrophils. Comparison of pI values for P47 (pI 6.1 to 6.5, Imaoka et al, 1983) and the CGD cytosolic proteins (pI 6.8 to 7.8, Okamura et al, 1988) suggests that they may not be identical. Despite this, no other phosphoproteins are visible in this region of 2D-PAGE autoradiograms of each cell type, with the possible exception of class I HLA antigens (Feuerstein et al, 1985). The patterns of successively phosphorylated forms of CGD protein and P47 are also quite similar (Imaoka et al, 1983; Okamura et al, 1988). Although platelets are incapable of a respiratory burst, they do contain other membrane-bound receptor-activated oxygenating systems, such as cyclo-oxygenase and lipo-oxygenase (Babior, 1987). Either of these could be controlled by P47.

An inherent drawback of the platelet system is that it is not amenable to molecular analysis since these cells contain no mRNA and do

not undergo protein synthesis. In order to help elucidate P47 structure and function, it is essential to identify this protein in a system where it can be radiolabeled with both ^{35}S and ^{32}P; molecular cloning of the P47 cDNA could also be achieved in such a cell type. The HL-60 cell line proved to meet these requirements.

1.7 SCOPE OF THIS WORK

Synergism between Ca^{2+} and PKC, as mimicked by ionophores and phorbol esters respectively, has been demonstrated for many short term responses. Lymphocyte activation and mitogenesis are the only long term responses known to be induced in a like manner. Thus to extend the concept of synergism in PI-based signalling to another class of long term response, the effect of phorbol ester and Ca^{2+} ionophore cotreatment on HL-60 differentiation was examined. The differentiation response to these agents was characterized by various cellular criteria and by hybridization studies with a number of known genes.

The second part of this thesis describes the molecular cloning of P47 from HL-60 cells. Not only was this significant for various aspects of platelet signal transduction and PKC function but it also fulfiled one of the original objectives of this work, that is isolation of differentiation-specific products. Regulation of both P47 protein and mRNA in HL-60 cells was extensively characterized. The implications of the P47 primary structure, tissue distribution, and conservation through evolution were considered with respect to the putative functions of this protein.

MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 Chemicals

The following sources were used for critical reagents:

A23187	Sigma Chemical Co.
cytosine arabinoside	Sigma Chemical Co.
dibutyryl cAMP	Sigma Chemical Co.
dimethylsulfoxide	BDH Chemicals
ionomycin	Calbiochem
hen egg white lysozyme	Sigma Chemical Co.
mezerein	Sigma Chemical Co.
Micrococcus lysodiekticus	Sigma Chemical Co.
nitro blue tetrazolium	Sigma Chemical Co.
p-nitrophenol	Sigma Chemical Co.
1-oleoyl-2-acetylglycerol	Sigma Chemical Co.
retinoic acid (all-trans-β)	Sigma Chemical Co.
phorbol-12,13-dibutyrate	Sigma Chemical Co.
4β-phorbol-12,13-didecanoate	Sigma Chemical Co.
4α-phorbol-12,13-didecanoate	Sigma Chemical Co.
12-O-tetradecanoyl-phorbol-13-acetate	Sigma Chemical Co.

All tissue culture reagents were obtained from from Gibco/BRL. Other chemicals and supplies were of standard laboratory grade.

2.1.2 Radiochemicals

[γ-^{32}P]-ATP (3,000 Ci/mmol) and $^{32}PO_4^{3-}$ (carrier free) were obtained from New England Nuclear through the laboratory of Dr. P. Branton, Department of Pathology, McMaster University. [^{35}S]-methionine (1,400 Ci/mmol) was also obtained from Amersham. [α-^{32}P]-dATP and

[α^{32}P]-dCTP (3,000 Ci/mmol) were purchased from either Amersham or ICN Radiochemicals. [^{125}I]-protein A was kindly supplied by the laboratory of Dr. R.J. Haslam, Department of Pathology, McMaster University.

2.1.3 Enzymes

Restriction endonucleases were purchased from Gibco/BRL, Pharmacia or New England Biolabs. AMV reverse transcriptase, DNA polymerase I, gene 32 protein, RNAse H, T4 polynucleotide kinase, mung bean nuclease and Klenow large fragment were obtained from Pharmacia. T4 DNA ligase was purchased from either Gibco/BRL or Pharmacia. EcoRI methylase was obtained from Promega Biotech. The Sequenase sequencing kit was bought from U.S. Biochemical.

2.1.4 Cell Lines

The HL-60 Golde subline was kindly supplied by Dr. G. Broman, Henderson Hospital, Hamilton and maintained in RPMI 1640 supplemented with 10% (v/v) FBS, 2 mM L-glutamine, 0.5% (v/v) antibiotic/antimycotic, 25 mM sodium bicarbonate and 5 mM HEPES buffer (pH 8.0) at a final pH of 7.6 ± 0.1. Original passage HL-60 cells (P13) were obtained from the American Type Culture Collection (ATCC) and maintained in the same medium except that 20% (v/v) FBS was added. All cell culture was carried out in either flasks or dishes obtained from commercial suppliers (Falcon Industries or Corning Plastics). K562, Raji and U937 cell lines were kindly provided by Dr. B.J. Clarke, Department of Pathology, McMaster University and maintained as for the HL-60 Golde subline.

2.1.5 Other Biological Materials

Purified platelet P47, anti-human P47 rabbit serum and pre-immune serum was provided by the laboratory of Dr. R.J. Haslam, Department of Pathology, McMaster University. OKM-1 mouse monoclonal antibody was obtained from Ortho Diagnostics Systems and fluoroisothiocyanate-conjugated goat anti-mouse antibody was kindly provided by the laboratory of Dr. D.N. Sauder, Department of Immunology, McMaster University. Materials supplied on a one time basis are cited under technical acknowledgements.

2.1.6 Cloning Vectors and Host Strains

The pUC 118/119 vectors and MV1193 host cells (Viera and Messing, 1987) were obtained from Dr. A.B. Futcher, Department of Biochemistry, McMaster University who originally obtained them from Dr. J. Viera. The lambda gt11 vector (Young and Davis, 1983) was obtained as the Protoclone system (de-phosphorylated lambda gt11 arms, Packagene *in vitro* packaging extract, 1089 and 1090 host cells and anti-rabbit mouse-alkaline phosphatase conjugate, BCIP and NBT) was purchased from Promega Biotech. Dephosphorylated lambda ZAP arms and BB4 and XL-1 Blue host strains were obtained from Stratagene. The pKK-233 bacterial expression vector and NcoI linkers were purchased from Pharmacia; JM 109 cells (obtained from Dr. S. Mak, Department of Biology, McMaster University) were used as the host strain. The pOTS-Nco12 bacterial expression vector and N5151 and AR 68 host cells (Schatzman and Rosenberg, 1987) were kindly supplied by the lab of Dr. G. Shore, Department of

Biochemistry, McGill University. Routine cloning was also carried out in DH5α competent cells obtained from Gibco/BRL. An amplified EMBL 3 human leukocyte genomic library was bought from Clontech and an unamplified EMBL 3 human lymphocyte genomic library was kindly provided by Francoise Fernandez-Rachubinski of Dr. M. Blajchman's laboratory, Department of Pathology, McMaster University. Both were propagated in LE392 cells as described by Frischauf et al (1983; 1987).

2.1.7 cDNA probes

Human c-myc (pHSR1) and c-fos (pc-fos-1) genomic clones were obtained from the ATCC. GRP78 (3A4) and GRP94 (4C3) clones were kindly provided by Dr. A.S. Lee, Department of Biochemistry, University of Southern California. The plasmid pHS1 containing the human $MTII_A$ promoter was obtained from Dr. M. Karin, Division of Pharmacology, School of Medicine, University of California, San Diego.

2.1.8 Oligonucleotides

The oligonucleotides listed in Table 2.1.1 were synthesized with an Applied Biosystems 381A Automated DNA Synthesizer (in laboratory of Dr. H.P. Ghosh, Department of Biochemistry, McMaster University) or obtained from the Central Facility of the Institute for Molecular Biology and Biotechnology, McMaster University. Strand polarity is indicated by (-) and (+) for complementarity to the non-template (i.e. mRNA) and template strands, respectively. Underlined bases indicate a mismatch with wild type sequence. Superscripted and subscripted bases separated by a slash indicate degenerate positions. Prior to synthesis

Table 2.1.1 List of Oligonucleotides

oligo/strand			sequence	ends		reference
myc		(-)	5'-GAGAAGCCGCTCCACATACAG-3'	565	549	Battey et al, 1983
actin		(-)	5'-GATCCACATCTGCTGGAAGGTGGACAG-3'	1112	1086	Ponte et al, 1984
fms		(-)	5'-GCTCTCGATGATCTTCCAGCG-3'	1965	1945	Coussens et al, 1986
MTAP		(-)	5'-GGGCAGCAGGAGCAGCAGCT-3'	689	670	Karin & Richard, 1982
IL-1β		(-)	5'-TCGCTTTTCCATCTTCTTCTTTGG-3'	728	705	Bell et al, 1987
$(dT)_{18}$		(-)	5'-TTTTTTTTTTTTTTTTTT-3'	---	---	Harley, 1987
P47	AB391	(-)	5'-CTCTCAGGCAACTCCTCTCAGCAGCTGGGC-3'	-59	-30	
	AB296	(-)	5'-ATGCTGGCTGGACAGG-3'	1	-14	
	AB207	(-)	5'-GCTAGTCAGAGTGCTC-3'	170	155	
	AB319	(-)	5'-GCGATTCCTAACAGAC-3'	521	506	
	AB317	(-)	5'-GTGAATTGCTCCCAGG-3'	872	857	
	AB316	(-)	5'-ACCTTGCACACCACAT-3'	1214	1199	
	AB119	(-)	5'-CAAGGCCAAGAGCAAG-3'	1608	1593	
	AB114	(-)	5'-GAAACAGAATCTCACA-3'	1929	1914	
	AB98	(-)	5'-AACTCAAGCAGGAGCTGCT-3'	2225	2207	
	AB82	(-)	5'-CAAGAGAGGATTTGAT-3'	2470	2456	
	AB321	(+)	5'-AGCGGATCAGAGAGGG-3'	10	25	
	AB320	(+)	5'-CGACTGCCAGAAACCA-3'	354	369	
	AB318	(+)	5'-GATGATGTGATTCTGA-3'	696	711	
	AB476	(+)	5'-TGGCCTCCCGAACTG-3'	1030	1044	
	AB477	(+)	5'-CTGCTGGTGTCCTTC-3'	1445	1459	
	AB120	(+)	5'-GGAGTAGCTGAGGGTC-3'	1764	1769	
	AB113	(+)	5'-ACCCTGACCACTAACTA-3'	2303	2319	
	AB336	(-)	5'-GTTCCATGGTGGCTGGA-3'	6	-10	
	AB453	(-)	5'-GAATGG$^{C}/_{A}$CCTGCGGG$^{C}/_{T}$AG$^{C}/_{A}$TTTCCT-3'	354	330	
	AB452	(-)	5'-CTCACTGA$^{C}/_{T}$CCTGCCAT-3'	929	912	

oligonucleotides were searched against current data bases for fortuitous similarity to other sequences. The actin oligonucleotide was complementary to α, β and τ actin with a maximum of 1 mismatch between the human and chicken sequences (Ponte et al, 1984). Similarly, the myc oligonucleotide was complementary to c, N and L myc sequences with at most a single mismatch between the human and chicken sequences (Nau et al, 1985).

2.2 CELL CULTURE AND DIFFERENTIATION

2.2.1 Growth of HL-60 Cells

Cells were maintained in suspension culture at a density of 2 x 10^5 to 2 x 10^6 cells/ml by subculturing every 2-3 days at a ratio of between 1:2 and 1:10, as required. Under these conditions, the Golde subline doubled every 24 h whereas early passage cells from the ATCC doubled every 48 h. Unless otherwise indicated, all experiments described employed cells obtained from the ATCC of passage number between 20 and 50. This corresponded to no more than 3 months of continuous culture. Cells earlier than P18 were not used because they underwent a crisis in which highly granulated cells were lost from the population. This has been noted by other investigators (Leglise et al, 1988). Other cell lines (U937, K562, Raji and HL-60/MT-myc), were maintained in the same media as the Golde HL-60 subline at similar densities. Cells were stored in liquid nitrogen at a density of 10^7 cells/ml in RPMI 1640 containing 20% (v/v) FBS and 10% (v/v) DMSO. Vials containing 1 ml of cells were initially frozen wrapped in paper

towels at -70°C or in the vapour of a liquid nitrogen tank.

Experiments were carried out with pooled populations to eliminate variation in the cell density of individual flasks. Prior to each experiment cells were visually inspected for viability; this was occasionally confirmed by trypan blue dye exclusion (Freshney, 1983). Small scale preparations used for determination of cell growth, morphology, and enzymatic activity were carried out on 5 ml of cell suspension culture in 60 mm dishes. Large preparations of cells for RNA extraction (typically 40-60 ml) were cultured in either 75 cm^2 flasks or 100 mm dishes. Drugs were added to cultures at either 1:300 or 1:1000 dilutions from stocks in ethanol (phorbol esters, RA), DMSO (A23187, ionomycin, $1,25\text{-}(OH)_2D_3$), or water (dbcAMP, cytosine arabinoside, $CdCl_2$). Adherent differentiated cells were removed by gentle scraping with a rubber policeman. The same procedure was applied to all experimental conditions. Once suspended, all cultures were pelleted either at 1,500 x g for 2 minutes in a clinical centrifuge or 15,000 x g for 10 s in an Eppendorf microfuge.

2.2.2 Assessment of Differentiation

Adherence to substratum was assessed qualitatively by shaking the culture vessel to identify suspension cells and then assigning the culture to the following range: no adherent cells (-) to all adherent with extensive clumping (+++). Attempts to quantitate adherence were not reproducible since methods for removing suspended cells dislodged a variable fraction of attached cells. Subcellular morphology was examined in cells stained with May-Grunwald-Geisma (carried out by the

laboratory of Dr. R. Barr, Department of Pathology, McMaster University). Nuclear to cytoplasmic ratios were estimated by light microscopy, using an eyepiece grid to measure the diameter of nuclei and cells. At 1000 x final magnification, 1 grid division corresponded to 1 μm.

Lysosomal enzymatic activity secreted into the medium by cells was quantitated according to Huberman et al (1982). Briefly, 1.3 ml of a 0.3 mg/ml suspension of lyophilized Micrococcus lysodeikticus in 67 mM K_2HPO_4 (pH 6.24) was inoculated with 0.5 ml of cell supernatant and a further 1.2 ml of the same buffer. Digestion of the turbid bacterial suspension was monitored at 450 nm every 3 min for at least 9 min; the rate of digestion was converted to lysozyme equivalents by interpolation of a standard curve derived from dilutions of a hen egg white lysozyme solution. Activity was then normalized to cell number. Cellular acid phosphatase activity was measured by hydrolysis of p-nitrophenol by cell extracts (modified from Schnyder and Baggiolini, 1978). The cell pellet from 1.5 ml of culture was washed once in Dulbecco's PBS and disrupted by addition of 100 μl 0.05% digitonin (w/v) and pipetting vigorously. The suspension was spun for 2 minutes in an Eppendorf microfuge and 50 μl was diluted into 150 μl of reaction mix which contained 5 mM p-nitrophenol in 100 mM sodium acetate (pH 4.5). The reaction was incubated for 30 min at 37°C and then terminated by the addition of 1 ml of 1 M NaOH. Absorbance was measured at 405 nm and an absolute rate of hydrolysis calculated based on an extinction coefficient of 18,800 $(M/cm)^{-1}$ for the nitrophenol product. This value was then normalized to cell number. The ability of cells to generate a superoxide burst was

assessed by reduction of nitroblue tetrazolium to a blue-black Formazan precipitate (Yen et al, 1984a). A 0.2 ml aliquot of a solution containing 160 nM TPA (100 ng/ml) and 0.2% NBT (w/v) in PBS was added to 0.2 ml of cell suspension and incubated at 37°C for 20 min. The suspension was then counted on a hemocytometer for cells containing intracellular black deposits. At least 200 cells (as independent sets of at least 100 cells) were counted for each sample.

OKM-1 reactivity of intact HL-60 cells was assessed by indirect immunofluorescence either visually or with a flow cytometer. Staining was carried out based on the method of Sauder et al (1981). Cells (1-5 x 10^6) were washed once in PBS containing 0.2% (w/v) NaN_3 and 2% (v/v) FBS and resuspended in 0.3 ml of OKM-1 mouse monoclonal antibody (diluted 1:24 in azide/PBS). After a 30 min incubation at 0-4°C, cells were washed with 1 ml PBS/azide/2% FBS, resuspended in 0.3 ml of goat anti-mouse IgG-FITC conjugate (diluted 1:10 in azide/PBS) and set on ice for an additional 30 min. Cells were again washed in PBS/azide/2% FBS and resuspended in 0.4 ml of the same solution. For visual experiments, cell viability was monitored by addition of 50 µl ethidium bromide (60 ng/ml in PBS) to the final suspension. Wet mounts for fluorescence microscopy were made by lining the edges of a coverslip with vaseline and dropping the coverslip over 50 µl of cell suspension. In this state, cells remained viable for several hours. Fluoresence was evaluated at 400 nm. Positive cells were scored as those with an intense halo of green fluorescence around their periphery. Inviable cells stained diffusely and had orange nuclei as a result of ethidium bromide penetration. At least 200 cells from each sample were scored by

naive observers. Flow cytometry on identically treated cells (except for the omission of ethidium bromide staining) was carried out by Barb Kurk (Department of Clinical Chemistry, McMaster University Medical Centre) on an Ortho System II laser flow cytometer. Non-specific fluorescence was corrected by subtracting readings from cells stained with secondary antibody only.

Cell cycle analysis of at least 10^7 cells was carried out with the same flow cytometer. Nuclei were isolated, stained with propidium iodide and evaluated according to standard methods (see Gray and Darzynkiewicz, 1987).

2.2.3 Measurement of Intracellular Ca^{2+}

HL-60 cell populations were measured using the quin2 fluorescent dye essentially as first described by Tsien et al (1982). The excitation and emission maxima of free quin2 in simplified medium (Tsien et al, 1982) were verified on a Perkin-Elmer 44 MPF Fluorescence Spectrophotometer; the former occured at 339 nm and the latter at 495 nm. These values were used for all subsequent experiments. Typically, 1-1.5 ml of HL-60 cells were resuspended in serum free RPMI 1640 at a density of 10^8 cells/ml. Quin2-AM was added to a final concentration of 50 μM. After incubation at 37°C for 20 min, the suspension was diluted 10-fold with serum free RPMI 1640 and held at 37°C for an additional 40 min. Cells were then pelleted at 800 x g for 5 min in a clinical centrifuge and resuspended at a final density of 1.5 x 10^7 cells/ml in RPMI 1640 at 37°C. Experiments were initiated with 1 ml of cells which were pelleted in an Eppendorf microfuge for 5 s and transferred to a

fluorometer cuvette in 2 ml of simplified medium. The cuvette was maintained at 37°C and the contents continuously stirred with a motor driven propellor. Drugs were added from stock solutions such that the final solvent concentration never exceeded 0.3%. Traces were terminated by addition of Nonidet P-40 to a final concentration of 0.1%. This released all intracellular quin2 to generate a maximum fluorescence signal. Background fluorescence was determined by addition of EGTA to 4 mM and adjusting the pH to 8.3 with 1 M KOH. Ca^{2+}-free medium was identical to simplified medium except for the absence of $CaCl_2$ (in the absence of EGTA this yields a nominal Ca^{2+} concentration of 10 μM (Tsien et al, 1982)). The following fluorometer settings were routinely employed: excitation slit width = 4 nm, emission slit width = 10 nm, chart speed = 10 cm/min, ADV = 7.5, sensitivity = 10.65, response = 1.5, pen range = 1 V.

2.3 ANALYSIS OF PROTEINS

2.3.1 Electrophoresis

One dimensional SDS polyacrylamide gel electrophoresis (SDS-PAGE) was carried out exactly as described by O'Farrell (1975). Cell pellets were lysed directly in sample buffer and boiled for 5 min just prior to loading. Gels were cast as either 11% (Figs. 4.1.7, 4.3.3 and 4.3.4) or 13% (Figs. 4.1.1-3, 4.1.5-6 and 4.1.8-9) acrylamide or as a 7-15% acrylamide gradient (Fig. 4.1.4) and run at either 250 V for 4 h or 80 V overnight, until the tracking dye reached the bottom of the separating gel. After electrophoresis, gels were stained for total

protein with 1% Coomassie Brilliant Blue. Gels of ^{35}S-labeled proteins were dried either directly or after fluorography (Fujiki et al, 1984) and exposed to Kodak XAR-5 film at -70°C. Gels of ^{32}P-labeled samples were dried directly and exposed to film at -70°C in the presence of an intensifying screen.

2.3.2 Immunoblot Analysis

Electrophoretic transfer of proteins to nitrocellulose was carried out according to Erickson et al (1982). Pelleted HL-60 cells were washed twice with ice cold PBS and resuspended in 1 ml; this was divided into two parts (between 1:1 and 4:1) and each fraction was TCA precipitated by addition of 1/3 volume of 30% (v/v) TCA. The smaller fraction was analyzed for protein content (Lowry et al, 1951) and used to calculated the amount of the larger fraction suspended in sample buffer required to load 80 µg of protein. Fractionation of HL-60 cells was achieved by sonicating a cell suspension (3×10^7 cells/ml, 4 x 15 s bursts at 0-4°C with a Heat Systems-Ultrasonics W140D probe sonicator at maximum power) in 20 mM MES (pH 6.5) containing 0.02% leupeptin and spinning the homogenate at 100,000 x g for 30 min at 4°C in a Beckman Airfuge. Membrane and cytosol protein content was determined on parallel TCA precipitated fractions as described above. Triton X-100 insoluble cytoskeletal fractions were prepared according to the method of Pontremoli et al (1987) and processed for electrophoresis in an identical fashion to other HL-60 samples.

Samples were electrophoresed in parallel with purified platelet P47 and pre-stained molecular weight markers (Sigma) on a 13% acrylamide

gel. Transfer to nitrocellulose (0.45 µm) was carried out with a BioRad Transblot apparatus at 100 mA for 16 h. After transfer, the filter was pre-washed with 100 ml of blocking solution (Blotto: 5% (w/v) non-fat milk powder in 0.3% (w/v) Tween 20/154 mM NaCl/2mM NaH_2PO_4 (pH 7.2)) for 30 minutes then incubated with a 1:100 dilution of P47 antiserum for 30 minutes. After washing with saline/phosphate/Tween 20 for 45 min, the filter was incubated with 1 µCi of ^{125}I-protein A in 100 ml Blotto. The filter was washed again and exposed to Kodak XK-1 film at -70°C in the presence of an intensifying screen. Immunoreactive bands were cut out and quantitated by gamma counting. Background radioactivity was accounted for by subtracting counts detected on representative areas that showed no specific immunoreactivity. Counts were converted to absolute amounts of protein by interpolation of P47 standard curves generated in the same manner.

Analysis of bacterial lysates was carried out in similar fashion with the following exceptions (based on the procedure of Fujiki et al, 1984). Equal volumes of saturated overnight cultures (typically 20 µl of a 1.5 ml culture lysed in 300 µl sample buffer, about 20 µg of bacterial protein) were electrophoresed on an 11% acrylamide gel. After transfer, blots were rinsed in 50-100 ml of blocking solution (1% (w/v) bovine hemoglobin in tris-buffered saline (TBS: 10 mM Tris-Cl (pH 7.4), 0.9% NaCl)) at 37°C for 30 min. The filters were then sealed in plastic bags and 10 ml of the blocking solution with a 1:300 - 1:1000 dilution of P47 antiserum was added. To reduce bacterial immunoreactivity, 100 µl of a bacterial lysate (made from a 50 ml overnight culture of the appropriate bacterial strain resuspended in 3 ml of TBS and sonicated at

maximum power for 3 x 2 min bursts) was added to the probe solution for 15-30 minutes before addition to the blot. Blots were gently rocked for 90 minutes at 22°C (or overnight at 4°C) then removed from bags and rinsed as follows: TBS 10 min, TBS plus 0.05% (w/v) NP-40 2 x 20 min, TBS 10 min. Blots were re-sealed in plastic with 5-10 ml of blocking solution containing 1 µCi of ^{125}I-protein A and rocked for 30 minutes. Blots were then washed as above and exposed to X-ray film at -70°C in the presence of an intensifying screen. Although each immunoblot procedure gave similar results, the latter appeared 2-3 fold more sensitive and required much less antiserum.

2.3.3 Immunoprecipitation

Cell lysates or *in vitro* translations were immunoprecipitated according to Fujiki *et al* (1984). Metabolic labelling of RA-differentiated HL-60 cells with [^{35}S]-methionine (10 µCi/ml, 1.6 x 106 cells/ml, 2 ml/timepoint) was carried out in Dulbecco's minimal essential medium (MEM) lacking methionine but supplemented with 10% (v/v) FBS. Labelling of cells with $^{32}PO_4^{3-}$ (0.5 mCi/ml, 1.5 x 10^6 cells/ml) was carried out in unsupplemented PO_4^{3-}-free Dulbecco's MEM in a 1.5 ml Eppendorf microfuge tube. Cells were washed twice with PBS and resuspended in 100 µl binding buffer (10 mM Tris-Cl (pH7.4), 1% (w/v) NP-40, 0.1% (w/v) SDS, 150 mM NaCl, 10 mM methionine, 2 mM EDTA, 25 µg/ml leupeptin, 25 µg/ml antipain, 12.5 µg/ml chymostatin and 12.5 µg/ml pepstatin). After pelleting out cell nuclei (30 s in Eppendorf microfuge) and adding 900 µl binding buffer and 20 µl P47 antiserum, the solution was rocked at 22°C for 90 min or overnight at 4°C.

Immunoglobulin was then absorbed onto 20 µl Staphlococcus aureus cells (prewashed as a 10% suspension in 2 x binding buffer) for 90 min at 22°C. The cells were then pelleted and sequentially washed with 1 ml each of the following; binding buffer (3 washes), binding buffer minus SDS and EDTA (2 washes), binding buffer minus SDS, EDTA and NP-40 (1 wash) and binding buffer minus SDS, EDTA, NP-40 and NaCl (1 wash). Cell pellets were then boiled for 5 min in sample buffer, electrophoresed on an 11% acrylamide gel, fluorographed and exposed to film at -70°C.

2.3.4 Protein Sequencing

This work was carried out at Smith Kline and French Laboratories by Angela Varrichio and Mark Strohsacker under the supervision of Dr. R.G.L. Shorr. Briefly, purified human platelet P47 (from the laboratory of Dr. R.J. Haslam) was denatured, alkylated and further purified by reverse phase HPLC on 330 Å Vydac C_4 column using a linear gradient of 0-70% acetonitrile in 0.1% trifluoroacetate. CNBr and tryptic fragments were resolved on the same C_4 column and a Beckman C_{18} column, respectively. Sequence analysis was carried out on Beckman 890M amino acid sequencer. Phenylthiohydantoin-amino acids were analyzed on a C_{18} column as described by Hawke et al (1982).

2.4 ANALYSIS OF NUCLEIC ACIDS

Unless stated otherwise all techniques used were carried out according to either product data sheets, Maniatis et al (1982) or Davis et al, (1986). Another source occasionally used was Current Protocols

in Molecular Biology (Ausubel et al, 1987). All standard buffers and solutions are also described in these sources. A brief overview of experimental strategy is presented with detailed modification of existing protocols or relevant comments as necessary.

2.4.1 Labelling and Hybridization of DNA Probes

i) Oligonucleotides

Desalted oligonucleotides proved adequate for all applications. Typically, 5' end labeling reactions were carried out in 30 µl of reaction buffer containing 10 µCi of γ-ATP (3,000 Ci/mmol), 6 pmol of oligonucleotide (i.e. 0.0015 A_{260} units or 33 ng, assuming 1 A_{260} = 20 µg of 16-mer) and 2-4 U of T4 polynucleotide kinase at 37°C for 40 min. Enzyme obtained from Pharmacia appeared more stable and gave better incorporation than that from BRL. Reactions were stopped on ice and incorporation of 5' $^{32}PO_4$ was estimated from Cerenkov counts remaining on DE81 paper after washing 3 x 100 ml of P wash solution (0.1 M Na_3PO_4, 10 mM $Na_7P_2O_7$). Typically, 10^6 cpm/pmol was obtained, corresponding to a specific activity of roughly 2 x 10^8 dpm/µg. Nitrocellulose filters containing bound nucleic acid were prehybridized in a plastic bag for at least 30 min in 5 x Denhardt's solution, 5 x SSC, and 1 x P wash in a volume of roughly 1 ml/10 cm^2. Labelled oligonucleotide was diluted into 1-3 ml of prehybridization solution and added to the bag to give a final probe concentration of 1-300,000 cpm/ml. If the expected number of bound target molecules exceeded the number of probe molecules added (e.g. for $(dT)_{18}$ or actin signals), probe was diluted with cold oligonucleotide such that at least a 2-fold excess over target sequences

was maintained. Hybridization was at 42°C overnight, except for $(dT)_{18}$, which was at room temperature for 6 h. Filters were washed in 2 x SSC at room temperature 4 x 5 min. Washing at 48°C gave similar signals with only slightly lower background. Non-specific $(dT)_{18}$ binding was removed by washing in 2 x SSC at room temperature, 4 x 5 min.

ii)- DNA fragments

The random primer method of Feinberg and Vogelstein (1983; 1984) was used. Digested DNA was electrophoresed in 1% low melting point agarose at 4°C and bands of interest were excised and diluted with 1.5 volumes of water. Sufficient DNA was loaded such that bands of 1 kbp in size contained approximately 1 µg; upon dilution this gave a final concentration of roughly 1 ng/µl. Alternatively, DNA fragments were isolated using the Gene Clean kit (Bio/Can Scientific) based on the procedure of Vogelstein and Gillespie (1979). The following conditions represent the final version of the random primer protocol: 20 µM each dNTP except the radionucleotide (usually [α-^{32}P]-dATP, 3000 Ci/mmol, 10 µCi/µl); 5 A_{260} units/ml of $(dN)_6$; 20 mM Tris-Cl (pH 7.5); 30 ng DNA fragment in 0.3% LMP agarose; 25 µCi dATP (1 µM). Practically, 10 µl of a 5 x stock solution of dNTP in Tris-Cl (pH 7.5) was combined with 30 µl of fragment solution, 3 µl of 90 A_{260} units/ml $(dN)_6$, 2.5 µl of [α-^{32}P]-dATP and 5 µl of sterile water. The reaction was initiated by addition of 2-5 U Klenow fragment (preferably Pharmacia enzyme) and allowed to proceed for 0.5-24 h. Despite the limiting concentration of [α-^{32}P]-dATP, a 2 min chase with 20 µM dATP had no effect on total incorporation or hybridization of the probe. The major modification in this method compared to that originally described is the increased pH of the

reaction buffer, from 6.6 to 7.5. The former was designed to reduce contaminating exonuclease activity in Klenow preparations at the expense of slower polymerization rates. However, most commercial enzymes are nuclease free and hence can be used at higher pH, allowing shorter reaction times if desired. Reactions were halted by addition of an equal volume of stop solution (Feinberg and Volgelstein, 1984). Free isotope was separated from labelled DNA by centrifugation through a Sephadex G-50 column for 1 min at speed 6 in a clinical centrifuge (Maniatis et al, 1982). Typically, 10^8-10^9 Cerenkov cpm/µg fragment was obtained. The probe solution was diluted to 0.3-0.4 ml with TE and boiled for 5 min before storage at -20°C. Unless otherwise indicated, hybridization conditions for cDNA probes were as follows: 5 x SSC, 5 x Denhardts, 50% formamide, 0.5 x P wash, 250 µg/ml sheared and denatured salmon sperm DNA, 0.1% SDS, and 2 x 10^5-10^6 cpm of probe/ml of hybridization mix. Prehybridization in the absence of probe was for at least 1 h at 48°C. After overnight hybridization at 48°C, filters were washed twice in 2 x SSC, 0.1% SDS for 15 min at 22°C, then twice in 0.1 x SSC, 0.1% SDS for 10 min at 48°C, unless stated otherwise. After washing, filters were blotted for 1 min on 3 MM paper, wrapped in Saran film while still moist and exposed to Kodak XAR film at -70°C in the presence of an intensifying screen. After appropriate exposures, bound probe was stripped off by heating for 15 min in 0.1 x SSC, 0.1% SDS at 85-90°C. Up to seven successive reprobings of the same filter could be achieved in this manner. If possible, blots were first probed with oligonucleotides since they were easily removed under less harsh conditions (60-70°C).

iii) DNA and RNA size markers

The standard marker employed in this work was the BRL 1 kbp ladder. DNA (0.5 µg) was 5' end-labelled with [^{32}P]-τ-ATP in an exchange reaction catalyzed by T4 polynucleotide kinase and separated from unincorporated radioisotope over a Sephadex G-50 spin column (Maniatis et al, 1982). Markers for Northern analysis were taken up in RNA sample buffer and boiled for 5 min prior to loading. Typically, 10,000 cpm of marker could be visualized by autoradiography after several hours exposure at -70°C.

2.4.2 RNA Analysis

RNA was isolated from all cell types and tissues by a modification of Chirgwin et al (1979) as described by Rachubinski et al (1985). Briefly, cells were pelleted (up to 5 x 10^7) and taken up with vigorous pipetting into 2 ml of 4 M guanidinium isothiocyanate, 0.25 mM Na citrate (pH 7.0), and 0.1 M mercaptoethanol. The viscous solution was layered over a 2.5 ml cushion of 5.7 M CsCl and spun in a Beckman SW50.1 rotor at 30,000 rpm or in a Sorvall TST 41.4 at 27,000 rpm for 8-24 h. The upper phase, interfacial DNA and lower phase were removed by aspiration. Tubes were inverted just as the last of the CsCl solution was removed to avoid contamination of the RNA pellet. After draining in an inverted position, tube walls were wiped dry with Kimwipe tissue wrapped around a sterile cotton swab. Pellets were dissolved in 0.05% SDS, heated at 56 °C for 5 min, chilled on ice and centrifuged in an Eppendorf microfuge for 5 min at room temperature. RNA was precipitated from the supernatant by addition of 33 µl of 2M KOAc (pH 5.5), 200 µl

water and 1 ml of absolute ethanol in an Eppendorf tube. Samples were precipitated at -20°C for more than 20 min and recovered by centrifuging for 5 min at 4 °C. The supernatant was aspirated and pellets rinsed with ice cold 70% ethanol, 100 mM NaOAC, pH 6.0. The pellet was then dried and resuspended in 100 µl of sterile water. Typically, 4 µg RNA was obtained per 10^6 cells; this value dropped to less than 1 µg per 10^6 RA-differentiated HL-60 cells. Large scale preparations of RNA for isolation of the poly(A)$^+$ fraction utilized identical conditions except that RNA was spun out of ethanol in a Beckman JS-13 rotor at 8,000 rpm for 20 min at 4°C.

Selection of poly(A)$^+$ RNA was carried out on an $(dT)_{12}$ cellulose column essentially as described (Maniatis et al, 1982). Typically 1-2 mg of total RNA in 1 ml of binding buffer was applied to a 0.5 ml column and the eluent recycled once after heating to 70°C. Elution of poly(A)$^+$ RNA in low salt buffer was followed by the fluorescence assay described below. Poly(A)$^+$ RNA containing fractions were pooled and precipitated with wheat germ tRNA carrier as described (Rachubinski et al, 1985). RNA was quantitated by the ethidium bromide fluorescence method of Morgan et al (1979). A Sequoia-Turner Model 450 fluorometer was calibrated with 1 µg of a standard DNA solution set to a value of 300 in pH 8.1 buffer. At this setting 1 µg RNA gave a reading of 150. Linearity was maintained from 0 to 700; above this samples were diluted and re-read. DNA contamination measured by fluorescence at pH 11.8 rarely occured and was always below 5%. DNA was quantified in a similar manner except that 0.5 µg was set to 100 in pH 11.8 buffer.

Specific RNA species were detected either by dot blot or

Northern analysis (Thomas et al, 1980). RNA (0.25 to 1 µg) was heated to 70°C for 5 min in 6 x SSC, 30% formaldehyde, then spotted under vacuum onto nitrocellulose that had been wetted first in water then in 10 x SSC for at least 15 min. After RNA dotting, filters were baked for 2-4 h in vacuo at 80°C. Alternatively, samples (15 µg total RNA, 0.5 µg poly(A)$^+$ RNA) were electrophoresed on a 1% agarose, 6% (v/v) formaldehyde gel in 20 mM borate (pH 8.0), 0.2 mM EDTA (pH 8.0) running buffer. Prior to loading, dried down samples were dissolved in 4.5 µl sterile water and added to 17.5 µl RNA sample buffer (composed of 2 µl 10 x running buffer, 3.5 µl formaldehyde, 10 µl deionized formamide, 2 µl formamide dye mix and 320 mg/ml sucrose) and heated at 70°C for 10 min. The 1 M sucrose prevented a vortex effect that occured frequently upon pipetting samples into the wells. Gels were run at 60 V for 5 h which brought the bromophenol blue tracking dye to the bottom of the gel. RNA was transferred to nitrocellulose in 10 x SSC by the wick method (Maniatis et al, 1982). Efficiency of transfer was checked by ethidium bromide staining of the gel after overnight transfer. Filters were baked in vacuo at 80°C for 2-4 h and probed as described in Section 2.4.1.

2.4.3 DNA analysis

Transformation of bacteria was carried out according to the $CaCl_2$ method (Maniatis et al, 1982) or the high efficiency protocol of Hanahan (1983) except that the pH of completed ligation reactions was buffered by the addition of 1 µl of 1 M MES (pH 6.3). This overcame the inhibition of transformation by alkaline pH (Dr. A.B. Futcher, personal

communication). Plasmid DNA was isolated from bacterial cells by the alkaline lysis miniscreen method (Birnboim and Doly, 1979) for screening purposes and according to Pulleyblank et al, (1983) for large scale preparations. Bacteriophage lambda DNA was isolated either on a small scale from 10 ml lysates (Davis et al, 1986) or from 500 ml lysates according to the following procedure modified from Maniatis et al (1982) and Davis et al (1986). Overnight host cell culture grown in the presence of 0.2% (w/v) maltose and 10 mM $MgCl_2$ was expanded (1:500) and grown for 1-1.5 h until turbidity was just visible. Typically, 500 µl of lysate from a small scale preparation was then inoculated into the culture; each clone and minilysate had different infectivity and titre so that more or less inoculum was sometimes used (between 200 µl and 1 ml). Lysis occured in 6-12 h and was completed by the addition of 10 ml of $CHCl_3$ and shaking for 10 min. After adding 60 ml 5 M NaCl and 5 ml 1 M $MgSO_4$, bacterial debris was spun out at 8,000 rpm in a Beckman JA-10 rotor for 20 min at 4°C. Then 60 g polyethylene glycol (PEG) 6,000 (formerly 8,000) was dissolved in the supernatant and phage were allowed to precipitate for 1 h to overnight at 4°C and subsequently pelleted in a Beckman JA-20 rotor at 8,000 rpm for 20 min. The precipitate was pipetted off the walls of the centrifuge bottle, suspended 15 ml TM and twice extracted with $CHCl_3$. The aqueous phase was removed avoiding the large interface and brought to a final volume of 32.8 ml with TM; to this 27.8 g of CsCl was added giving a final concentration of 4.13 M. The solution was centrifuged at 40,000 rpm in a Beckman VTi 50 rotor for 20-36 h. The bacteriophage band was visualized against a dark background by shining an intense light on top of the tube and extracted

through the side of the tube with a 3 ml syringe fitted with an 18-gauge needle. Phage DNA was then extracted as described (Maniatis et al, 1982). Up to 1 mg of lambda DNA, suitable for further manipulation, was obtained.

Southern analysis was carried out with 20 µg of digested genomic DNA isolated from tissues (liver) or cells (HL-60, lymphocyte, chicken embryo fibroblast) by proteinase K digestion as described by Maniatis et al (1982). Electrophoresis was in 0.8% agarose and 1 x TBE at 25-30 V for 24 h. Alternatively, for analysis of genomic clone DNA (0.5-1.0 µg) field inversion gel electrophoresis was carried out using a Bio-Rad Pulsewave Model 760 switcher set at a forward time of 0.7 seconds and a forward to reverse ratio of 3:1. Electrophoresis at 170-200 V for 6-12 h resolved bands below 50 kbp. Transfer to nitrocellulose was carried out in 10 x SSC by the sponge method (Maniatis et al, 1982). Blots were probed as described in Section 2.4.1.

2.5 CLONING AND SEQUENCING

2.5.1 Construction of cDNA Libraries

P47 clones were obtained by the strategy first described by Young and Davis (1983) using the lambda gt11 expression system (Promega Biotech) and a polyclonal P47 antiserum provided by Dr. R. J. Haslam. The method of Wickens et al (1978) as modified by Rachubinski et al (1985) was used to construct double-stranded cDNA. Poly(A)$^+$ RNA was isolated from HL-60/MT-myc cells that had been differentiated with 1 µM

RA for 5 days. The first strand was synthesized in the following reaction buffer: 50 mM Tris-Cl (pH 8.3), 100 mM KCl, 6 mM $MgCl_2$, 10 mM dithiothreitol, 1 mM each dNTP, 200 pmol $(dT)_{18}$, 10 µg poly A^+ RNA in a final volume of 105 µl. The reaction was initiated by addition of 50 U AMV reverse transcriptase and incubated at 42°C for 1 h. A radioactive side reaction was carried out by adding 1 µCi of [^{32}P]-α-dCTP to 5 µl of the above mix immediately after reverse transcriptase. The reaction was stopped on ice water and labeled cDNA analyzed on a 5% acrylamide denaturing gel (Maniatis et al, 1982). RNA template was heat denatured away from cDNA by boiling for 2 min and the second strand synthesized in the same reaction tube by adding an equal volume (95 µl) of: 200 mM HEPES (pH 6.9), 145 mM KCl, 26 mM $MgCl_2$, 4 mM dithiothreitol and 0.75 mM of each dNTP. The reaction was initiated with 30 U of E. coli DNA polymerase I and incubated at 15°C for 2 h. Double-stranded cDNA synthesis was also monitored with a radioactive side reaction and analyzed along with single stranded cDNA. Approximately 30 µl of the poly A+ RNA was converted to first strand cDNA, and 30% of the latter to double stranded cDNA; this gave a final yield of approximately 10%. The size range of the cDNA extended beyond 3 kbp. Instead of cleaving the hairpin loop with S1 nuclease (Wickens et al, 1978), mung bean nuclease (MBN) was tested on part of the radiolabelled second strand. Titration revealed that 40 U MBN per µg of cDNA reduced the second strand size range to that of the first strand. Up to 160 U/µg did not further degrade the cDNA, confirming the specificity of this nuclease. The remaining cDNA (0.5 µg in 100 µl) was diluted into 500 µl of MBN reaction buffer and cleaved with 45 U of MBN at 37°C for 30 min. The

reaction was stopped by addition of EDTA to 20 mM. It was assumed that DNA ends were left in blunt form by MBN treatment. The cDNA was ethanol precipitated, resuspended in 100 µl TE and passed over a Sephadex G-50 spin column. Blunt ended cDNA was methylated with 5 U EcoRI methylase in 110 µl of reaction buffer (Promega Biotech) at 37°C for 1 h. The reaction mix was phenol-chloroform extracted, ethanol precipitated and resuspended in 12 µl water. Kinased EcoRI linkers (5'-CGGAATTCCG-3', 300 ng) were ligated to 9 µl of cDNA (about 1 µg = 1 pmol) in 20 µl of ligation buffer with 2 U T4 DNA ligase. The reaction was followed with 10^6 cpm of ^{32}P 5' end labeled linkers (0.3 pmol) and allowed to proceed overnight at 16°C. The solution was then diluted up to 200 µl with EcoRI reaction buffer and digested for 4 h with 100 U of EcoRI. Digested linkers were separated from cDNA by chromatography over a 5 ml Sepharose CL-4B column (Maniatis et al, 1982). The cDNA peak eluted first (in about 3 x 0.3 ml fractions) and contained 8% of the counts of the linker peak. After ethanol precipitation in the presence of 10 µg carrier wheat germ tRNA, half the cDNA (approximately 0.5 µg) was ligated to 1 µg of dephosphorylated lambda gt11 arms (Promega Biotech) in 10 µl at 16°C for 2 hours. Ligated products (0.5 µl) were observed as high M_r radioactive species on a 1% agarose gel. The ligated cDNA (9.5 µl) was packaged in vitro and titered on Y1090 cells in top agarose containing 10 µl of 100 mM IPTG and 10 µl of 10% (w/v) XGAL. Two thirds of the plaques contained recombinant inserts and 10^6 independent clones were obtained. One third of the library was plated at a density of 5 x 10^3-10^4 plaques/dish and screened with P47 antiserum at a dilution of 1:500 exactly as recommended by the supplier (Promega Biotech).

Titration of the alkaline phosphatase conjugate on purified platelet P47 showed that 0.1 ng could be detected at this antibody dilution. Approximately 30 immunoreactive clones of varying size and intensity were obtained. Initially 4 of these (clone 23, 34A, 34B and 37) were plaque purified and DNA prepared by the mini-lysate method (Davis et al, 1986). Three recombinants (34A, 34B and 37) liberated inserts when digested with EcoRI. These were subcloned into pUC118 (Viera and Messing, 1987) as both EcoRI fragments and as the complete KpnI-SacI inserts flanked on each side by approximately 1 kb of lambda DNA. Subsequently, all the remaining immunoreactive plaques from the initial plating were picked and frozen in simplified medium containing 7% (v/v) DMSO after various stages of purification. Subsequent to the isolation of all clones, the library was amplified by the plate lysate method and stored at 4°C and -70°C.

Two additional libraries were made subsequent to the first lambda gt11 expression library. The mRNA source in this case was parental HL-60 cells (from the ATCC) differentiated with 1μM RA for 5d. Synthesis of full length cDNA was optimized by the method of Gubler and Hoffman (1983), which was followed exactly as described in Davis et al (1986). Blunt-ended cDNA was methylated and EcoRI linkers were added as described above. After digestion with EcoRI, half the cDNA was ligated to dephosphorylated lambda ZAP and half to lambda gt11 arms. Each was packaged with the same extract (Packagene, Promega Biotech) but the lambda ZAP library contained only 10^4 independent clones, whereas the lambda gt11 library had at least 5×10^5 primary recombinants. All of the former and 10% of the latter were plated for screening. The

remainder of the lambda gt11 library was frozen at -70°C. Recombinants were identified by hybridization first to a 2 kb PvuII P47 cDNA fragment then to ^{32}P end labelled oligonucleotide (AB206) complementary to the extreme 5' end of P47 cDNA. Full length clones thus obtained were plaque purified. All 3 lambda ZAP clones (3A1, 2A1 and 2A3) were excised as pBluescript plasmid exactly according to the supplier (Stratagene). Full length clones from the lambda gt11 library were digested with EcoRI and analyzed by a fill-in reaction with [^{32}P]-α-dATP and electrophoresed on a 5% acrylamide gel (Maniatis et al, 1982). The longest recombinant (3-1) was subcloned into pUC118 and pUC119 as the KpnI-SacI insert.

2.5.2 DNA Sequencing

Single stranded DNA template from pUC118/119 was obtained by infection of MV1193 cells bearing the plasmid of interest (Viera and Messing, 1987). An overnight culture was diluted 1:10 (0.3 ml) and inoculated with 3 x 10^9 M13KO7 phage. After 1 h further growth, cells were diluted 1:4 into media containing 70 µg/ml kanamycin in order to select for infected cells. Cells were allowed to grow for an additional 8 h before phage were harvested by PEG precipitation (Davis et al, 1986). 1.5 ml of supernatant proved sufficient for up to 10 sequencing reactions (roughly 10 µg). DNA sequencing was originally carried out with Klenow fragment according to the BRL sequencing manual. This was superceded by the chemically modified T7 DNA polymerase method (Tabor and Richardson, 1987) using the Sequenase kit (United States Biochemical). The only modification to the suppliers protocol was to

carry out all reactions in drawn out capillary pipettes held tip down in Eppendorf microfuge tubes. DNA was sequenced either from M13 universal primers (15 and 17 nucleotide, BRL) or from specific primers based on sequence obtained from previous primers. The entire sequence of two clones (34A, 34B) was determined from nested sets of Exo III nuclease deletion mutants, generated exactly according to Henikoff (1984). The opposite strand was sequenced from subcloned EcoRI fragments and specific primers. Clones from the lambda ZAP library were converted to the single stranded form exactly as described by the supplier (Stratagene) and sequenced from the BRL reverse primer and specific primers. Occasionally sequencing was done by the double stranded method of Zhang et al (1988).

2.5.3 Expression of P47 Sequences

An NcoI site that contained the first in-frame ATG was created by site-directed mutagenesis of full length single stranded template originally used for sequencing. Two clones (34A and 3A1) were mutated using the single primer method (Zoller and Smith, 1984). The following conditions were adapted from the Bio-Rad Muta-Gene protocol. 2-3 µg of single stranded DNA template (either 34A or 3A1) was annealed to 70 pmol of kinased AB336 in 10 µl annealing buffer (20 mM Tris HCl (pH 7.4), 10 mM $MgCl_2$, 50 mM NaCl, 1 mM dithiothreitol), heated to 65°C and allowed to cool to room temperature in the water bath. The reaction was made 1 x in synthesis buffer (0.5 mM dNTP, 1 mM ATP, 30 mM Tris HCl (pH 7.4), 12 mM $MgCl_2$) and 1 µg of gene 32 protein, 2 U of T4 DNA polymerase and 2 U of T4 DNA ligase were added. The reaction was incubated at 22°C for 5

min then at 37°C for 90 min at which time it was stopped by the addition of 30 µl TE and 1 µl MES (pH 6.3). This mixture (2 µl) was used to transform competent DH5α cells, thereby yielding several thousand colonies. The G-G mismatch created by the mutagenic oligonucleotide was insufficiently destabilizing to screen colony lifts by differential hybridization (Zoller and Smith, 1984). Therefore, 10-fold excess cold primer complementary to the same region (AB206) was added to the colony hybridizations (based on Miyada and Wallace, 1987). Mutant colonies were then easily identified by washing in 6 x SSC at increasing temperatures (Zoller and Smith, 1984). A further modification permitted direct identification after a single room temperature wash. Colonies (200) were randomly picked onto a grid plate and grown for 24 hours such that they were very large. The corresponding colony lift contained much more target plasmid than mutagenic probe; hybridization to equilibrium (18 h) in the presence of 10-fold excess wild type primer allowed DNA from mutant colonies to anneal with most of the mutagenic primer and thus be detected after a single wash at 22°C. Positive clones detected by either method were digested with NcoI to confirm the mutation but only less than half released the expected 1.1 kb insert, probably because of incomplete synthesis from the single primer. The NcoI fragment from 34A was subcloned into pKK-233 (Amann and Brosius, 1985) as was the corresponding wild type PvuII fragment after in-frame NcoI linker (5'-CAGCCATGGCTG-3') addition and digestion with NcoI. Ligation reactions were used to transform JM109 cells and colony hybridization was used to detect recombinants. Overnight cultures of freshly transformed colonies were grown in the absence and presence of 0.1 mM IPTG. Cell pellets

from 1.5 ml of culture were lysed directly in 300 µl of sample buffer and 20 µl of this was electrophoresed on 11% acrylamide and stained with Coomassie Brilliant Blue. Immunoblot analysis was carried out on 2-4 µl of such lysates.

NcoI fragments from mutated 34A and 3A1 clones were also inserted into the NcoI site of pOTS Nco12 (of the pAS vector series (Schatzman and Rosenberg, 1987)) and transformed into N5151 host cells. Overnight cultures grown at 30°C were temperature induced in a 42°C shaking water bath and cultures analyzed as above. Alternatively, the unknown insert in pOTS Nco12 was dropped out by digestion with SacI and partial digestion with NcoI. The appropriate fragment comprised of vector and P47 NcoI insert was blunt-ended and re-circularized. The construct was transformed into AR68 cells and temperature-induced in the same manner.

In vitro transcription and translation of the P47 coding region was carried out in the pGEM-3Z vector (Promega Biotech). The 2 kbp PvuII fragment of P47 was cloned into the SmaI site. A mini-preparation of the resultant recombinant plasmid was linearized with BamHI and 1 µg used as a template in the Promega Biotech T7 RNA polymerase/rabbit reticulocyte system. Products of the in vitro translation were electrophoresed on an 11% acrylamide gel and exposed to film without fluorography. Similar reactions carried out with the pBluescript 3A1 clone yielded identical results.

2.5.4 Sequence Analysis

Current databases (EMBL, Genbank, PIR Swiss) were searched against the P47 protein and nucleic acid sequence using PIR software based on the FASTN, FASTP, and FASTA algorithms (Lipman and Pearson, 1985; Pearson and Lipman, 1988). Recent (later than May 1988) sequences of interest were entered manually and tested against P47 using the same algorithms. Theoretical analysis of P47 protein and nucleic acid sequence employed the University of Wisconsin Genetics Computer Group (UWGCG) software package (Devereux et al, 1984). PIR and UWGCG were accessed on the Ottawa SND Vax system. Recording and preliminary analysis of sequence data were done with Beckman Microgenie software installed on a Zenith 158 microcomputer.

Multiple sequence alignment was done with the ALIGN program (Bacon and Anderson, 1986) as modified by Dr. C.B. Harley, E. Harley and Vivian Li. Alignment of EF-hand domains was over the 30 residue stretch defined from crystallographic data by Kretsinger (1980). Conserved residues were weighted twice that of similar but non-conserved matches. The core set was tested against various sequences with or without known Ca^{2+} binding activity. Similarly, PKC phosphorylation sites were aligned over an empirically determined 10 residue stretch for 8 known PKC sites 4 at a time for all possible combinations. Matches between basic (arginine and lysine) or serine and threonine residues were weighted twice that of matches in the standard matrix. The best 4 of the 8 known sites were designated as a core set and used to test against other sequences.

RESULTS AND DISCUSSION

3. SYNERGISTIC EFFECTS OF PHORBOL ESTERS AND Ca^{2+} IONOPHORES ON HL-60 CELL DIFFERENTIATION

Results presented in this section show that the PKC and Ca^{2+} branches of PI-based signal transduction can act in a synergistic fashion to induce macrophage-like differentiation of HL-60 cells. The various cellular criteria used to assess differentiation induced by either a high concentration of TPA or a low concentration of TPA in conjunction with Ca^{2+} ionophore A23187 suggested that similar but not identical phenotypes were achieved. The generality of synergistic differentiation was shown with various biologically active phorbol esters, Ca^{2+} ionophores and myeloid leukemia cell lines. Synergistic and TPA-induced differentiation were assessed at a molecular level by hybridization analysis with 8 reporter genes, including the proto-oncogenes c-myc and c-fos. The implications of these results are discussed with respect to known and putative mechanisms of interaction between PKC and Ca^{2+}.

3.1 CELLULAR PARAMETERS OF SYNERGISTIC HL-60 DIFFERENTIATION

3.1.1 Alterations of HL-60 Cell Proliferation and Morphology

Initial establishment of the dose-response relationship for synergistic HL-60 cell differentiation relied on visual assessment of cell morphology. An interaction between phorbol esters and Ca^{2+} ionophores was obvious at a TPA concentration of 0.5 nM, which itself caused no morphological alterations (Fig. 3.1.1). In the presence of 300 nM Ca^{2+} ionophore A23187 this low concentration of TPA evoked marked adherence to substratum, cell clumping, formation of dendritic processes and a nearly complete inhibition of proliferation. This response was similar to cells treated with 10 nM TPA. For the sake of brevity in subsequent discussion the following notation will be used unless otherwise indicated: low TPA refers to 0.5 nM TPA, TPA/A23187 to co-treatment with low TPA and A23187, and finally high TPA to 10 nM TPA.

Analysis of cells stained with May-Grunwald-Giesma revealed that a similar phenotype was evoked by TPA/A23187 and high TPA (Fig. 3.1.1, e and f insets). HL-60 cells incubated with either low TPA or A23187 alone exhibited no change in nuclear to cytoplasmic ratio or azurophilic granulation when compared to either untreated or solvent treated cells (Fig. 3.1.1, a-d). However, the Ca^{2+} ionophore did stimulate degranulation and vacuole formation (Fig. 3.1.1, c). In contrast, TPA/A23187 caused a marked decrease in nuclear to cytoplasmic ratio and a loss of granulation, similar to treatment with high TPA (Fig. 3.1.1, e vs. f). Nuclear to cytoplasmic ratios after each drug treatment were quantitated by light microscopy (Table 3.1.1). Compared to untreated

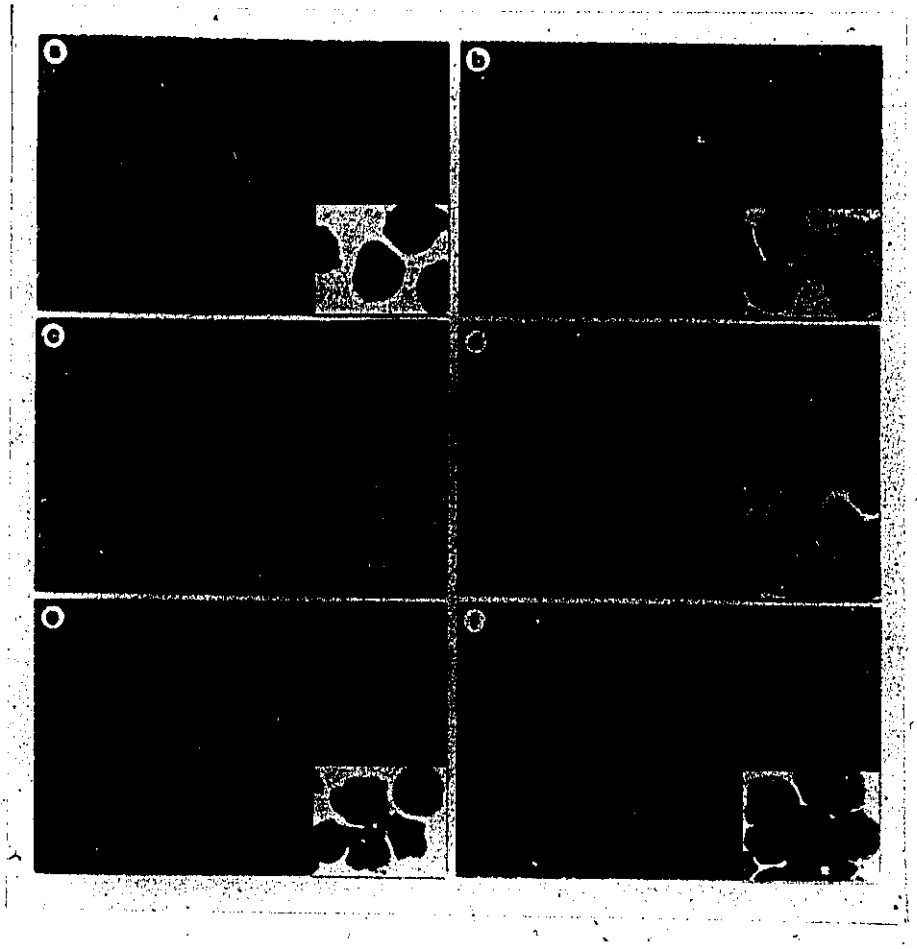


Fig. 3.1.1 Morphological changes in HL-60 cells after treatment with various combinations of A23187 and TPA. a, no treatment; b, carrier solvents alone; c, 300 nM A23187; d, 0.5 nM TPA; e, 0.5 nM TPA and 300 nM A23187; f, 10 nM TPA. Insets show corresponding May-Grunwald-Giemsa stained cells. All fields were chosen randomly by a naive observer. Final magnification is 75 X and 400 X for insets.

Table 3.1.1 Quantitation of HL-60 nuclear to cytoplasmic ratios

	untreated	solvent	0.5 nM TPA	300 nM AZ3187	TPA/AZ3187	10 nM TPA
nuclear diameter (µm)	14.4 ± 2.1	14.3 ± 1.7	15.5 ± 1.4	16.4 ± 2.5	11.3 ± 2.1	11.5 ± 1.7
cytoplasmic diameter (µm)	20.2 ± 3.4	19.2 ± 2.4	21.3 ± 1.7	22.9 ± 3.5	21.7 ± 3.8	22.7 ± 3.4
N/C ratio	0.73 ± 0.1	0.77 ± 0.1	0.73 ± 0.1	0.71 ± 0.1	0.49 ± 0.1*	0.53 ± 0.1*

HL-60 cells were exposed to various drug combinations for 72 h (solvent = 0.3 % (v/v) DMSO, 0.3 % (v/v) ethanol) and stained with May-Grunwald-Geimsa. Measurements ($n > 20$) and nuclear to cytoplasmic (N/C) ratios ($n > 40$) were determined by naive observers and are shown as mean ± SD. Ratios significantly less than that of untreated cells are indicated by * ($p < 0.05$, t-test).

cells only high TPA and TPA/A23187 induced a significant change in this criterion of differentiation.

In order to prove a synergistic (i.e., more than additive) effect occured it was necessary to demonstrate a leftward shift of log dose-response curves for differentiation. Cell growth and cell adherence were thus monitored over a range of TPA and A23187 concentrations for a 96 h time period (Fig. 3.1.2, e and f). Low concentrations of TPA caused a slight increase in cell proliferation whereas 8 nM TPA caused essentially complete growth arrest. Below 200 nM, the Ca^{2+} ionophore had no effect on proliferation but cytotoxic growth inhibition began to occur at 300 nM A23187. An ionophore concentration of 900 nM caused nearly complete cell lysis and dispersion of cells into small blebs. Despite its effect on proliferation, A23187 did not cause any coordinated changes in HL-60 phenotype indicative of monocytic differentiation (see Section 3.1.2-4). More to the point, 200 nM A23187 caused a leftward shift in the TPA dose-response curve by approximately a factor of 2. The A23187 dose-response relationship was also shifted leftward in a non-cytotoxic manner by 0.5 nM TPA. In each case the effect on cell growth and adherence was clearly more than additive, particularly as A23187 itself did not induce differentiation at any concentration.

Two other experimental parameters affected HL-60 cell growth and differentiation. Early experiments showed considerable variability in cell viability after treatment with A23187. Other investigators have reported that their attempts to observe synergistic HL-60 cell differentiation were unsuccessful because of A23187 cytotoxicity (Dr. R.

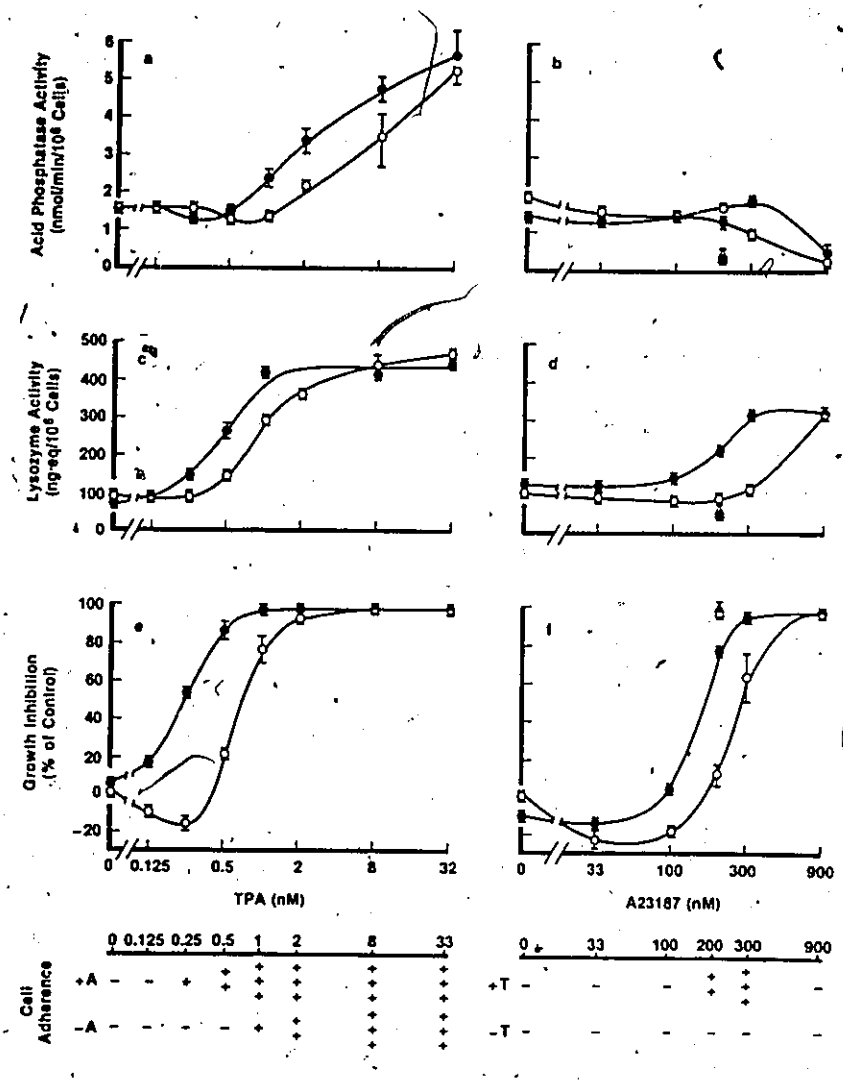


Fig. 3.1.2. Dose response curves for synergistic differentiation of HL-60 cells induced by TPA and A23187. Maturation was assayed by cellular acid phosphatase activity (a,b), secreted lysozyme activity (c,d) and growth arrest and morphology (e,f). a,c,e, TPA in the presence (●,+A) or absence (○,-A) of 200 nM A23187. b,d,f, A23187 in the presence (●,▲,+T) or absence (○,△,-T) of 0.5 nM TPA. Adherence was assessed visually (no adherent cells (-) to essentially all cell adherent with extensive clumping). Triangles represent cells grown in media buffered to pH 7.2 with 10 mM sodium bicarbonate. The scale for lysozyme activity should be multiplied by a factor of 5 because of an initial error in plotting the standard curve. Experimental points are for duplicate dishes and curves are representative of several experiments (n>3).

Dougherty, personal communication). Cell passage number, FBS lot number, the density of cell passage and media pH were varied to see if cytotoxicity could be ameliorated. Each parameter was without effect, except for media pH. A titration experiment revealed that A23187 caused cell lysis in media that was below pH 7.3. In one experimment at pH 7.2, 200 nM A23187 caused a greater than 80% reduction in cell growth when compared to pH 7.6 (Fig. 3.1.2, f, triangles). Subsequently, the pH of unsupplemented RPMI 1640 was found to vary considerably at the source (Central Media Facility, Department of Immunology, McMaster University). For this reason media was obtained from a commercial supplier, titrated to pH 7.6 and further buffered at this pH with HEPES. This consistently minimized the cytotoxicity of A23187.

A second parameter which improved the degree of synergistic differentiation was the use of carrier solvents. Although alone these diluents had no overt influence on HL-60 growth and differentiation by any criteria used (Fig. 3.1.1, panel b; see also Section 3.1.2-4), typically 20-30% more inhibition of cell growth and increased adherence were obtained compared to when drugs were delivered in aqueous diluent. It was thus possible that the concentrations used for much of this work (0.3% (v/v) DMSO, 0.3% (v/v) ethanol) augmented the synergistic response. However, synergistic differentiation was obtained in later experiments with solvent levels of 0.1% (v/v) or lower, suggesting that these agents were not crucial to the effect (see section 3.2).

A final caveat of synergistic differentiation was phenotypic variability in the HL-60 line. HL-60 cell cultures passaged for various periods of time exhibit heterogeneity in morphological, enzymatic and

molecular characteristics (see Section 1.2.2). Initially, an HL-60 subline of unknown passage number (the "Golde" subline) was used to investigate synergistic differentiation. These cells grew well in 10% (v/v) FBS/RPMI 1640 and had a doubling time of approximately 24 h. When a cell density of 5 x 10^5/ml was used, 0.5 nM TPA and 100 nM A23187 together gave a response similar to that seen with 5 nM TPA. Phenotypic drift in this subline with respect to synergistic differentiation was addressed by obtaining early passage HL-60 cells from the American Type Culture Collection. These cells required a 20% (v/v) FBS supplement for cell maintenance and growth, and had a doubling time of roughly 48 h. Although slightly higher drug concentrations were required, a similar response to TPA and A23187 was obtained with the parental HL-60 cell line. Except were noted (Section 4.2.2), all experiments reported in this work used HL-60 cells from the American Type Culture Collection.

3.1.2 Enzymatic Activities Increased During HL-60 Cell Differentiation

Growth arrest and morphological alterations were substantiated by two enzymatic activities characteristic of monocytic differentiation, namely acid phosphatase (Vorbrodt et al, 1979) and extracellular lysozyme (Polansky et al, 1982). The optimum length of exposure for induction of each marker was determined in preliminary time course experiments; acid phosphatase activity had the longest induction period as it was not evident until 4 d post-treatment (data not shown). This timepoint was therefore used for all dose-response determinations (Fig. 3.1.2). As for growth inhibition, a leftward shift in TPA and A23187 dose-response profiles was observed for each enzyme activity (Fig.

3.1.2, a-d). Likewise, the cytotoxic effects of A23187 were very marked at pH 7.2 but not at pH 7.6 (Fig. 3.1.2, b and d, triangles). High concentrations of A23187 caused some accumulation of extracellular lysozyme, possibly due to a stimulated release of granule contents as suggested by the highly vacuolated nature of HL-60 cells treated with A23187 (Fig. 3.1.1, c). Acid phosphatase activity was not altered by A23187 alone, suggesting it was an indicator of more complete cell maturation. Thus, using criteria classically used to define monocytic differentiation, Ca^{2+} ionophore A23187 itself caused little or no HL-60 maturation but strongly augmented differentiation in response to the phorbol ester TPA.

3.1.3 Induction of the OKM-1 Reactive Cell Surface Antigen

Human monocytes and granulocytes exhibit strong immunoreactivity with the OKM-1 mouse monoclonal antibody (Talle et al, 1983). Differentiated HL-60 cells exhibited an intense pattern of green fluorescence about their periphery, as expected for a cell surface antigen. In contrast, non-viable cells were labeled with diffuse green fluorescence over the entire cell body and had fluorescent orange nuclei indicative of cell permeation by ethidium bromide (Sauder et al, 1981). The degree of specific immunoreactivity observed after various treatments clearly reflected the synergistic effect of A23187 and TPA (Table 3.1.2). Visual assessments were corroborated by flow cytometry, which yielded similar results. Representative flowcytometer print-outs for each drug treatment are shown in Fig. 3.1.3. Although TPA/A23187 appeared to evoke a slightly less differentiated phenotype, the effects

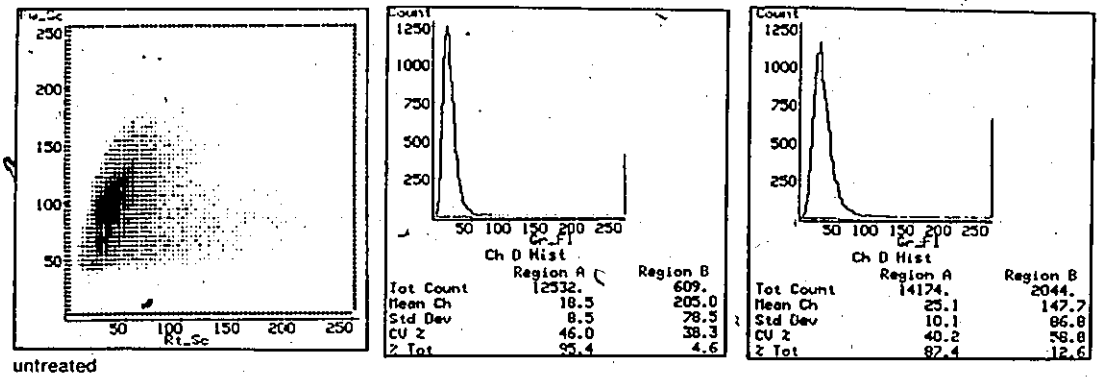
Table 3.1.2. OKM-1 reactivity of HL-60 cells after various treatments

Condition	Positive (%)
untreated	6 +/- 2
solvent	8 +/- 3
300 nM A23187	19 +/- 7
0.5 nM TPA	12 +/- 4
0.5 nM TPA + 300 nM A23187	77 +/- 4
10 nM TPA	87 +/- 7

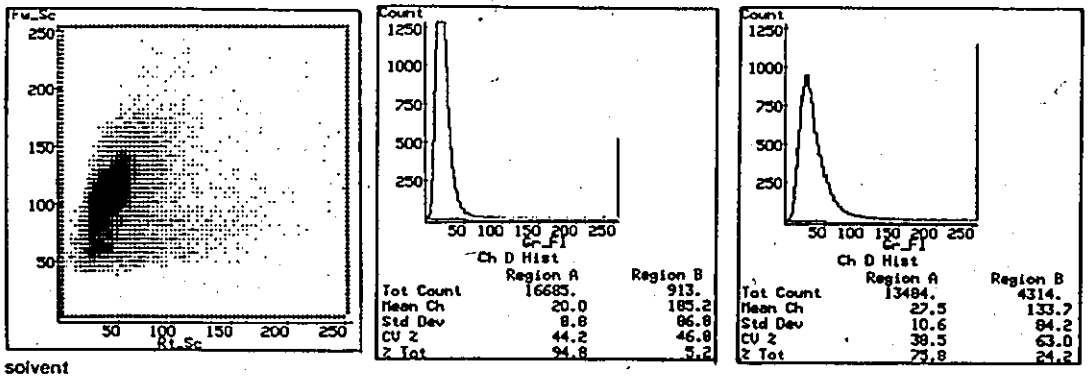
Expression of the macrophage-granulocyte cell surface glycoprotein detected by OKM-1 antibody was determined visually and by flow cytometry on HL-60 cells after 3 days continuous treatment. Five independent experiments (3 visual, 2 flow cytometric) were pooled and expressed as mean ± SEM. Cotreatment with TPA and A23187 had a greater effect than the sum of individual drug effects ($p < 0.01$, t-test).

Fig. 3.1.3. Representative printouts of OKM-1 reactivity in variously treated HL-60 cell populations: untreated, solvent (0.3% DMSO, 0.3% ethanol), low TPA (0.5 nM), A23187 (300 nM), low TPA/A23187 co-treatment and high TPA (20 nM). Left panels (1) show cellular complexity in terms of light scattering, middle panels (2) show control fluorescence after incubation with secondary FITC-goat anti-mouse and right panels (3) show OKM-1 reactivity. Threshold intensity for green fluorescence, determined from the trigger region, was set to a value of 50 (open bar along horizontal axis). Cells to the left of this value are compiled as region A, whereas cells to the right make up region B. Immunoreactivity was calculated as total fluorescent cells in region B (% value) corrected for blank fluorescence divided by 100 minus blank fluorescence.

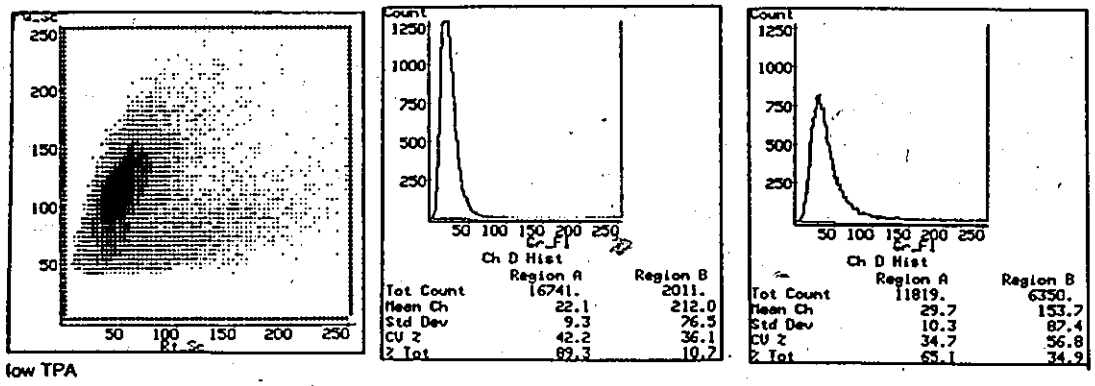
1 2 3



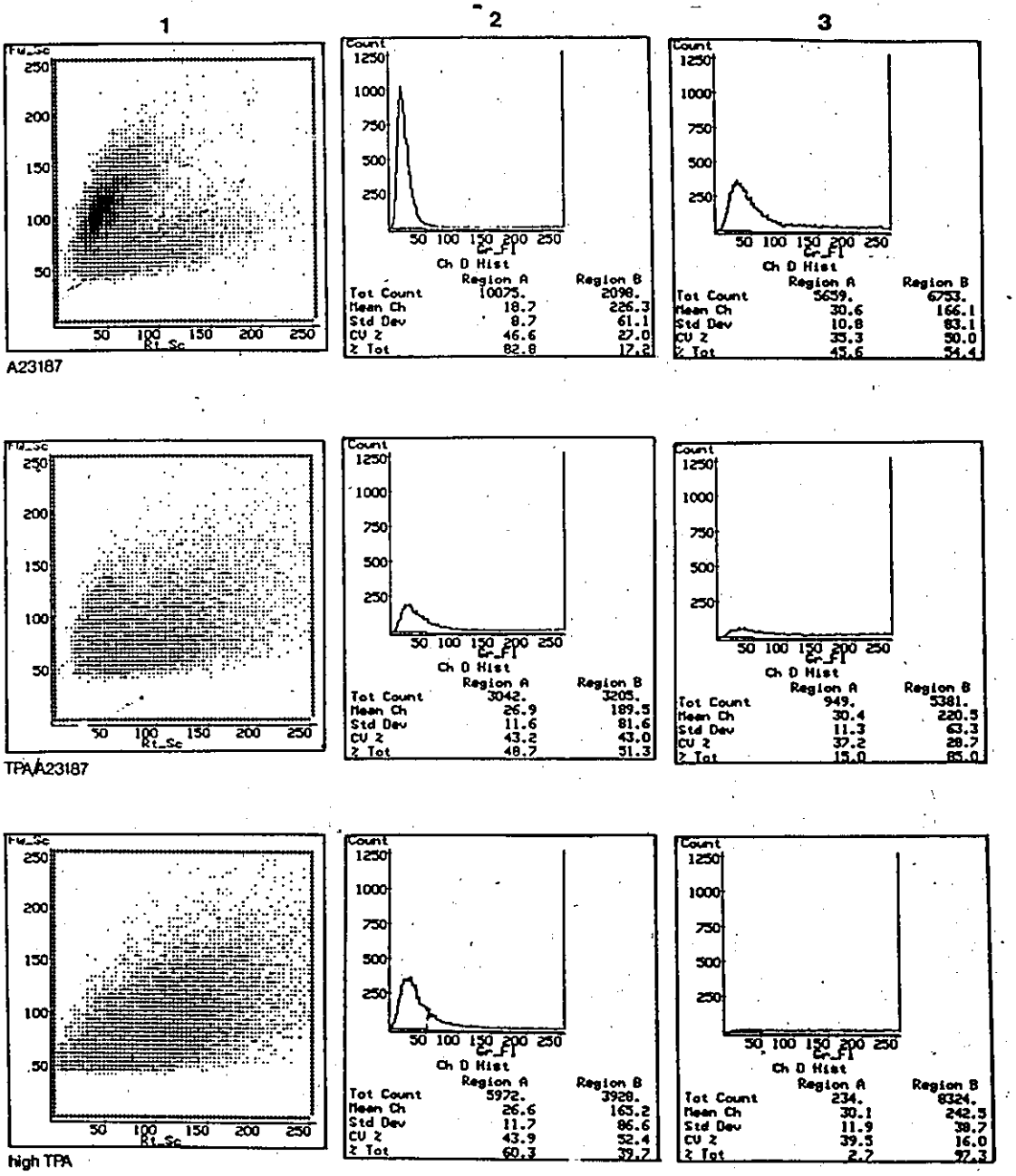
untreated



solvent



low TPA


1
2
3
Fw_Sc
Rt_Sc
Count
Ch D Hist
Region A
Region B
Tot Count 10075. 2098.
Mean Ch 18.7 226.3
Std Dev 8.7 61.1
CV % 46.6 27.0
% Tot 82.8 17.2
Tot Count 5659. 6753.
Mean Ch 30.6 166.1
Std Dev 10.8 83.1
CV % 35.3 50.0
% Tot 45.6 54.4
A23187
Tot Count 3042. 3205.
Mean Ch 26.9 189.5
Std Dev 11.6 81.6
CV % 43.2 43.0
% Tot 48.7 51.3
Tot Count 949. 5381.
Mean Ch 30.4 220.5
Std Dev 11.3 63.3
CV % 37.2 28.7
% Tot 15.0 85.0
TPA/A23187
Tot Count 5972. 3928.
Mean Ch 26.6 165.2
Std Dev 11.7 86.6
CV % 43.9 52.4
% Tot 60.3 39.7
Tot Count 234. 8324.
Mean Ch 30.1 242.5
Std Dev 11.9 38.7
CV % 39.5 16.0
% Tot 2.7 97.3
high TPA

of TPA/A23187 and high TPA on HL-60 cells were also evident in the complexity of light scattering (Fig. 3.1.3, panel 1).

The absence of OKM-1 reactivity in cells exposed to carrier solvent alone suggested that the enhancement of synergistic effects by solvent was not due to an overt induction of differentiation. interestingly, A23187 by itself induced a small increase in OKM-1 immunoreactivity (Fig. 3.1.3, d and Table 3.1.2). In conjunction with elevated lysozyme activity, this may indicate that A23187 caused slight differentiation.

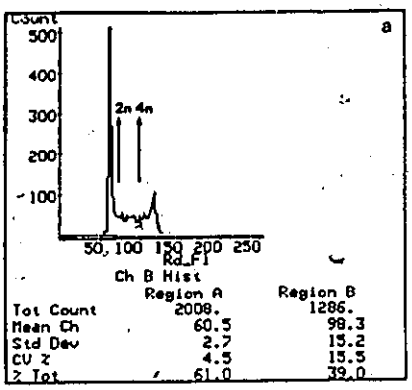
3.1.4 HL-60 Cell Cycle Distribution

The growth inhibition caused by treatment with A23187 and TPA was further characterized in a preliminary set of experiments by determining the DNA contents of various cell populations. Nuclei were stained with propidium iodide and analyzed for red fluorescence by flow cytometry (Table 3.1.3). Representative printouts for each drug treatment are shown in Fig. 3.1.4. Untreated and solvent treated HL-60 cells contained DNA corresponding to approximately 60% of cells in G1, 24% in S phase and 16% in G2/M. A notable increase in S phase cells and decrease in G1 phase cells was caused by treatment with low TPA. This was consistent with the observed increase in cell growth over a 96 h period (Fig. 3.1.2, e). Two concentrations of Ca^{2+} ionophore were used in these experiments; 200 nM A23187 did not alter cell cycle distribution, whereas in 2 of 3 experiments 300 nM A23187 caused a dramatic reduction in S phase cells and a concomitant increase in G1 phase cells (Table 3.1.3). This occurred without any visible decrease

Table 3.1.3. Cell cycle distribution of variously treated HL-60 cells. Nuclei from treated cells were stained with propidium iodide and analyzed on a flow cytometer, as in Fig. 3.1.4. Raw data for 3 independent experiments is shown. Not all parameters were investigated in each experiment (indicated by -).

CONDITION (expt #)	TIME (h)	GROWTH INHIB.	G1 PHASE	S PHASE	G2/M PHASE
UNTREATED					
1	72	0	60	24	16
2	48	-	61	23	16
3	48	0	61	-	-
SOLVENT					
1	72	-16	62	25	13
2	48	-	61	23	16
3	48	8	60	-	--
0.5 nM TPA					
1	72	-69	54	33	13
2	48	-	58	24	18
3	48	-65	57	-	-
200 nM A23187					
1	72	-23	61	26	13
2	48	-	65	21	14
3	48	-2	59	-	-
300 nM A23187					
1	72	51	85	6	9
2	48	-	88	7	5
3	48	-9	63	-	-
0.5 nM TPA + 200 nM A23187					
1	72	74	78	12	10
2	48	-	82	6	13
3	48	-34	65	-	-
0.5 nM TPA + 300 nM A23187					
1	72	104	80	6	14
2	48	-	75	7	18
3	48	73	80	-	-
10 nM TPA					
1	72	97	89	3	8
2	48	-	93	2	5
3	48	89	90	-	-

Fig. 3.1.4. Representative printouts of HL-60 cell cycle distribution after various treatments. Cell nuclei were isolated, stained with propidium iodide and analyzed on a flowcytometer: a, untreated; b, solvent (0.3% DMSO, 0.3% ethanol); c, 0.5 nM TPA; d, 200nM A23187; e, 300 nM A23187; f, 0.5 nM TPA and 200nM A23187; g, 0.5 nM TPA and 300 nM A23187; h, 20 nM TPA. Threshold fluorescence corresponding to G1 DNA content (2n) was set at a value of 75 using trout erythrocytes as an internal standard. Region A thus corresponds to G1 phase nuclei and region B to S+G2+M phase nuclei. Determination of G2/M DNA (4n) represented by the second peak is not shown because printouts were not obtained at the time.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Count
2n 4n
Rd_FI
Ch B Hist
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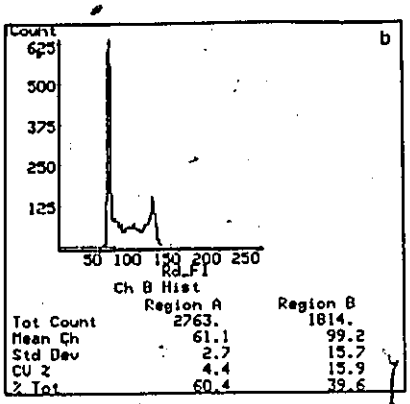
Std Dev 2.7 15.2
CV % 4.5 15.5
% Tot 61.0 39.0


b
Count
Rd_FI
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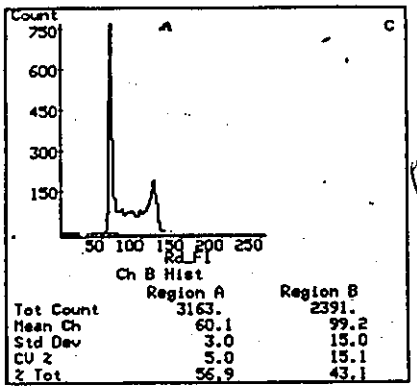
Mean Ch 61.1 99.2
Std Dev 2.7 15.7
CV % 4.4 15.9
% Tot 60.4 39.6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Rd_FI
Ch B Hist
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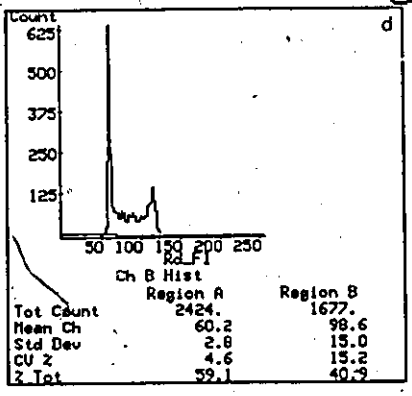
Mean Ch 60.1 99.2
Std Dev 3.0 15.0
CV % 5.0 15.1
% Tot 56.9 43.1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Rd_FI
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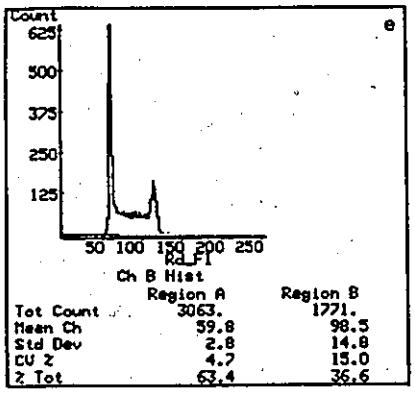
% Tot 59.1 40.9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Count
Rd_FI
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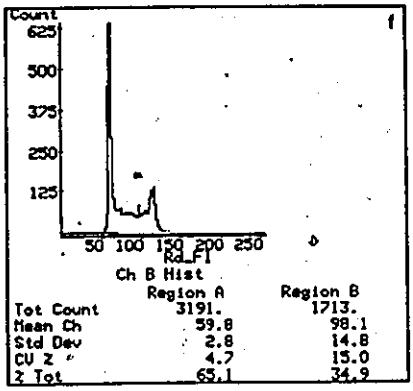
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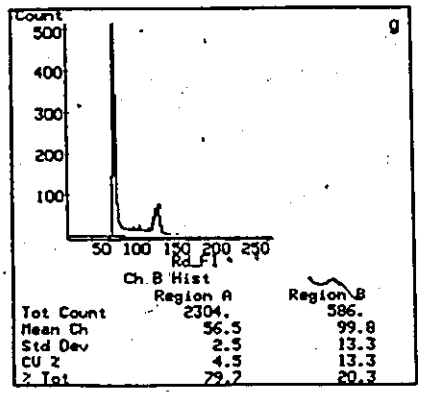
CV % 4.7 15.0
% Tot 65.1 34.9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Rd_FI
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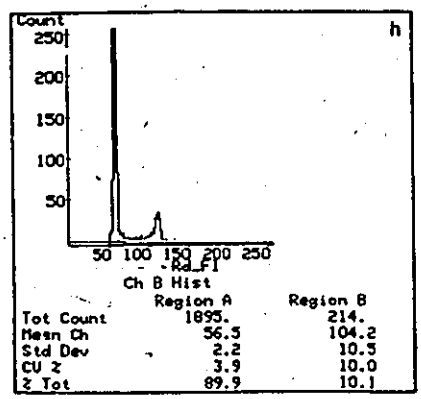
CV % 4.5 13.3
% Tot 79.7 20.3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Rd_FI
Ch B Hist
Region A Region B
Tot Count 1895. 214.
Mean Ch 56.5 104.2
Std Dev 2.2 10.5
CV % 3.9 10.0
% Tot 89.9 10.1

in cell viability. However, in each instance where A23187 alone had no effect on cell cycle distribution, co-treatment with 0.5 nM TPA caused a marked accumulation of cells in G1. This response was qualitatively similar to that obtained with 10 nM TPA, which itself induced virtually complete cell cycle exit. Although not carried out in sufficient replicates to quantitate changes in cell cycle distribution, these results are corroborated by other studies on HL-60 cells (eg. Palis et al, 1988; Yun and Sugihara, 1986).

3.1.5 Effects of Other Phorbol Esters and Ionomycin

Among other criteria, implication of PKC in a biological process requires a demonstration of appropriate structure-activity relationships for a series of different phorbol esters (Vandenbark et al, 1984). In addition, the specificity of A23187 is best confirmed with another structurally unrelated ionophore (Tsuda et al, 1985). Synergistic HL-60 cell differentiation was tested with the biologically active phorbol esters TPA, PDBu and PDD, an inactive phorbol derivative, 4-α-PDD and the Ca^{2+} ionophore ionomycin (Fig. 3.1.5). Differentiation was assessed using the same enzymatic, morphological and growth criteria as described above. The optimal concentration of each phorbol ester required to manifest synergistic differentiation was determined from effects on growth inhibition and cell morphology in preliminary experiments. The activity of each phorbol derivative matched that shown previously for the HL-60 system, the rank order of potency being TPA > PDD > PDBu >>> 4α-PDD (Vandenbark et al, 1984). Both A23187 and ionomycin were tested for synergism with each phorbol ester. Unlike A23187, which has equal

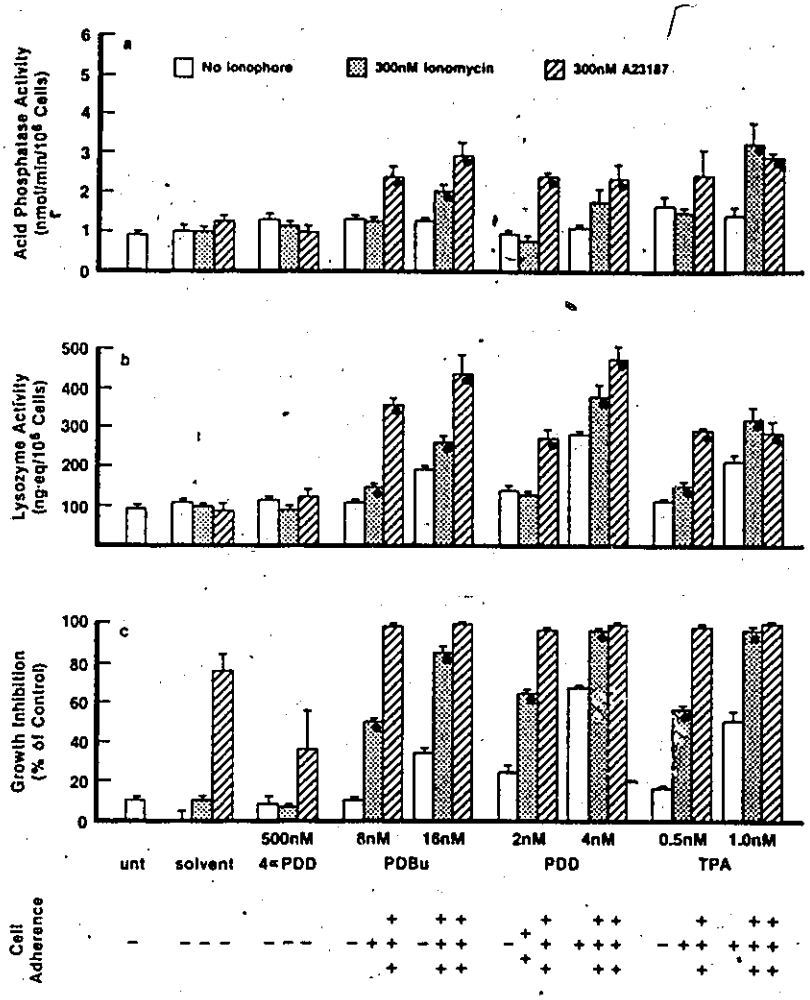


Fig. 3.1.5. Synergistic differentiation of HL-60 cells induced by various phorbol esters and Ca^{2+} ionophores. Concentrations of active phorbol esters used approximate the inflection point of dose response curves. Untreated (unt) and solvent (0.3% (v/v) DMSO, 0.3% (v/v) ethanol) represent control conditions without phorbol esters. Phorbol esters were added to cells either alone (open bars), or with 300 nM ionomycin (stipled bars), or with 300 nM A23187 (hatched bars). As for Fig. 3.1.2, the lysozyme scale should be multiplied by a factor of 5. Bars with dots in the upper right corner indicate that phorbol ester and Ca^{2+} ionophore co-treatment had a greater effect than the sum of individual drug effects ($p<0.05$, t-test). Similar data were obtained in at least 3 separate experiments.

selectivity for Ca^{2+} and Mg^{2+} (Chapman et al, 1987), ionomycin strongly prefers Ca^{2+} over other divalent cations (Liu and Hermann, 1978). Although the concentration of A23187 used in this set of experiments (300 nM) caused significant growth inhibition, morphological differentiation was markedly enhanced by A23187 for each concentration of active phorbol derivative (Fig. 3.1.5). Enzymatic markers, which were not altered by A23187 alone, also showed statistically significant increases compared to the summation of phorbol ester and ionophore individually. Thus each of the active phorbol esters synergistically interacted with A23187 to cause HL-60 cell differentiation. As expected, 4-α-PDD either in the presence or absence of Ca^{2+} ionophore had no effect on differentiation.

In contrast to A23187, ionomycin (300 nM) did not alter cell growth itself; nonetheless, it did cause a marked synergistic inhibition of proliferation with each active phorbol ester (Fig. 3.1.5). Enhanced expression of secreted lysozyme activity by ionomycin was observed with PDBu and TPA at both concentrations tested, and for the higher concentration of PDD used. Synergistic induction of acid phosphatase activity was observed only with 1 nM TPA. This discrepancy between ionomycin and A23187 is discussed in the next section.

3.1.6 Quantitation of Intracellular Ca^{2+}

In order to confirm that the two ionophores used in these studies elevated intracellular Ca^{2+} (denoted $[Ca^{2+}]_i$) in HL-60 cells and also to define the extent and time course of their effects, $[Ca^{2+}]_i$ was monitored with the fluorescent indicator quin2 (Tsien et al, 1982).

Uptake of quin2/AM and its conversion to quin2 by cytoplasmic esterases in HL-60 cells was estimated from F_{max} after cell disruption by NP-40 (Tsien et al, 1982); the signal obtained corresponded to a concentration of 20 μM quin2 in simplified medium. Based on a mean HL-60 volume of 800 μm^3 (Palis et al, 1988), the intracellular concentration of quin2 was therefore approximately 2-4 mM. This is towards the high end of loading in other cells types and may be sufficient to substantially buffer Ca^{2+} transients (reviewed in Rink and Pozzan, 1985). Tracings reproduced in Figure 3.1.6 and 3.1.7 show that both A23187 and ionomycin increased $[Ca^{2+}]_i$ in undifferentiated HL-60 cells. Basal Ca^{2+} levels were calculated as 106 ± 16 nM (n = 11, range = 64-123 nM); this compares with published values for undifferentiated HL-60 cells of 117 ± 6 nM (Levy et al, 1988), 102 ± 6 nM (Hrsuka et al, 1988) and 131 ± 19 nM (Okazaki et al, 1986). Typically, 100 nM A23187 caused a transient rise from basal levels up to a peak concentration of 809 ± 232 nM (range = 530-1,230 nM, n=6). After 5 min $[Ca^{2+}]_i$ decayed to 274 ± 78 nM (range = 182-431 nM, n=6), approximately 2-3 fold higher than resting levels (Fig. 3.1.7 a). Elevation of $[Ca^{2+}]_i$ was largely dependent on extracellular Ca^{2+}. In Ca^{2+}-free buffer, A23187 increased $[Ca^{2+}]_i$ to only 158 nM and even this returned to near basal values by 5 min (Fig. 3.1.7 b). The slight increase may have been due to release from intracellular stores or a nominal amount of Ca^{2+} in the buffer (estimated at 10 μM in the absence of EGTA, Tsien et al (1982)). Finally, it has been reported that A23187 exhibits significant fluorescence at the wavelengths used for quin2 and must therefore be used judiciously (Rink and Pozzan, 1985). When A23187 at 200 nM was

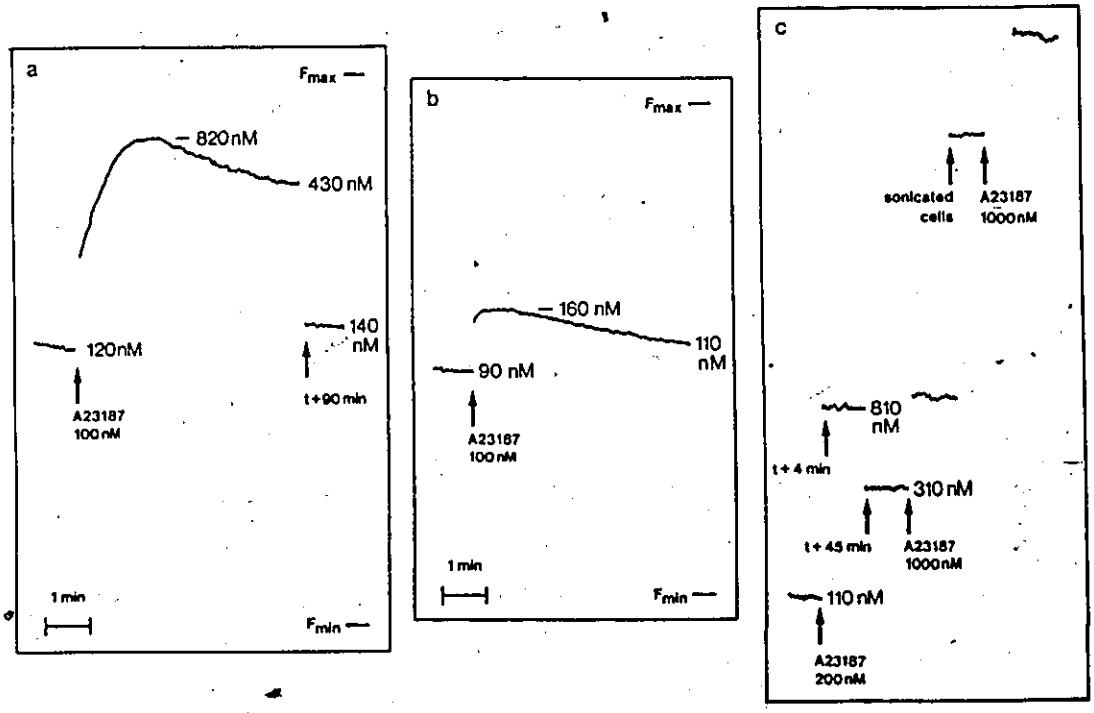


Fig. 3.1.6. Measurement of $[Ca^{2+}]_i$ in HL-60 cells treated with A23187. Undifferentiated HL-60 cells loaded with quin2 were resuspended in simplified medium and fluorescence recorded before and after addition of A23187; a, typical $[Ca^{2+}]_i$ trace immediately after addition of A23187 and at equilibrium 90 min later; b, as for (a) but in nominally Ca^{2+} free buffer; c, effect of high A23187 concentration on fluorescence. Numbers indicate calculated $[Ca^{2+}]_i$ rounded to 2 significant digits. F_{min} and F_{max} used to calculate Ca^{2+} concentrations were taken as the signal after cell disruption by NP-40, in the presence and absence of EGTA respectively.

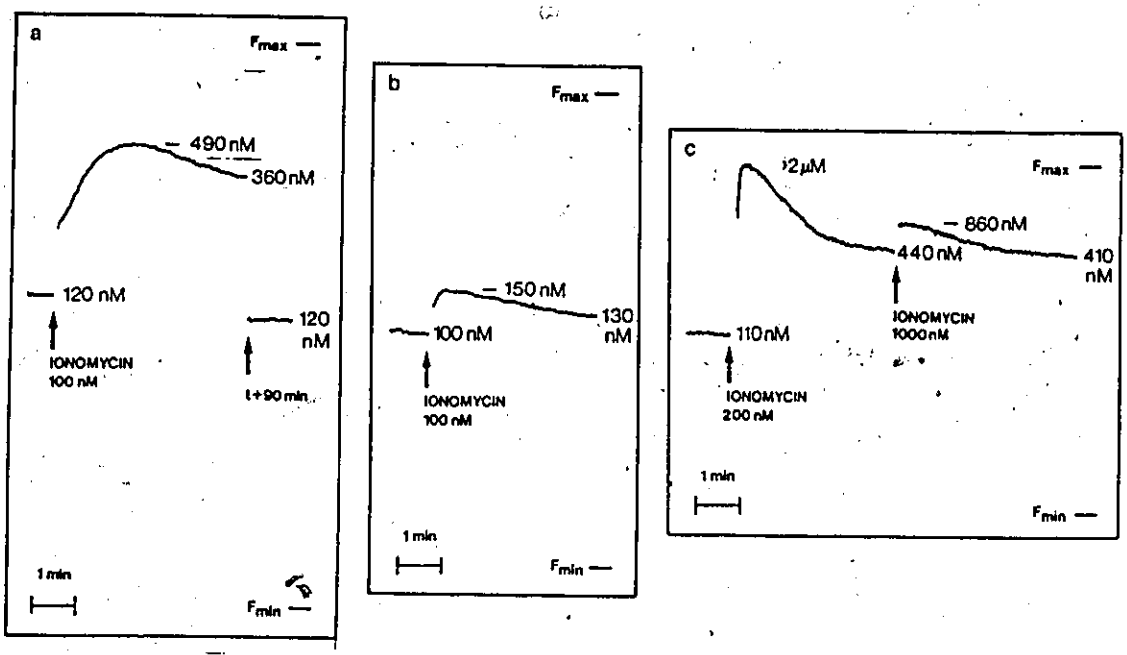


Fig. 3.1.7. Measurement of $[Ca^{2+}]_i$ in HL-60 cells treated with ionomycin. Experiments were carried out as described in Fig. 3.1.6: a, typical $[Ca^{2+}]_i$ trace immediately after ionomycin addition and at equilibrium 90 min later; b, as for (a) but in nominally Ca^{2+} free buffer; c, effect of high ionomycin concentration on $[Ca^{2+}]_i$.

added to lysed cells no appreciable increase in fluorescence occured. However, addition of 1,000 nM A23187 caused a significant increase in F_{max} (Fig. 3.1.7 c). If this was taken into account, the increase in $[Ca^{2+}]_i$ beyond that caused by 200 nM A23187 was negligible.

Ionomycin was also able to increase $[Ca^{2+}]_i$, but was less active than A23187. Treatment with 100 nM ionomycin caused a peak value of 394 ± 151 nM (range = 220-491 nM, n = 3). By 100 min this had decayed to basal concentrations (Fig. 3.1.7 a). The reduced ability of ionomycin to synergize with phorbol esters thus correlated with its lower Ca^{2+} mobilizing activity. Although ionomycin forms a 1:1 complex with divalent cations compared to the $(A23187)_2Ca^{2+}$ complex (Liu and Hermann, 1978), the ionomycin chelate is highly pH dependent and at pH 7.6 less than 5% partitions into an organic phase (Liu and Hermann, 1978). Despite this, Ca^{2+} transport by ionomycin appeared to saturate at 200 nM (Fig. 3.1.7 c). As for A23187, elevation of $[Ca^{2+}]_i$ by ionomycin depended strongly on extracellular Ca^{2+} (Fig. 3.1.7 b).

Cooperativity between TPA and A23187 in the elevation of $[Ca^{2+}]_i$ was tested but the phorbol ester had no effect either in the presence or absence of A23187 (not shown). Carrier solvents alone also did not affect $[Ca^{2+}]_i$ over the concentration range 0.3-2.0% (v/v) as reported by Hrsuka et al, (1988). In one experiment DMSO appeared to depress the activity of A23187 in a dose dependent manner. However, this effect was apparent only at concentrations of greater than 1% (v/v) DMSO. In any event, neither DMSO nor ethanol enhanced Ca^{2+} influx by the ionophore.

The concentration of Ca^{2+} ionophore required to sustain elevated $[Ca^{2+}]_i$ was somewhat lower than that needed for synergistic

differentiation, particularly since a 25 fold higher cell density was used in quin2 assays. This may reflect differences between the culture medium and the simplified medium used in quin2 assays. In particular, the 20% (v/v) FBS supplement to RPMI 1640 probably reduced the effective ionophore concentration (Drummond et al, 1987).

3.1.7 Synergistic Differentiation of U937 Histiocytic Leukemia Cells.

To test whether synergistic differentiation was peculiar to the HL-60 cell line, trial experiments were carried out with U937 cells. This monoblastic leukemia line also differentiates into macrophage-like cells when treated with phorbol esters (reviewed in Harris and Ralph, 1985). U937 cells were unaffected by low TPA concentrations (0.5 and 1.0 nM), but 20 nM TPA caused cell adherence and moderate growth arrest (Fig 3.1.8). Low TPA concentrations in combination with 400 nM A23187 also caused inhibition of cell proliferation and morphological alterations, similar to high TPA. Unlike HL-60 cells, the U937 response appeared more transient in that populations which were largely adherent by 12 h had detached by 48 h. In any event, it appeared that synergistic differentiation of myeloid leukemia cells was a general phenomenon.

3.2 GENE REGULATION DURING MONOCYTIC HL-60 CELL DIFFERENTIATION

3.3.1 Dot Blot Analysis of c-myc and Actin RNA

Initially, differentiation induced by TPA/A23187 and high TPA were compared by 2D-PAGE; however, no obvious differences in protein

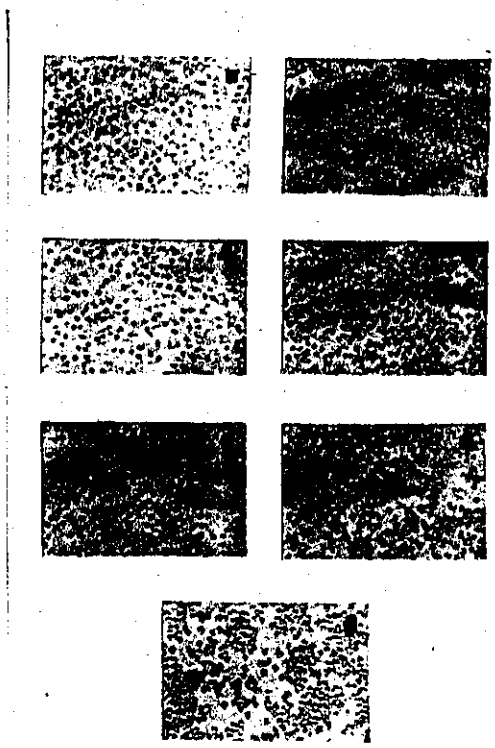


Fig. 3.1.8. Synergistic differentiation of U937 cells. Photomicrographs were taken 24 h after after treatment with various combinations of A23187 and TPA: a, untreated; b, 0.5 nM TPA; c, 1nM TPA; d, 400 nM A23187; e, 0.5 nM TPA and 400 nM A23187; f, 1 nM TPA and 400 nM A23187; g, 20 nM TPA. Final magnification is approximately 50 X.

expression were observed. Molecular analysis of synergistic HL-60 differentiation was therefore carried out with cDNA and oligonucleotide probes complementary to mRNAs either known or likely to be regulated during differentiation. By employing several different marker genes, a detailed comparison between TPA-induced differentiation and synergistic differentiation was made. It should be stressed that all subsequent discussion refers only to stable mRNA levels; transcription, mRNA stability, translational efficiency and protein turnover were not examined. Similar conditions and control treatments to cellular studies were used with the exception that cell density was increased from 2 x 10^5/ml to 1 x 10^6/ml. This improved RNA yields and allowed multiple assessments to be carried out with the same samples. It was necessary to increase drug concentrations to maintain the effect of synergistic differentiation: low TPA was increased from 0.5 nM to 1 nM and high TPA from 10 nM to 20 nM, whereas A23187 was increased from 200 nM to 400 nM. The abbreviations low TPA, high TPA and TPA/A23187 now refer to these drug concentrations. Differentiation-specific effects at these higher concentrations were confirmed by cell morphology, growth inhibition, OKM-1 immunoreactivity and cell cycle distribution. Differentiation was also evidenced by the changes in gene expression described below.

Initial investigations were carried out by dot blot quantitation of c-myc mRNA levels since repression of this abundant transcript correlates closely with the onset of differentiation (reviewed in Collins, 1987; see section 1.3.1 for specific references). Hybridization of myc oligonucleotide to total HL-60 RNA was normalized to either signals from a conserved actin oligonucleotide (complementary

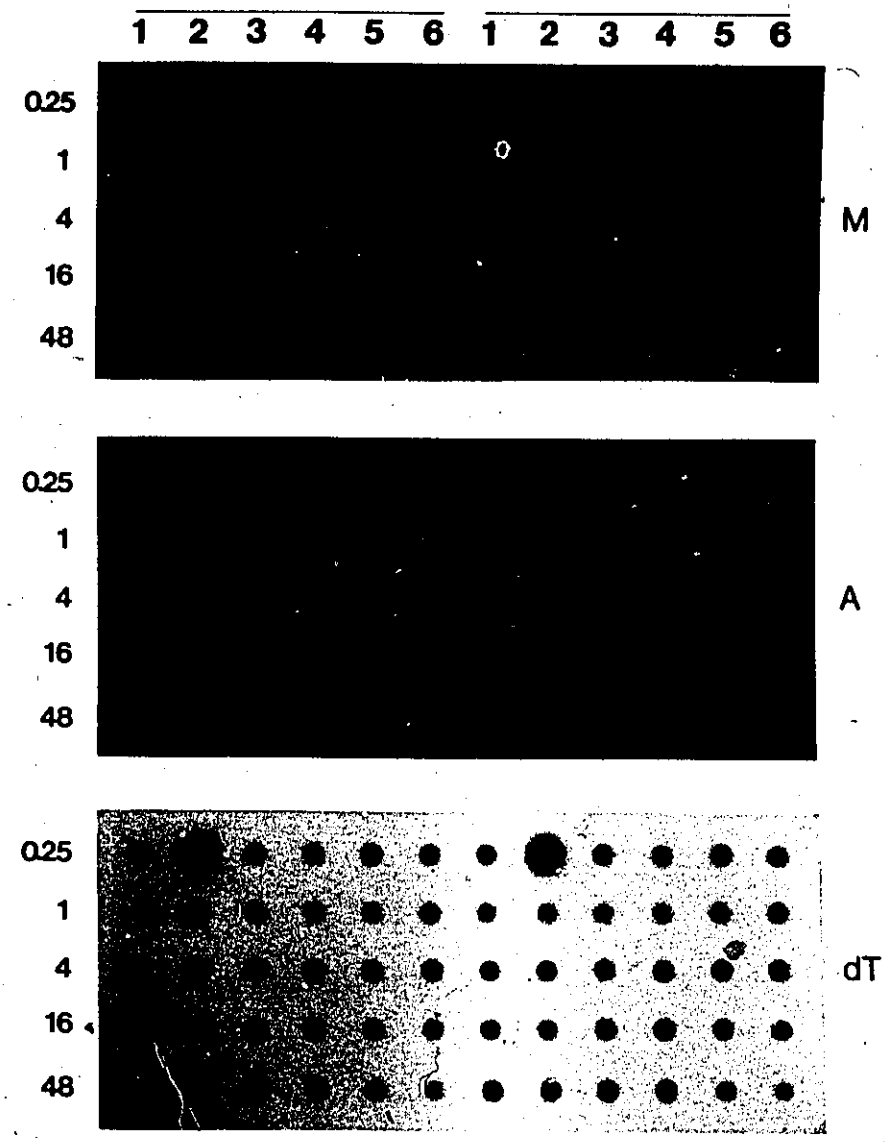


Fig. 3.2.1. Dot blot analysis of c-myc, actin and poly(A) RNA signals in HL-60 cells. Oligonucleotides complementary to c-myc (M), actin (A) and poly(A) (dT) were used to probe total RNA (1 µg) from variously treated cultures: 1, untreated; 2, solvent; 3, 1 nM TPA; 4, 400 nM A23187; 5, 1 nM TPA and 400 nM A23187; 6, 20 nM TPA. Length of each treatment before RNA isolation is indicated to the left in h. Duplicate dots are shown but RNA was actually spotted in triplicate. The intense spot arose from a dilution error.

to α, β and γ-actins) or from $(dT)_{18}$ (complementary to total poly(A)$^+$ mRNA). Autoradiographic signals were measured by densitometry for successive hybridizations with myc, actin, and $(dT)_{18}$ oligonucleotides. Representative autoradiograms are shown in Fig. 3.2.1. Absolute measurements for each signal averaged over a number of independent experiments are recorded in Table 3.2.1. Undifferentiated HL-60 cells were estimated to contain approximately 400 c-myc mRNA molecules/cell, based on cDNA standards dotted on the same filter. Although a transient decline in c-myc levels occured with all drug regimes including the solvent control (see below), only TPA/A23187 and high TPA permanently depressed c-myc expression. Typically either of these two treatments reduced c-myc transcript abundance to 10% of that in undifferentiated cells. Thus, as for cellular markers of differentiation, A23187 and TPA regulated c-myc mRNA in a synergistic fashion.

Precise quantitation was difficult because both normalization signals were unsatisfactory in some regards. Actin levels remained relatively constant, except at 16 h when a slight elevation occured in cells which eventually differentiated (Table 3.2.1). When normalized to actin, c-myc transcripts were therefore underestimated at this timepoint such that c-myc abundance sometimes appeared to increase after 48 h (Table 3.2.1). Normalization to $(dT)_{18}$ suffered a similar setback in that weaker hybridization occured at the 48 h time point when cells were fully differentiated (Fig. 3.2.1). This also masked the decline in c-myc levels. A further problem with the original $(dT)_{18}$ procedure was that filters containing many dots theoretically contained more target

Table 3.2.1. Summary of c-myc and actin RNA abundance in variously differentiated HL-60 cells. Total RNA was isolated from HL-60 cells after the indicated treatments and probed with myc and actin oligonucleotides. Values are averages of densitometric scans that were first normalized to signals from all untreated samples in each individual experiment. Each experimental condition was dotted and scanned in triplicate; the number of individual experiments contributing to each value is indicated by n. Ratios were determined within each individual experiment then averaged; n indicates the number of experiments for which this was possible. Values for c-myc abundance that were significantly less than that of corresponding untreated sample are indicated by * ($p < 0.05$).

CONDITION (time in h)	c-myc RNA (x ± SD, n)	actin RNA (x ± SD, n)	myc/actin ratio (x ± SD, n)
UNTREATED			
0.25	1.01 ± 0.04, 4	1.03 ± 0.04, 3	0.97 ± 0.06, 3
1	1.02 ± 0.17, 3	0.87, 1	1.11, 1
4	0.92 ± 0.07, 2	1.04, 1	0.93, 1
16	0.99 ± 0.17, 3	1.09, 1	0.78, 1
48	1.03 ± 0.30, 4	0.96 ± 0.05, 2	1.17 ± 0.25, 2
SOLVENT			
0.25	0.89 ± 0.45, 5	0.94 ± 0.08, 3	1.21 ± 0.29, 3
1	0.46 ± 0.19, 6 *	0.95 ± 0.05, 4	0.46 ± 0.23, 4
4	0.30 ± 0.14, 5 *	0.93 ± 0.05, 4	0.26 ± 0.06, 4
16	0.45 ± 0.14, 6 *	1.08 ± 0.02, 4	0.37 ± 0.13, 4
48	0.89 ± 0.30, 6	1.05 ± 0.18, 4	0.99 ± 0.27, 4
1 nM TPA			
0.25	1.08 ± 0.49, 5	0.99 ± 0.09, 4	1.16 ± 0.46, 4
1	0.59 ± 0.14, 6 *	0.98 ± 0.06, 4	0.56 ± 0.13, 4
4	0.61 ± 0.13, 5 *	0.82 ± 0.14, 4	0.69 ± 0.06, 4
16	0.51 ± 0.19, 6 *	1.13 ± 0.24, 4	0.41 ± 0.15, 4
48	0.81 ± 0.25, 6	1.29 ± 0.26, 4	0.73 ± 0.22, 4
400 nM A23187			
0.25	1.44 ± 0.54, 5	0.99 ± 0.19, 4	1.57 ± 0.63, 4
1	1.04 ± 0.46, 6	0.99 ± 0.11, 4	0.99 ± 0.38, 4
4	0.41 ± 0.19, 5 *	0.83 ± 0.07, 4	0.40 ± 0.11, 4
16	0.41 ± 0.17, 6 *	0.96 ± 0.18, 4	0.37 ± 0.07, 4
48	0.83 ± 0.34, 6	1.37 ± 0.15, 4	0.70 ± 0.07, 4
TPA/A23187			
0.25	1.56 ± 0.43, 5	1.02 ± 0.15, 4	1.60 ± 0.38, 4
1	1.75 ± 1.05, 6	0.93 ± 0.12, 4	1.64 ± 0.63, 4
4	0.29 ± 0.21, 5 *	0.58 ± 0.12, 4	0.34 ± 0.09, 4
16	0.14 ± 0.09, 6 *	1.22 ± 0.21, 4	0.08 ± 0.02, 4
48	0.12 ± 0.07, 6 *	0.78 ± 0.24, 4	0.11 ± 0.03, 4
20 nM TPA			
0.25	0.82 ± 0.15, 2	1.04, 1	0.69, 1
1	0.54 ± 0.13, 3 *	0.79, 1	0.65, 1
4	0.32 ± 0.14, 2 *	0.71, 1	0.31, 1
16	0.20 ± 0.10, 3 *	1.65, 1	0.07, 1
48	0.24 ± 0.10, 3 *	1.05, 1	0.13, 1

poly(A) tail (upto 40 pmol) than $(dT)_{18}$ probe (18 pmol). Under these conditions, non-equilibration binding of probe caused irreproducible normalization signals. Addition of excess cold $(dT)_{18}$ to the hybridization solution corrected the problem and allowed its use in determination of metallothionein expression (see Section 3.3). Myc/actin ratios are reported in Table 3.2.1 since most experiments were carried out with these 2 oligonucleotides.

Unlike for cellular parameters of differentiation, carrier solvent treatment caused a marked alteration of c-myc transcripts (Table 3.2.1). A 4 h exposure of cells to 0.3% (v/v) DMSO and 0.3% (v/v) ethanol resulted in a 50% decline of myc hybridization compared to untreated controls. By 16 h, however, the myc signal had nearly returned to its basal level. Given the qualitative observation that DMSO and ethanol enhanced synergistic differentiation (see Section 3.1.1), it was possible that an interaction between all three control treatments was necessary to induce differentiation. The effects of DMSO on HL-60 cell differentiation and c-myc mRNA are well documented (Westin et al, 1982a); in particular, pretreatment with 1.5% (v/v) DMSO sensitizes cells to TPA (Fibach et al, 1982). Titration of the solvent effect on c-myc revealed that at 0.1% (v/v) the diluents had little effect but that at 0.3% (v/v), the concentration used for many early experiments, a reproducible decline in c-myc transcripts occured (Table 3.2.2). It was thus unclear if the solvent effect masked changes in c-myc caused by either TPA or A23187 or if these agents alone induced a transient decline in c-myc abundance. Dissection of the effects of DMSO and ethanol indicated that, at 0.3% (v/v) for each solvent, most of the

Table 3.2.2 Influence of carrier solvent on c-myc RNA abundance

	DMSO	Ethanol	DMSO + Ethanol
0.0% (v/v)	1.0 ± 0.2		
0.1% (v/v)	0.97	1.0	1.0
0.3% (v/v)	0.48	0.90	0.35
1.2% (v/v)	0.04	0.29	----

Total RNA (1 µg) isolated from undifferentiated HL-60 cells treated for 4 h with solvent was spotted onto nitrocellulose and probed with myc oligonucleotide. Densitometric values were normalized to signal from untreated controls (shown as mean ± SD for 6 dots on the filter) and reported as the mean of duplicate dots from a single experiment.

decline was due to DMSO (Table 3.2.2). At concentrations at or below 0.1% (v/v) neither solvent had any significant effect on c-myc expression, either alone or in combination.

Although the solvent-induced decline in c-myc RNA was not accompanied by diminished cell proliferation (Fig. 3.1.2), there was an obvious concern about the validity of synergistic differentiation. Experiments to investigate mRNA levels during differentiation were thus carried out under identical conditions except that solvent levels were maintained at or below 0.1% (v/v) final concentration of each solvent. Under this condition, synergistic differentiation was phenotypically indistinguishable from that in the presence of 0.3% (v/v) carrier solvents. This is discussed in the next section.

3.2.2 Northern Analysis of mRNAs Regulated During Monocytic Differentiation

Although c-myc and actin were of sufficiently high abundance to quantitiate by dot blot analysis, attempts to use this method with other genes (e.g. c-fos) proved futile because signals were overwhelmed by non-specific background hybridization. Northern analysis was thus used for all genes studied. In addition to increased sensitivity, this method permitted signals arising from distinct transcripts to be distinguished. Given the effect of 0.3% (v/v) carrier solvents on c-myc mRNA abundance, it was established that synergistic differentiation did not depend on diluent concentration. Results shown in all figures from this point were obtained at concentrations of less than or equal to 0.1% (v/v). With the exception of c-myc, these results matched data for

identical treatments under previous solvent conditions. Each molecular marker of differentiation will be discussed individually.

i) Re-analysis of c-myc expression

To confirm the effects of TPA and A23187 on c-myc mRNA identified by dot blotting, Northern blots of total cellular RNA from various treatments at various times were probed with the myc oligonucleotide. Specificity of the myc oligonucleotide was confirmed by its hybridization to the expected 2.2-2.4 kb transcript (Westin et al, 1982a). As revealed by dot blot analysis, solvent treatment either at 0.3% (v/v) alone or in the presence of 1 nM TPA or 400 nM A23187 caused an immediate but transient depression of c-myc that was clearly evident on autoradiographs of Northern blots (not shown). In marked contrast, treatment with identical drug concentrations in the presence of only low carrier solvent concentrations (less than or equal to 0.1% v/v) did not decrease c-myc hybridization. Rather, c-myc mRNA was elevated within 15 min of exposure to either A23187 or TPA and by 4 h had increased 2-3 fold above basal levels in untreated cells (Fig. 3.2.2). By 48 h c-myc mRNA abundance returned to the pre-treatment level. The early rise in c-myc mRNA was even more dramatic during differentiation induced by high TPA or TPA/A23187. In both instances, the elevation was greater (4-5 fold induction) and occured faster (maximal by 1 hr) than for either A23187 or low TPA. Densitometric scans of autoradiograms revealed that after differentiation c-myc had fallen by 50-100 fold compared to maximal abundance and 10-20 fold compared to that in undifferentiated cells. This complex regulation of c-myc mRNA fortuitously indicated that synergistic differentiation

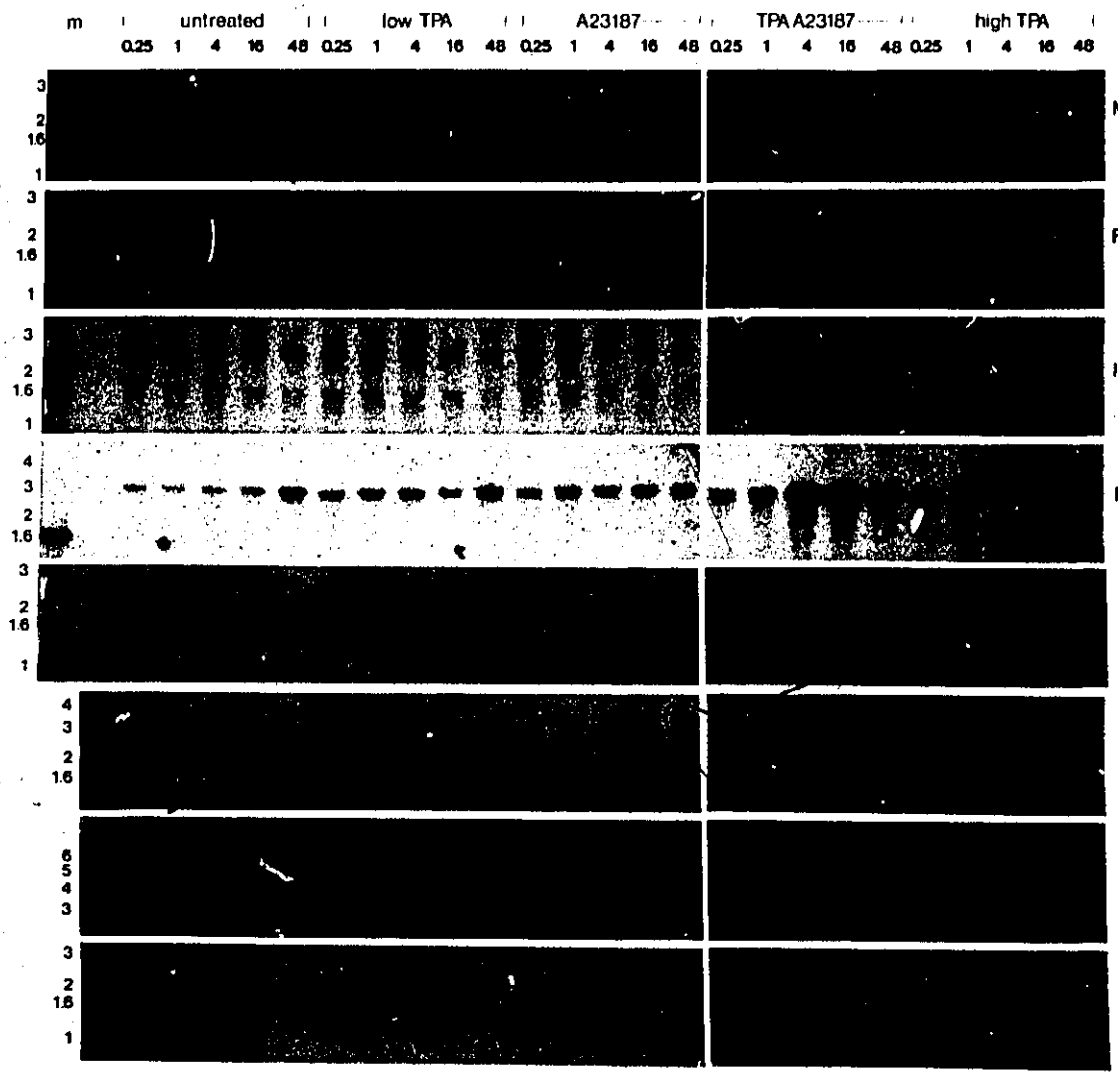


Fig. 3.2.2. Regulation of various mRNA's during synergistic HL-60 differentiation. Total RNA was isolated from cells that were either untreated or treated with low TPA (1 nM), A23187 (400 nM), TPA/A23187 (1 nM and 400 nM, respectively) or high TPA (20 nM) for the indicated periods of time (in h) and probed with radiolabeled oligonucleotides and DNA fragments. Marker (m) sizes in kb are indicated to the left and the hybridization prode for each row to the right (M, myc oligonucleotide; Fo, 2.7 kb c-fos genomic DNA NcoI fragment; I, IL-1β oligonucleotide; P, 2 kb P47 cDNA PvuII fragment; A, actin oligonucleotide; G, 2.5 kb GRP78 cDNA BamHI-EcoRI fragment; Fm, c-fms oligonucleotide). The first 5 rows originate from one blot and the last three from an independent duplicate; actin signals are shown for each. Representative of 2 independent experiments.

matched the response to phorbol ester quite closely.

ii) Differential induction of c-fos

Phorbol esters induce immediate c-fos transcription in many cell types (reviewed in Verma and Sassone-Corsi, 1987), including the HL-60 line (Mitchell et al, 1985; Muller et al, 1985). When cells were exposed to 20 nM TPA, the 2.2 kb c-fos transcript was detectable by 15 min. and maximal by 1 h (Fig. 3.2.2). Although only minimal hybridization occured to RNA from undifferentiated cells, induction was estimated to be at least 15 fold above basal levels. In marked contrast, low TPA/A23187 did not cause any early rise in c-fos mRNA although by 16 h transcripts were detected at approximately the same level as in TPA-differentiated cells (roughly 2 fold above basal). This slight increase probably represents the mature monocytic phenotype as c-fos mRNA is present in normal peripheral blood monocytes (Bravo et al, 1987) as well as in HL-60 cells induced to differentiate by $1,25(OH)_2D_3$ (Muller et al, 1985). Neither low TPA nor A23187 much influenced c-fos transcription. In point of fact, greater induction of c-fos occured in response to 1 nM TPA than during cotreatment with 400 nM A23187. Thus, TPA-induced differentiation and synergistic differentiation differed dramatically in terms of c-fos regulation.

iii) Expression of c-fms

Although c-myc was regulated almost identically in response to TPA/A23187 and TPA, this did not establish that a specific program of gene expression was induced, particularly as c-fos responded in an unexpected manner. In order to assess the extent of monocytic

differentiation, RNA samples were probed with an oligonucleotide complementary to human c-fms mRNA. This gene is slowly upregulated in HL-60 cells treated with either TPA or 1,25-$(OH)_2D_3$ (Sariban et al, 1985). As expected, 4.3 kb c-fms transcripts were present 48 h after addition of TPA/A23187 or high TPA (Fig. 3.2.2), similar to previous results on c-fms in HL-60 cells (Mitchell et al, 1986; Horiguchi et al, 1986; Sariban et al, 1984). Although TPA/A23187 initiated c-fms transcription in HL-60 cells, it was at a level somewhat reduced compared to high TPA. Moreover, the onset of c-fms expression was delayed as transcripts were apparent only after 48 h. Thus, while the synergistic combination of phorbol ester and Ca^{2+} ionophore clearly induced monocytic HL-60 differentiation, this stimulus appeared not as strong as a high concentration of TPA alone. This may reflect the degree of cell maturation in each treatment.

A subtle effect in c-fms expression was found to correlate with both types of monocytic differentiation. Undifferentiated HL-60 cells contained a weak, diffuse signal centered at about 5 kb that appeared specific for the oligonucleotide probe. Upon acquisition of monocytic characteristics, this region of hybridization was lost with the concomitant appearance of discrete c-fms transcripts (Fig. 3.2.2). All control treatments failed to induce expression of a discrete mRNA for the CSF-1 receptor, nor was the diffuse c-fms hybridization lost under these conditions. The nature of the heterogenous c-fms like RNA is unknown (i.e. it is not apparent in unstimulated monocytes (Horiguchi et al, 1988)) but incomplete RNA processing in undifferentiated HL-60 cells could explain the larger size and broad region of hybridization. This

could be resolved by comparison of c-fms hybridization in nuclear and cytoplasmic RNA fractions.

iv) Regulation of IL-1β like transcripts

A systematic investigation of IL-1β regulation during monocytic HL-60 cell differentiation has not been reported. Thus, although IL-1 transcripts were expected, the pattern of regulation could not be anticipated. Northern transfers were probed with an oligonucleotide complementary to a conserved region of human IL-1β. Surprisingly, two transcripts of 2.7 kb and 1.6 kb were detected (Fig. 3.2.2). The latter corresponded to authentic size IL-1β mRNA, but this was less abundant in undifferentiated cells than the larger transcript. High TPA induced a slight shift in the signal intensity of each mRNA such that they were of equal abundance by 16 h. By 48 h both transcripts had diminished somewhat. In contrast, TPA/A23187 evoked a greater accumulation of the 1.6 kb mRNA by 16 h. This difference in regulation of IL-1β like transcripts confirms the observation made with c-fos that the two modes of differentiation are not identical. Low TPA and A23187 caused only a slight transient increase in the 2.7 kb transcript without altering the abundance of the 1.6 kb mRNA.

The nature of the larger IL-1β transcript is at present unknown. It may be related to an epidermal cell-derived cytokine known as ETAF (epidermally-derived thymocyte activating factor, Bell et al 1987). If this transcript is shown to encode IL-1 like activity, the HL-60 system will be useful for the investigating the differential regulation of the two transcripts and for elucidating a functional role for the cytokine encoded by the 2.7 kb mRNA.

v) Induction of P47 mRNA

Initial characterization of P47 mRNA revealed a marked induction that peaked after 4 h exposure to TPA (see Section 4.4.1). This was reproduced in separate studies on synergistic HL-60 cell differentiation in which the transcript was used as a marker of differentiation. TPA/A23187 and high TPA evoked similar induction patterns, although the effect was somewhat more pronounced during synergistic differentiation. (Fig. 3.2.2). By 48 h differentiated cells contained only slightly more P47 mRNA than untreated cells. Abundance of the P47 transcript was only slightly affected by either A23187 or low TPA, again confirming the synergistic effect of these agents on HL-60 gene regulation. Further details of P47 mRNA regulation are discussed in Section 4.4.1.

vi) Induction of GRP78 mRNA

Originally the GRP78 cDNA was acquired to confirm the anticipated effects of A23187 on transcription (reviewed in Lee, 1987). Unexpectedly, the 2.7 kb GRP78 transcript was transiently elevated by high TPA and TPA/A23187. The former caused an approximate 6 fold induction of the mRNA after 4 h whereas the latter caused a 3 fold increase (Fig. 3.2.2). Thereafter levels declined to well below that of untreated cells, particularly in cultures differentiated with high TPA. Treatment with low TPA also induced the GRP78 transcript slightly. Even more surprising was the observation that A23187 itself caused only a very slight transient increase on GRP78 mRNA abundance after 4 h. This result was in spite of an apparent increase GRP78 protein synthesis detected by 2-D PAGE (not shown).

The discrepancy between HL-60 cells and other cell types is

difficult to resolve. In fibroblasts, induction of GRP78 mRNA can be completely accounted for at the transcriptional level (Lin and Lee, 1984). The GRP78 promoter contains a Ca^{2+}-responsive enhancer element between nucleotide -100 and -150 (Resendez et al, 1988). This region confers responsiveness to all known GRP78 inducers (Lin et al, 1986; Chang et al, 1987; Kim and Lee, 1987), implying that many apparently independent stimuli act through a mechanism involving the same trans-acting factor(s). It is also difficult to explain the induction of GRP78 mRNA by both high TPA and TPA/A23187. GRP78 transcription in fibroblasts is not responsive to phorbol esters; moreover, the upstream region of the GRP78 gene does not contain any of the known TPA responsive elements (Resendez et al, 1986; 1988). It is possible that the accumulation of GRP78 mRNA actually reflects an alteration in the cell cycle distribution of HL-60 cells as they differentiate. Although GRP78 has been classified as a cell cycle regulated protein that increases slightly in G1 (Bravo and Celis, 1980), its induction upon serum starvation probably reflects glucose deprivation (Lee et al, 1984). Analysis of GRP78 mRNA in serum starved HL-60 cells and/or cells non-specifically arrested in G1 with hydroxyurea could test this idea.

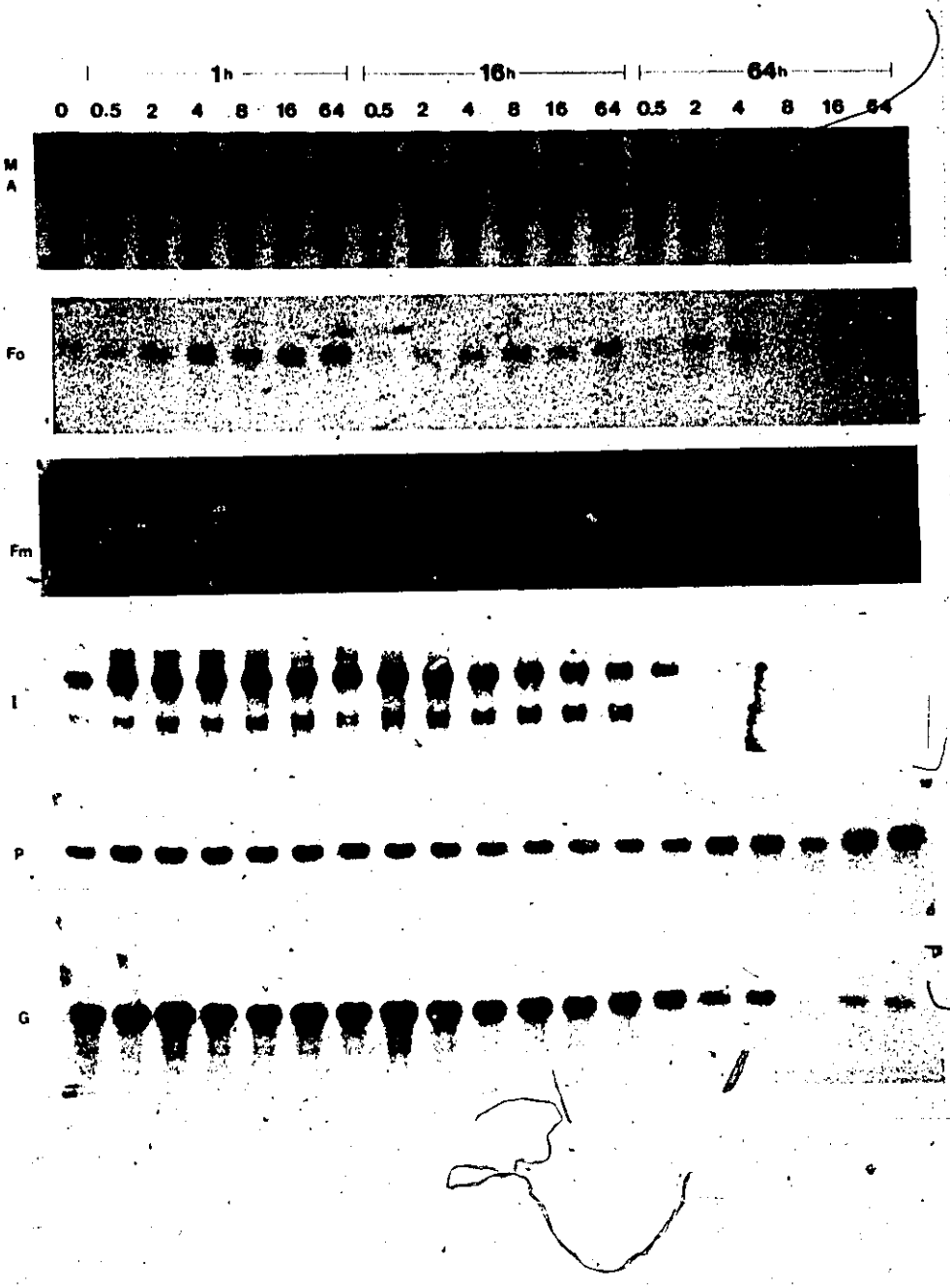
Several trivial explanations for these results can be ruled out. Although higher concentrations of A23187 have been used to induce GRP78 in some other cell types (7-10 μM is optimal in hamster fibroblasts), HeLa cells require only 500 nM A23187 (Resendez et al, 1988). Significantly, HeLa cells exhibit a similar cytotoxic response to A23187 as HL-60 cells (Resendez et al, 1988). Since the concentrations of A23187 used in HL-60 cell cultures caused a substantial elevation of

$[Ca^{2+}]_i$ (see Section 3.1.6), there seemed little chance that A23187 was not added or that non-equivalent concentrations of A23187 affected results. Finally, both restriction enzyme analysis and transcript size indicated that the correct clone was used (Lee et al, 1981).

3.2.3. Regulation of Various mRNAs by TPA

Although the results presented in section 3.1 suggested the contrary, it was important to confirm that the incomplete induction of some molecular markers by TPA/A23187 was not because this condition merely mimicked a low concentration of TPA. Time course studies for a range of TPA concentrations were therefore analyzed for each mRNA investigated in Section 3.2.2. Between 2-4 nM TPA caused a sustained repression of c-myc mRNA (Fig. 3.3.3). Longer exposure of filters probed with the myc oligonucleotide by itself showed that c-myc mRNA was still detectable in cultures treated with 2 nM TPA. This was in accord with continued growth and visually unaltered morphology (data not shown). The decline in c-myc mRNA was slightly preceded by expression of the 4.3 kb c-fms transcript, which was readily apparent at 2 nM TPA (Fig. 3.2.3). This suggested slight uncoupling of growth and differentiation. Induction of c-fos expression was also quite evident at 2 nM TPA (Fig. 3.2.3), supporting the idea that it was induced by TPA only as an ancillary event to differentiation (Mitchell et al, 1986; Calabretta, 1987). Differences between the response to TPA and TPA/A23187 treatment were underscored by the fact that induction of the lower IL-1β transcript was not observed at any TPA concentration (Fig. 3.3.3). Overt P47 and GRP78 regulation were not observed in this dose

Fig. 3.2.3. TPA dose-response of mRNA regulation in HL-60 cells. Total RNA was isolated from HL-60 cells treated with the indicated concentrations of TPA (in nM) for the indicated periods of time and analysed as in Fig. 3.2.2 except that a single filter was used with all probes (M, myc oligonucleotide; Fo, 2.7 kb c-fos genomic DNA NcoI fragment; I, β-IL-1 oligonucleotide; P, 2 kb P47 cDNA PvuII fragment; A, actin oligonucleotide; G, 2.5 kb GRP78 cDNA BamHI-EcoRI fragment; Fm, c-fms oligonucleotide). Hybridization to myc and actin oligonucleotides was carried out simultaneously. The actin signal revealed that effective binding of RNA decreased towards the right side of the filter. Transcript sizes are as in Fig. 3.2.2. Representative of 3 independent experiments.


1h
16h
64h
0 0.5 2 4 8 16 64 0.5 2 4 8 16 64 0.5 2 4 8 16 64
M
A
Fo
Fm
I
P
G

response study, in large part because the time points selected did not include the narrow window in which induction of these mRNAs occured. In summary, gene regulation observed during synergistic differentiation was not reproduced by any of the TPA concentrations tested.

3.3 METALLOTHIONEIN REGULATION IN HL-60 CELLS

3.3.1 Effect of Differentiation on Metallothionein mRNA

Changes in metallothionein (MT) mRNA levels during HL-60 differentiation were investigated to see if MT mRNA expression correlated with alterations in PKC activity upon differentiation and to test whether MT expression was increased in cells capable of a respiratory burst (see Section 1.3.2). In addition, the correlation between MT abundance and growth state was of interest (Karin, 1985). Finally, MT served as another gene system with which to compare synergistic and TPA-induced differentiation.

MT mRNA was quantitated by hybridization of an MT-specific oligonucleotide (MTAP) to RNA dot blots. Autoradiographic signals were normalized to those obtained with $(dT)_{18}$ (Harley, 1987). Representative autoradiograms for each hybridization condition are shown in Fig. 3.3.1. As summarized in Table 3.3.1 and Fig. 3.3.2, neither basal nor Cd^{2+}-inducible MT expression changed appreciably upon differentiation with TPA, TPA/A23187, RA, or 1[redacted]-$(OH)_2D_3$. Only prolonged culture (96 h) in the presence of TPA reduced the MTAP signal (Table 3.3.1). This may have resulted from an extreme terminally differentiated state in which many genes were inactive and cell viability was decreased. Induction of

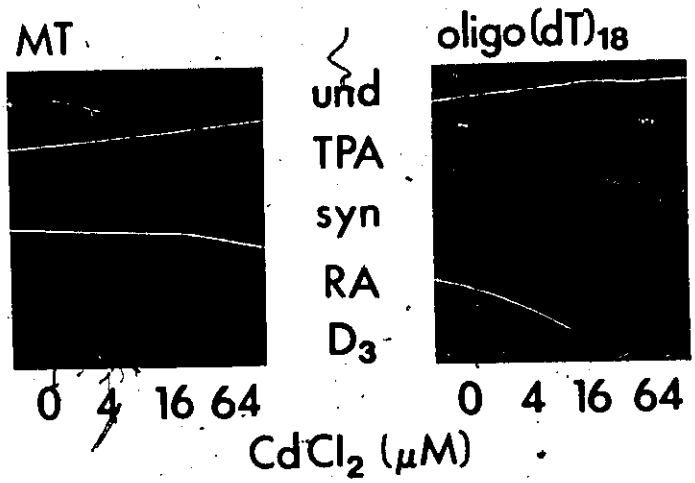


Fig. 3.3.1. Dot blot analysis of MT RNA in variously differentiated HL-60 cells. Total RNA from Cd^{2+} induced cultures was probed with the MTAP oligonucleotide (MT) or with $(dT)_{18}$. Cells were either undifferentiated (und) or differentiated with 20 nM TPA for 2 d (TPA), 1 nM TPA plus 400 nM A23187 for 2 d (syn), 1 μM RA for 5 d (RA) or 100 nM 1,25-$(OH)_2D_3$ for 5 d (D_3) prior to incubation with the indicated concentration of $CdCl_2$ for 6 h. Representative autoradiograms are shown.

Table 3.3.1. Basal MT RNA expression in differentiated HL-60 cells

Differentiated State	Relative Basal Level
high TPA (2 d)	0.87 ± 0.38 (n = 8)
high TPA (4 d)	0.26 ± 0.06* (n = 2)
TPA/A23187 (2 d)	0.68 ± 0.28 (n = 2)
$1,25(OH)_2D_3$ (5 d)	0.68 ± 0.17 (n = 2)
RA (5 d)	0.80 ± 0.28 (n = 7)
RA (7 d)	1.06 (n = 1)

Uninduced levels of MT RNA levels were determined in HL-60 cells differentiated as in Fig. 3.3.1. Values are mean ± SD of replicate determinations relative to uninduced cells. Levels significantly different ($p<0.05$) from undifferentiated cells are marked with *.

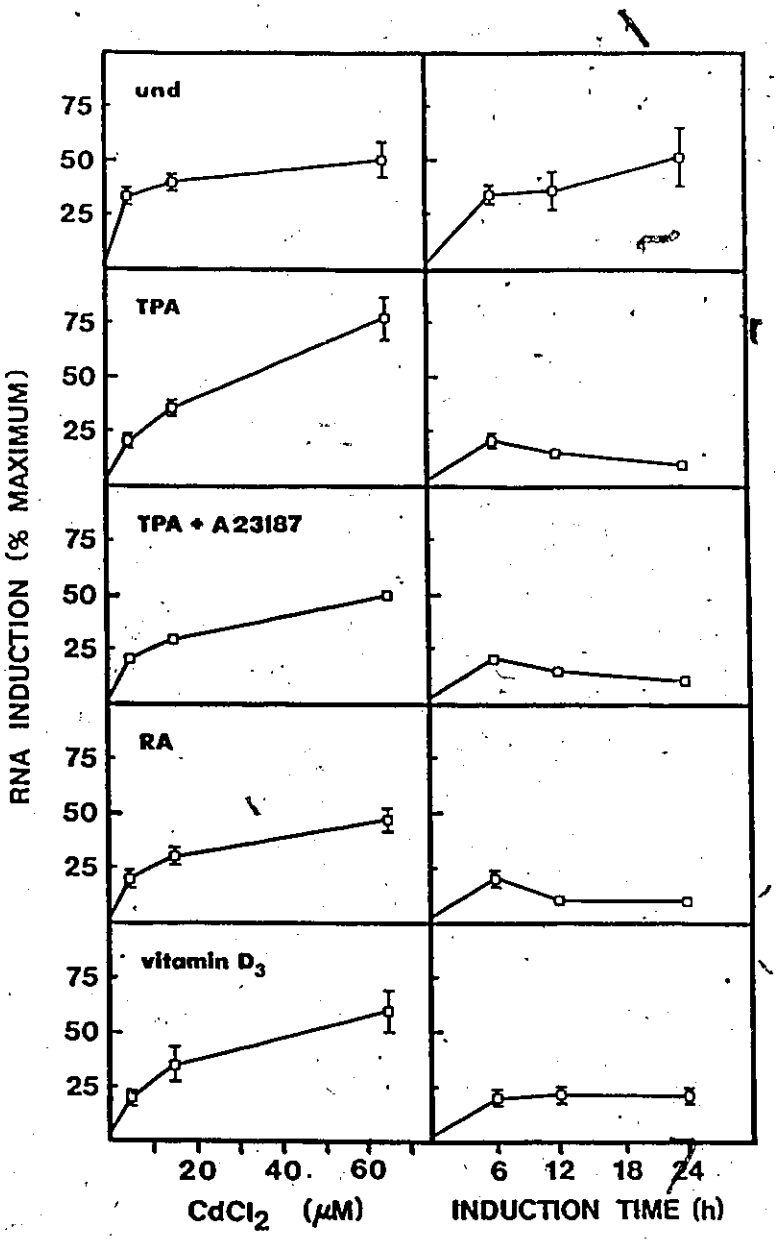


Fig. 3.3.2. Dose and time response of Cd^{2+} induction of MT RNA. Undifferentiated or differentiated HL-60 cell cultures (as described in Fig. 3.3.1) were incubated with indicated concentrations of $CdCl_2$ for 6 h (left panel) or with 4 μM $CdCl_2$ for indicated periods of time (right panel) and analyzed as in Fig. 3.3.1. In each experiment the autoradiographic signals from MTAP were quantitated by densitometric scanning, normalized to the signal from $(dT)_{18}$ and expressed as a percentage of the maximum induction. Results are the means (± SEM) of 2 experiments dotted in triplicate.

MT mRNA by Cd^{2+} exhibited a similar dose-response relationship for all of the variously differentiated HL-60 cells but saturated at a lower Cd^{2+} concentration in undifferentiated cells (Fig. 3.3.2, left panels). Although peak induction levels of MT mRNA were quite uniform in all types of HL-60 cells, the induction period was shortened considerably after differentiation. If differentiated HL-60 cells were challenged with 4 μM Cd^{2+}, MT mRNA was induced for 6 h but then returned to basal levels by 24 h (Fig. 3.3.2, right panels). In contrast, undifferentiated cells maintained MT mRNA at maximal abundance for the entire 24 h period examined. Other than this, however, alterations in HL-60 cell phenotype that occured upon differentiation appeared not to affect the response of the MT genes to Cd^{2+}. Thus, neither acquisition of the respiratory burst pathway nor permanent cell cycle exit influenced MT gene expression. In HL-60 cells, the level of MT mRNA did not correlate with a purported increased demand for Zn^{2+} in proliferating cells (Imbra and Karin, 1987).

With regard to synergistic differentiation, HL-60 cells differentiated either with TPA/A23187 or TPA were quite comparable in both Cd^{2+} dose-response profile and the attenuated induction period characteristic of differentiated cells (Fig 3.3.2). The only aspect of MT mRNA regulation in which TPA/A23187 differed from high TPA was the maximum level of induction. TPA-differentiated cells had the greatest degree of MT mRNA induction whereas TPA/A23187-treated cells had a maximum Cd^{2+}-response more like that of cells differentiated with RA or 1,25-$(OH)_2D_3$. The variability between differentiated states, however, amounted to less than 2 fold. Thus, both constitutive and Cd^{2+}-

inducible MT gene expression substantiated the monocytic phenotype of synergistically differentiated HL-60 cells.

3.3.2 TPA Does Not Induce MT mRNA in HL-60 Cells

Given the varied PKC activities in each different HL-60 state (Zylber-Katz et al, 1985), yet similar MT basal expression and induction profiles, it seemed unlikely that PKC played a role in MT transcription in HL-60 cells. This hypothesis was tested directly by examining the effect of TPA on MT mRNA in undifferentiated and RA-differentiated HL-60 cells. The results summarized in Table 3.3.2 clearly demonstrated that activation of PKC did not increase MT transcription in either cell type. Since this was contrary to findings in HepG2 hepatoma cells (Imbra and Karin, 1987), a preliminary experiment using HepG2 cells was carried out. In this case, TPA (160 nM) was found to increase MT mRNA less than 1.5 fold (data not shown). However, unlike the serum-free conditions employed by Imbra and Karin (1987), cells were maintained in media supplemented with 10% (v/v) FBS. This suggested that much of the MT response to TPA in HepG2 cells is due to either mitogenic effects that are manifest only in serum-free conditions, or that basal level enhancer activity, which maps to AP-2 binding sites in the $hMTII_A$ promoter (Imagawa et al, 1987), is shut down in serum-starved cells. Endogenous serum proteolipids may activate PKC (Ways et al, 1986) so that in the presence of serum, phorbol esters have no additional effect on MT mRNA transcription. By analogy, it is possible that MT transcription in HL-60 cells may respond to PKC activation under serum free conditions.

Table 3.3.2. Effect of TPA on MT RNA expression

A	Undifferentiated cells exposed to TPA (nM)							
time (h)	0.5	2	4	8	16	64	100	200
1	1.0	0.9	0.8	0.7	0.9	0.8	-	-
6	1.0	-	-	0.8	0.6	0.5	0.5	0.6
16	0.8	0.9	0.9	0.8	0.9	0.8	-	-
48	0.9	1.0	1.0	1.4	0.9	0.9	-	-

B	RA-differentiated cells exposed to TPA (20 nM)							
time (h)	0	0.08	0.33	1	4	6	24	48
RNA	1	1.5	1.6	1.4	1.1	1.4	1.3	1.2

MT RNA was quantitated (as in Fig. 3.3.2) in undifferentiated (A) and RA-differentiated (1 μM, 5 d) HL-60 cells (B) after treatment with TPA as indicated. Values are relative to untreated cells and are reported as means from two experiments.

3.4 CONCLUSIONS ON MONOCYTIC HL-60 CELL DIFFERENTIATION

3.4.1 Parameters Influencing Synergistic HL-60 Differentiation

By many different criteria, phorbol esters and Ca^{2+} ionophores were found to induce monocytic HL-60 differentiation in a synergistic manner. This was in spite of 2 caveats in the experimental conditions used. Media pH strongly influenced the cytotoxicity of A23187 such that consistent differentiation was only obtained in cultures buffered to pH 7.6. Similar effects have been observed in rat basophilic leukemia cell cultures (Fewtrell et al, 1978). In human erythrocytes, outside-to-in proton gradients facilitate A23187-mediated Ca^{2+} influx (Vestergaard-Bogind and Stampe, 1984); this effect is half maximal at pH 7.3, the pK_a for the carboxylic acid moiety of A23187. Elevation of extracellular pH above the pK_a causes preferential ionization of A23187 at the external face of the plasma membrane; this in turn increases the efficiency of Ca^{2+} influx because ionized carriers are available to form the 2:1 electroneutral Ca^{2+} complex (Vestergaard-Bogind and Stampe, 1984). Based on these arguments, it was unlikely that the cytotoxic effect of A23187 on HL-60 cells was due to entry of Ca^{2+} into the cytoplasm. Rather, the elevated external pH may have reduced partitioning of A23187 to intracellular membrane compartments by sequestering the ionophore at the external leaflet of the plasma membrane. One possible target for A23187 is the mitochondrial inner membrane, which maintains a positive pH gradient of 1.4 with respect to the cytoplasm (see Fillingame, 1980). Disruption of mitochondrial Ca^{2+} sequestration and proton motive force by A23187 could lead to metabolic death.

The role of pH in HL-60 differentiation has been investigated to a limited extent. One of the earliest effects of TPA in HL-60 cells is elevation of intracellular pH (pH_i) via activation of the Na^+/H^+ exchanger (Besterman and Cuatrecasas, 1984). Alkalinization of the cell interior is also caused by RA, 1,25-$(OH)_2D_3$, dbcAMP and τ-IFN, but in each case this is not essential for differentiation (Ladoux et al, 1987; 1988). Since A23187 acts as a Ca^{2+}/H^+ exchanger, it too can elevate pH_i (Muldoon et al, 1985). However, in a single experiment monensin had no effect on TPA-induced HL-60 differentiation (data not shown).

It may also be relevant that HL-60 cells acquire eosinophilic characteristics when maintained for long periods of time at pH 7.8-8.0 (Fischkoff et al, 1984). Aside from the slightly lower pH employed in this work, an effect on synergistic differentiation cannot be ruled out, particularly since inducers of HL-60 differentiation can often cooperate with each other (see Section 1.2.4). However, eosinophilic HL-60 differentiation is not associated with permanent cell cycle exit (Fischkoff and Condon, 1985). Above all, the effects of Ca^{2+} ionophores on HL-60 differentiation were dramatically different from the responses to phorbol ester alone under otherwise identical culture conditions.

Although synergistic differentiation was ultimately proved independent of carrier solvents, these effects warrant further comment. DMSO could have directly influenced HL-60 cell differentiation, particularly as it caused a significant decline in c-myc mRNA. In spite of the fact that 1.2% (v/v) DMSO is required to induce granulocytic maturation (Collins et al, 1978), low concentrations could have predisposed cells towards differentiation. An interaction between DMSO

and TPA has been noted (Fibach et al, 1982), but this required 1.5% (v/v) DMSO. Aside from the observed effect on c-myc in this work, low concentrations of DMSO diminish the neutrophil respiratory burst (Wong and Chew, 1982). Coincidentally, both processes are regulated by PKC.

Carrier solvents may also have served to displace drug from serum protein and, through increased water solubility, provided better access to the cell membrane (Sharkey and Blumberg, 1985). This was suggested by the fact that solvent effects were less pronounced in cultures used for RNA isolation; the higher cell density in these experiments (10^6/ml compared to 2 x 10^5/ml for experiments on cellular parameters) would have increased the ratio of cellular sites to non-specific sites approximately 5 fold. In addition, the higher drug concentrations employed under these conditions would have been less affected by non-specific binding. As indicated in section 3.2.2, however, synergistic differentiation was ultimately independent of carrier solvents.

3.4.2 Trends in Macrophage-Like HL-60 Differentiation

Induction of HL-60 differentiation occured as a graded response that depended on the strength of inducing stimulus. Inhibition of cell growth was the most sensitive quantitative marker of differentiation; cell cycle distribution reflected the proliferative arrest, although the complete absence of population growth was somewhat at odds with accumulation of cells in G1, since cells in later stages of the cycle must have divided to arrive at G1. The two other cellular parameters for which dose-response studies were systematically quantitated,

extracellular lysozyme and intracellar acid phosphatase, required a more potent differentiation-inducing signal. Of the mRNAs examined, c-myc was most responsive to inducer while c-fms appeared to require a strong phorbol ester stimulus for maximum induction. A definition of cell differentation obviously depended upon what indicators were used and when differentiation was assessed.

In some regards TPA/A23187 evoked a weaker phenotype than high concentrations of TPA. Cell cycle arrest, acid phosphatase activity, OKM-1 reactivity, and c-fos, c-fms and Cd^{2+}-resporsive MT expression all occured to a lesser extent. Despite this, cells treated with TPA/A23187 clearly differentiated, particularly as evidenced by morphological alterations and permanent repression of c-myc and cellular proliferation. Poor expression of some markers in TPA/A23187-differentiated cells may reflect a requirement for intense short term stimulation of PKC for maximal induction. This has been noted for CSF-1 expression in normal monocytes (Horiguchi et al, 1986; 1988). Such an effect could be easily tested by treating synergistically differentiated cells with TPA and re-examining various markers. Alternatively, downregulation of PKC may be required for induction of some markers; specific phorbol ester binding in TPA/A23187-treated could be compared to that in cells differentiated with TPA (Solanki et al, 1981). Interestingly, A23187 by itself caused slight induction of some indicators of differentiation. It could be argued that variable accumulation of cells in G1, increased OKM-1 immunoreactivity, release of lysozyme and growth inhibition caused by A23187 was partial differentiation rather than non-specific toxic effects. Whether this

occured via a direct Ca^{2+} signal or by ionophore-mediated DAG release is unclear (Preiss et al, 1987; see also Section 3.4.4).

Finally, the response to weak differentiation-inducing signals was not merely a linear interpolation from overt differentiation. Low concentrations of TPA caused an unexpected increase in growth rate. Although this amounted to only 10-20% above untreated controls, the effect was reproducible. One unsubstantiated report corroborates this observation (Collins, 1987). Accordingly, the cell cycle distribution after exposure to 0.5 nM TPA showed an increase in S phase at the expense of G1. The proliferative burst of cell populations treated with either A23187 or low TPA may be related to the transient elevation of c-myc transcripts observed in the same populations. Subthreshold levels of inducing agent could trigger cell growth as part of the overall response to differentiation induction. Transient elevation of c-myc mRNA also occured in HL-60 cell populations which differentiated in the absence of proliferation; the results of Sariban et al (1985) suggest a similar 4-5 fold increase in c-myc mRNA shortly after exposure to 3.3 nM TPA. RA also transiently elevates c-myc, possibly as part of the pre-commitment state that occurs within 24-48 h of exposure to the drug (Yen and Guernsey, 1986). In vivo, expansion of a given cell population may precede terminal differentiation so that increased numbers of differentiated cells arise in response to a given stimulus or gradient of stimulation.

3.4.3 The Role of c-myc and c-fos in HL-60 Differentiation

It is obvious from the above results that there is not a tight correlation between proliferation and c-myc mRNA expression in HL-60 cells. Just as induction of c-myc transcripts preceded differentiation in response to TPA/A23187 or high TPA, repression of c-myc RNA levels by carrier solvent coincided with increased cell growth in cultures treated with low TPA or A23187. Thus, alterations in c-myc expression were clearly separable from mitogenic stimulation. Rather than a requirement for proliferation, it may be that c-myc confers a non-terminal phenotype which must be completely eliminated before terminal differentiation can occur (see Section 1.3.1). In the TPA-resistant HL-60-1E3 subline, c-myc is only transiently repressed by TPA, implying that re-expression of c-myc is associated with defective commitment to differentiation (Ely et al, 1987). The lack of c-myc repression by bryostatin also suggests that this event is crucial for terminal HL-60 differentiation (Kraft et al, 1987). Downregulation of c-myc during synergistic differentiation of HL-60 cells supports this, if only as another variation on a long list of inducing agents. Finally, it is perhaps significant that c-myc was the only reporter gene directly influenced by A23187. It may be that Ca^{2+} enhances HL-60 differentiation by cooperating with TPA in the downregulation of c-myc mRNA (see section 3.4.4).

It is clear that the rapid induction of c-fos by TPA can be dissociated from macrophage-like HL-60 differentiation (see Section 1.3.1). In part this is based on the absence of c-fos expression in 1,25-$(OH)_2D_3$ treated cells (Mitchell et al, 1986; Calabretta, 1987). A caveat in this conclusion is the marked phenotypic difference between

cells differentiated with TPA as opposed to $1,25(OH)_2D_3$ (Murao et al, 1983). Synergistic differentiation more closely mimics the effect of phorbol esters, if only because it depends on low concentrations of TPA. The complete absence of c-fos induction in this situation thus confirms the previous notion. It has also been suggested that c-fos expression may be necessary for the rapid cell cycle exit that is observed with TPA but not $1,25-(OH)_2D_3$ (Mitchell et al, 1986). Since HL-60 cells treated with TPA/A23187 showed similar cell cycle exit and proliferative arrest as cells differentiated with TPA this hypothesis is not tenable. Rather, it seems that c-fos induction is a peripheral effect of high phorbol ester concentrations. Recently, c-fos induction has also been found to be separable from SH-neuroblastoma differentiation (Jalava et al, 1988). In terms of the apparent role of Fos as a transcription factor this is perhaps disappointing (see Section 1.3.1 and 1.4.2). Fos appears to regulate gene expression during adipocyte differentiation (Speigelman et al, 1988) and may play a causal role in F9 teratocarcinoma stem cell differentiation (Muller and Wagner, 1984). HL-60 cells may not express the appropriate transcription cofactors to utilize Fos in the induction of differentiation (see Section 3.4.4).

Finally, in many cell types the c-fos gene is responsive to other stimuli, including Ca^{2+} ionophores (reviewed in Verma and Sassone-Corsi, 1987). It was anticipated that cotreatment with A23187 and TPA might activate c-fos through an additive or synergistic mechanism, much as c-myc was transiently activated. It was therefore somewhat surprising that Ca^{2+} ionophore treatment of HL-60 cells did not cause any detectable increase in c-fos mRNA despite clearly elevating

intracellular Ca^{2+}. More than any other result, this implied that A23187 did not just directly augment the effects of low levels of phorbol esters.

3.4.4 Mechanisms of Cooperativity Between Ca^{2+} and PKC in HL-60 Cells

The role of PKC in HL-60 differentiation has been disputed because two activators of PKC, OAG and bryostatin, do not induce HL-60 differentiation (see Section 1.5.1). However, OAG in combination with A23187 causes partial monocytic differentiation of HL-60 cells (Yamamoto et al, 1985); this was substantiated by preliminary experiments not reported in this work. Since synergistic differentiation depended absolutely on a low concentration of phorbol ester, it too implied an essential role for PKC in macrophage-like HL-60 differentiation. Moreover, effects attributable to higher concentrations of TPA, such as altered membrane fluidity (Hoffman and Huberman, 1982) or ADP-ribosylation (Singh et al, 1985), could be ruled out. Since Ca^{2+} enhanced differentiation, it could be argued that one of the more Ca^{2+}-sensitive forms of PKC is involved in HL-60 differentiation. α-PKC is the predominant subspecies in HL-60 cells (Makowske et al, 1988) and strongly depends on Ca^{2+} for activity (Nishizuka, 1988). Superfluous gene induction by TPA could arise from activation of irrelevant PKC isoforms which are less sensitive to Ca^{2+} and/or lipid activators. For instance, the β_1/β_2 subspecies (also present in HL-60 cells) and a PKC isoform recently described in platelets have substantial activity in the absence of Ca^{2+} (Nishizuka, 1988).

As outlined in Section 1.4.2, cooperativity between PKC and Ca^{2+}

can take place at the level of the kinase itself, by proteolytic modification of PKC and by separate pathways which converge only in the ultimate biological response. As an example of the first mechanism, A23187 enhances TPA-mediated phosphorylation of the transferrin receptor in HL-60 cells (Wolf et al, 1985a,b). Cooperative translocation of PKC to the plasma membrane by TPA and A23187 has been demonstrated in K562 cells with PKC monoclonal antibodies (Ito et al, 1988). It is also probable that correct subcellular localization of PKC is crucial for generating a biological response (see Section 1.5.1); elevated Ca^{2+} may help partition PKC to the required intracellular sites for the initiation of HL-60 differentiation. However, since TPA/A23187 and high TPA did not elicit identical differentiation programs, the interaction between phorbol esters and Ca^{2+} ionophores must have occured at least in part beyond the level of PKC itself. Again, the absence of c-fos induction during synergistic differentiation illustrated this point.

Proteolytic regulation of cell function is an emerging theme in signal transduction (see Section 1.4.2). Conversion of PKC to PKM by calpain I appears essential for a complete neutrophil response (Pontremoli et al, 1987). With regard to differentiation, treatment of U937 cells with TPA causes the appearance of PKM activity (Ways and Earp, 1985). More convincingly, PKM is an essential early mediator in MEL differentiation induced by HMBA (Melloni et al, 1987). It is thus quite possible that a similar event occurs in HL-60 differentiation. It has been noted that TPA causes a marked increase in phospholipid/Ca^{2+}-independent phosphorylation at the expense of bona fide PKC activity in a line of adriamycin-resistant HL-60 cells (Aquino et al, 1988).

However, monocytic differentiation achieved by transient exposure to TPA followed by long term RA treatment does not result in downregulation of PKC (Zybler-Katz and Glazer, 1986). In synergistic HL-60 differentiation, Ca^{2+} ionophores may nonetheless act in part by generating PKM.

In a final mechanism of cooperativity, Ca^{2+} may initiate events independently of PKC which predispose HL-60 cells to differentiation. The slightly altered phenotype of A23187 treated cells may reflect this. As indicated in Section 1.5.2, the role of Ca^{2+} in HL-60 differentiation is equivocal. With regard to this work several reports indicate that various Ca^{2+} ionophores affect neither growth nor maturation of HL-60 cells (Kreutter et al, 1985; Yamamoto et al, 1985; Okazaki et al, 1986). In one instance, partial differentiation in response to A23187 and a synergistic effect with RA has been reported, although the criteria used to assess this effect were not convincing (Chapekar et al, 1987). An interaction between ionomycin and 1,25-$(OH)_2D_3$ has also been tested, but enhancement of monocytic differentiation was not detected (Okazaki et al, 1986). By contrast, Ca^{2+} influx during γ-IFN-induced HL-60 differentiation appears to be necessary for the expression of HLA class II (DR) molecules (Koide et al, 1988). There are many examples of interactions between independent inducers of HL-60 differentiation (see Section 1.2.2); such results lend support to the idea that different programs of gene expression contain common elements necessary for the induction of differentiation. Ca^{2+} may induce a subset of such elements.

Ca^{2+} and PKC independently regulate the same target gene in many

cell types. In 3T3 fibroblasts, A23187 or ionomycin increase c-myc expression as do phorbol esters (Tsuda et al, 1985). Similarly, both A23187 and low TPA alone were able to elevate c-myc levels in HL-60 cells. In cells treated with high TPA, c-myc mRNA rose transiently before falling to undetectable levels; it may therefore be argued that A23187 and low TPA together caused a large increase in c-myc that was necessary to evoke the subsequent decline associated with differentiation. In this sense it can be imagined that each signal acted independently on c-myc. TPA and Ca^{2+} could regulate c-myc in HL-60 cells by independent mechanisms; in human T lymphocytes, ionomycin increases the rate of c-myc transcription initiation, whereas TPA relieves a block to transcription elongation (Lindsten et al, 1988).

With regard to normal myelopoeisis it is unclear what role Ca^{2+} may play. TPA causes normal precursors to acquire macrophage-like characteristics (Griffin et al, 1985) and at least some cytokines appear to act via DAG in a Ca^{2+}-independent manner (see Section 1.5.1). On the other hand, synergistic HL-60 cell differentiation strongly suggests a role for Ca^{2+} in monocytic differentiation. It may be that Ca^{2+} modulates the lineage decision in a differentiating cell, depending on the strength of DAG stimulation and on other Ca^{2+}-generating signals impinging on the cell.

As a final note on gene regulation, compared to other cell types several unexpected responses occured during macrophage-like HL-60 differentiation. Genes often activated by phorbol esters (i.e. MT and actin) responded only minimally or not at all in HL-60 cells. Similarly, the anticipated induction of c-fos and GRP78 by A23187 did

not occur in HL-60 cells. In addition, GRP78 mRNA was induced by TPA and TPA/A23187, a response not documented in other cell types (Resendez et al, 1986). Unlike T cells, in which Ca^{2+} and phorbol ester cooperatively stimulate c-fos transcription (Grinstein et al, 1988), c-fos in HL-60 cells did not respond to A23187 treatment, despite induction by even relatively low levels of TPA. The two signaling pathways initiated by PI turnover obviously regulate various target genes in a very cell-specific manner.

Although based on only a small sample of genes in this study, it is possible to envisage a "transcription toggle" whereby an entire set of responses to a given stimulus can be turned either on or off according to cell type. Recently it has been reported that in mouse fibroblasts cAMP switches c-myc and c-fos induction from the PKC signalling pathway to the EGF receptor pathway (Kan et al, 1988). At the level of gene regulation, F9 teratocarcinoma cells activate transcription from AP-1 elements only if they are co-transfected with a c-jun expression vector (Chiu et al, 1988). Naively, in HL-60 cells it appears that the A23187-responsive element(s) which control c-fos and GRP78 may be switched off. Likewise, MT and actin, but not c-fos, are non-responsive to PKC activation. The same switch pattern could also have conferred GRP78 induction. Alternatively, the similar induction time courses of c-myc, GRP78 and P47 could reflect generalized transcriptional activation as cells commit to differentiation and accumulate in the G1 phase of the cell cycle. In terms of promoter elements identified thus far, HL-60 cells may not express the appropriate AP-1 or AP-2 binding factors for MT induction, if these are

in fact responsible for phorbol ester-responsiveness of the $hMTII_A$ promoter (Imagawa et al, 1987). Given the complexity of transcriptional control that has already emerged from a few model systems, an understanding of how HL-60 cells differ from non-terminal cells in their state of transcriptional readiness is obviously not at hand. The HL-60 cell system itself may be useful for elucidating interactions between different transcription factors and their various binding sites.

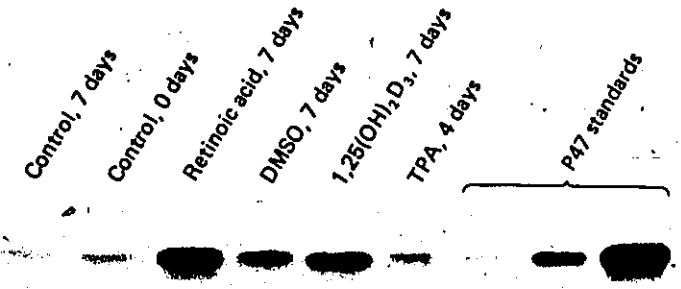
4. STUDY OF THE MAJOR PKC SUBSTRATE OF PLATELETS (P47) IN HL-60 CELLS

P47 is quantitatively the most important substrate of PKC in platelets (see Section 1.6). Despite this, its function remains unknown. Preliminary immunological screening of various human cell lines with a rabbit antiserum to human P47 indicated that it was present in HL-60 cells. The regulation of P47 protein during HL-60 cell differentiation was therefore investigated in detail. Since RA-differentiated HL-60 cells contained the greatest amount of P47, they were chosen as the mRNA source from which to clone the P47 cDNA. A detailed analysis of P47 structure, distribution and regulation was made possible with the P47 cDNA.

4.1 ANALYSIS OF P47 EXPRESSION IN HL-60 CELLS

4.1.1 Induction of P47 During HL-60 Cell Differentiation

A survey of human cell lines and rat tissues with a rabbit antiserum raised against human P47 revealed a low abundance of P47 in HL-60 cells (Stewart *et al*, in preparation). HL-60 was one of the few cell lines expressing P47 and therefore represented a potentially useful mRNA source for cloning. On the chance that P47 was up-regulated during differentiation, HL-60 cells were treated with several inducing agents and subjected to immunoblot analysis. As shown in Fig. 4.1.1, P47



P47 content (ng/80 μg sample) ...	14	14	59	22	51	15	10	30	100
NBT reduction (% positive) ...	2	3	47	50	52	12	–	–	–
Secreted lysozyme (μg-equivalent/10^6 cells) ...	0.5	0.5	0.6	4.1	9.9	4.1	–	–	–
Cell proliferation (% of control) ...	100	6	65	89	49	12	–	–	–

Fig. 4.1.1. Expression of P47 in HL-60 cells. Cells were induced to differentiate by various agents and then subjected to immunoblot analysis with P47 antiserum. Treatments were: 7 d without subculture (control 7 d); initial seeding (control 0 d); 7 d with 1 μM RA; 7 d with 1.3 % (v/v) DMSO; 7 d with 100 nM $1,25(OH)_2D_3$; 4 d with 10 nM TPA. Standards from left to right were 10, 30, and 100 ng of pure platelet P47. Corresponding values for HL-60 P47 content (calculated from standards), marker enzyme activities and cell growth are shown below each lane of the autoradiograph. All values are means of duplicate determinations.

abundance increased upon granulocytic differentiation, especially in response to 1 μM RA. The prevalence of P47 also increased during monocytic differentiation induced by 1,25-$(OH)_2D_3$. Only a slight increase in P47 content occured when cells were treated with DMSO or TPA (Fig. 4.1.1). Treatment with either cytosine arabinoside (0.5 μM) or dibutyryl cAMP (500 μM) had no effect on P47 abundance (not shown). Thus the increase in P47 abundance during differentiation was variable and apparently not restricted to any one lineage.

Although the P47 antiserum was quite specific, 2 other immunoreactive species were detected. Samples containing large amounts of P47 had a leading trail which may in part arise from P42, a probable degradation product that co-purifies with P47 (Stewart et al, in preparation); reactivity with P47 antiserum was also occasionally visible in the 10-20 kDa range (see Section 4.1.2). In contrast, immunoblots probed with pre-immune serum showed no reactivity whatsoever (not shown). P47 immunoreactivity was quantitated by γ-counting of cut out bands that corresponded to autoradiographic signals. In undifferentiated HL-60 cells, P47 accounted for 0.01 - 0.02% of total cellular protein; this was increased several fold by RA and 1,25-$(OH)_2D_3$ (Fig. 4.1.1). Differentiation in response to each agent was confirmed by inhibition of cell proliferation, acquisition of the ability to reduce nitroblue tetrazolium (NBT) by production of O_2^- and the amount of lysozyme released into the culture medium (Fig. 4.1.1). Roughly the same marker profile as reported in the literature was found for each drug treatment. Only the number of cells capable of reducing NBT was less than some previous reports (e.g. Yen et al, 1984a); this probably

reflects differences in the qualitative assessment of the extent of reduced formazan precipitate deposited in cells. Thus, an increase in P47 abundance was not necessarily associated with HL-60 cell differentiation.

Time course and dose response relationships for P47 induction in RA treated cells were established. As little as 1 nM RA increased the cellular content of P47, well before effects on cell growth and overt differentiation were observed (Figure 4.1.2). Induction plateaued at 1 μM RA; this concentration was thus used for subsequent experiments on differentiated HL-60 cells. Maximal induction of P47 occurred after 7 days exposure to 1 μM RA (Fig. 4.1.3). At this time, P47 abundance was calculated to have increased by 4.0 ± 0.5 fold (n=3) above that in untreated cells. A slight decline in this peak value occurred after 9 days exposure. Both time course and dose response studies indicated that increased P47 synthesis was a relatively early event in RA-induced differentiation. After 3 days, P47 reached 3.0 ± 0.1 fold its initial abundance. Induction thus preceded the inhibition of cell growth and the ability of cells to generate O_2^- (Fig. 4.1.3). Cell cycle exit and commitment to differentiation does not occur until at least 48 h after the addition of RA (Yen et al, 1984a). Since P47 is clearly up-regulated before this event (Figure 4.1.3), it may be a useful probe for elucidating primary events in the differentiation program.

Control of P47 synthesis at the mRNA level was investigated by in vitro translation of total RNA isolated from the same cell cultures as the protein samples used to generate Fig. 4.1.3. Electrophoresis and fluorography of total translation products did not reveal any bands

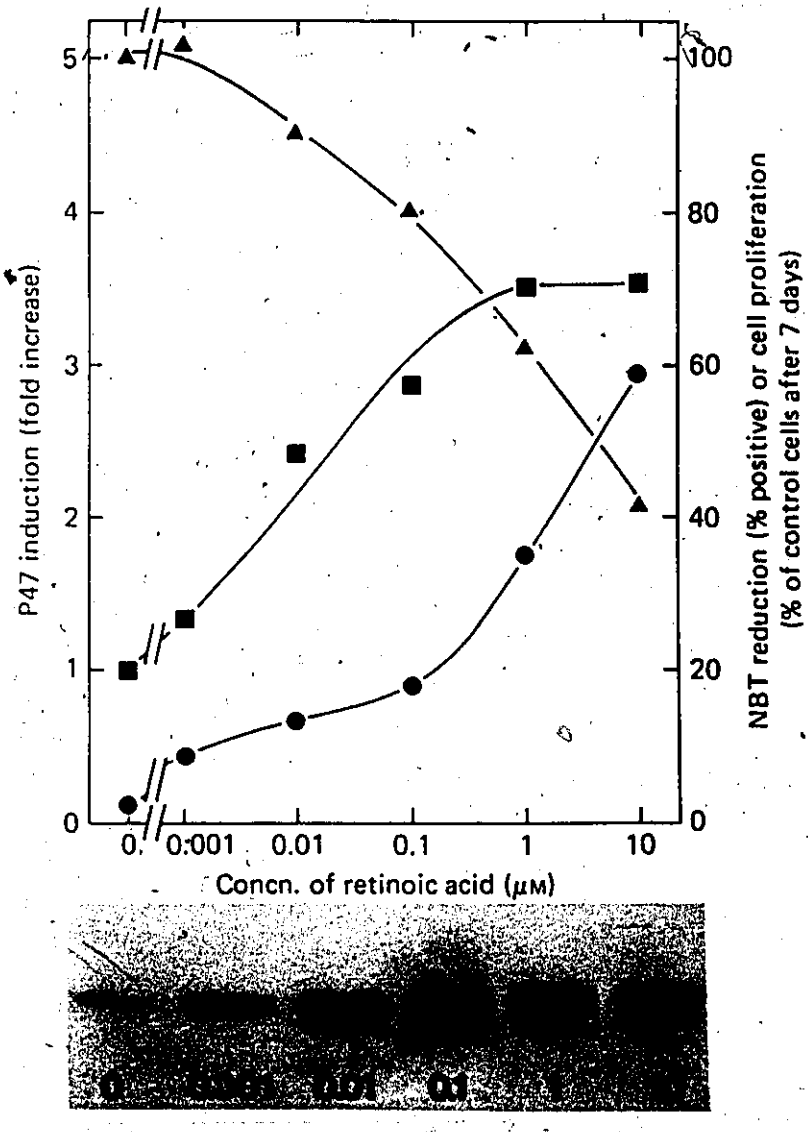


Fig. 4.1.2. Dose-response relationship of P47 induction. HL-60 cells were treated for 7 d with various concentrations of RA. P47 content was determined by quantitative analysis of immunoblots (mean of duplicates) and expressed in multiples of that found in untreated cells after 7 d of culture. NBT reduction and cell growth are means of duplicate determinations. ■ , relative P47 level; ● , NBT reduction; ▲, cell growth. A representative immunoblot is shown below.

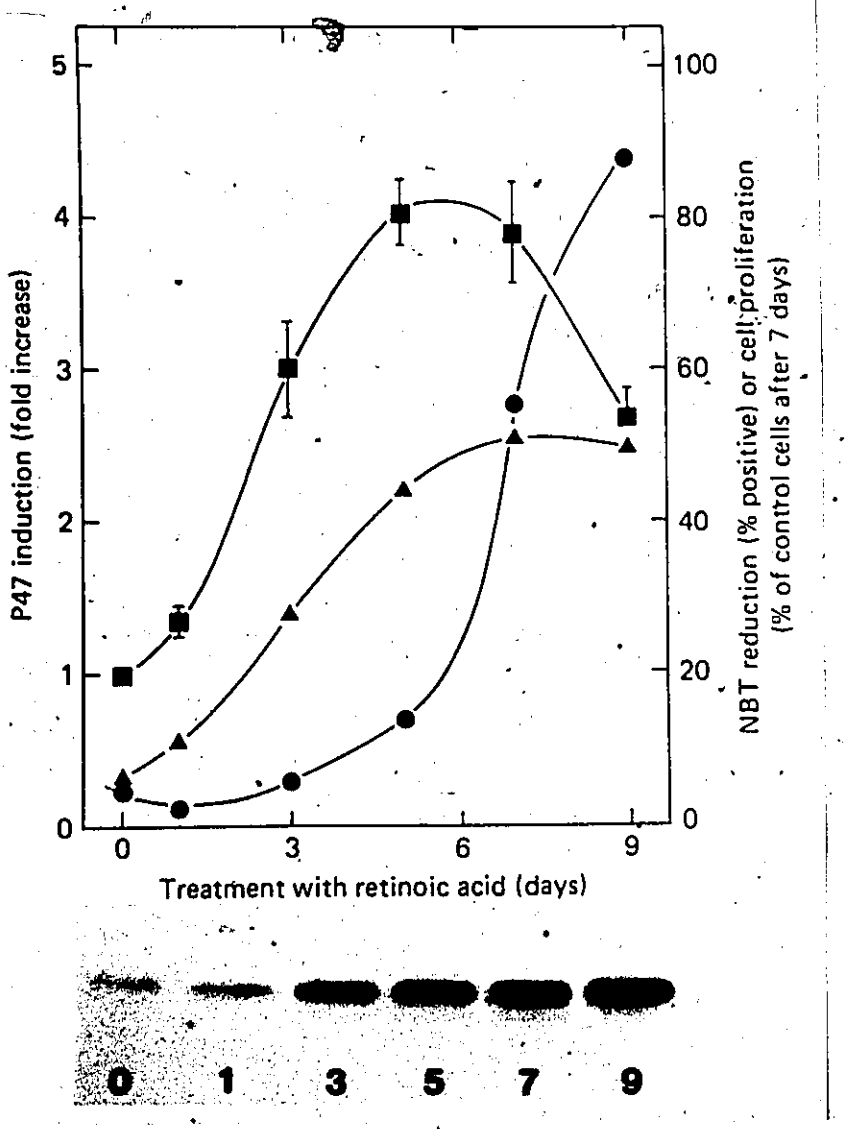


Fig. 4.1.3. Time course of P47 induction. HL-60 cells were exposed to 1 μM RA for various periods of time and then subjected to immunoblot analysis. P47 content is expressed in multiples of that found in cells harvested at 0 d (mean ± S.E.M. for 3 determinations). NBT reduction and cell growth are means of duplicate determinations. ■, relative P47 level; ● , NBT reduction; ▲ , cell growth. A representative immunoblot is shown below.

which paralleled the observed increase in P47 protein (not shown). Immunoprecipitation of translation lysates, however, revealed an immunoreactive species of approximately 44 kDa that had a nearly identical time course of induction as P47 protein (Fig. 4.1.4, panel a). The apparent M_r of P47 varies with the acrylamide concentration of the separating gel; at 7.5% (w/v) P47 clearly migrates below actin at 40kDa (e.g. Lapetina and Seigel, 1983), whereas at 13% (w/v) it migrates ahead of actin at 47 kDa (Imaoka et al, 1983). Co-migration of the translated immunoreactive product and P47 in the same sample was established by marking the position of the purified platelet protein (visualized by Coomassie blue stain) with a radioactive pen (Fig. 4.1.4 b, lane e). A low M_r species from translations of HL-60 RNA was also immunoprecipitated. Appearance of this product was inversely correlated with differentiation; except for a 1 d exposure to RA, which dramatically increased its presence, expression fell throughout the differentiation time course (Fig. 4.1.4, panel a). The low M_r translation species was not related to P47 since it was also immunoprecipitated by pre-immune serum (Fig. 4.1.4 panel b, lane a).

P47 labeled with [^{35}S]-methionine from the translation lysates was used to demonstrate the specificity of the rabbit antiserum and the co-identity of HL-60 P47 with platelet P47. Immunoprecipitation in the absence and presence of 2 μg of purified platelet P47 demonstrated that the labeled immunoreactive material at 45 kDa could be out-competed by the purified protein (Fig. 4.1.4 panel b, lanes c and d). In contrast, the low M_r species was not out-competed by excess cold P47; in fact, the apparent immunoreactivity of this material was enhanced under this

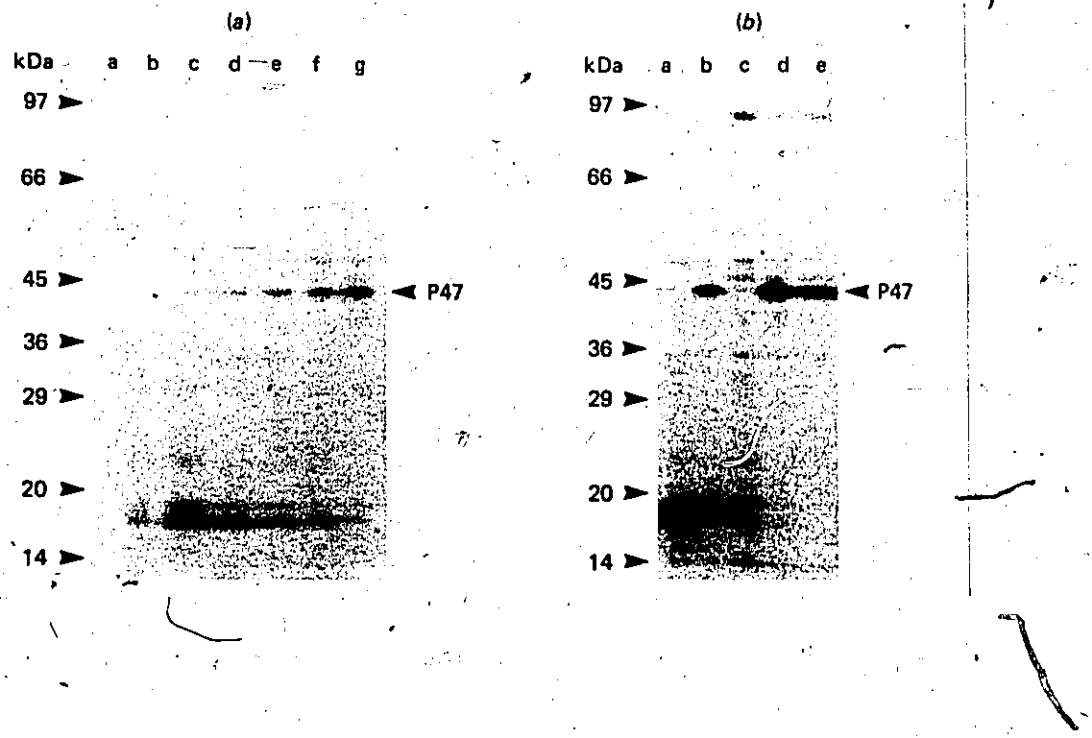


Fig. 4.1.4. Time course of P47 mRNA induction in HL-60 cells. (a), Products from in vitro translations precipitated by P47 antiserum. Total RNA preparations from cells in the experiment shown in Fig. 4.1.3 were used. a, reticulocyte lysate control with no exogenous RNA; b-g: immunoprecipitations corresponding to 0, 1, 3, 5, 7 and 9 d exposures to RA, respectively. (b), Control immunoprecipitations. a,b: translation products of total RNA (from cells exposed to RA for 1 d) precipitated with either pre-immune or immune serum, respectively; c,d,e: translation products of poly(A)$^+$ RNA (from cells exposed to RA for 5 d) precipitated with P47 antiserum in the presence (lane c) or absence (lane d) of 2 µg pure platelet P47 or with 1 µg pure platelet P47 added just prior to loading (lane e). M_r values of standard proteins electrophoresed in parallel are indicated on the left, and the position of pure platelet P47 is shown on the right of each autoradiograph.

condition (Fig. 4.1.4 panel b, lane c). A control immunoprecipitation with pre-immune serum also proved the specificity of of the P47 antiserum (Fig. 4.1.4 panel b, lane a versus lane b).

4.1.2 Effects of TPA on P47 in HL-60 Cells

Although P47 abundance was only marginally altered upon differentiation with TPA, given the close functional relationship between P47 and PKC in platelets a detailed time course of P47 abundance TPA treatment was warranted (Fig. 4.1.5). Although there was a slight transient increase in P47 content between 2 and 16 h, a more dramatic finding was the appearance of a 15-17 kDa immunoreactive species after 48 h (Fig. 4.1.5, lane 6). Induction of this product (hereafter called P15) was not always observed to the same extent (e.g. Fig. 4.1.6 panel a, lane 3). Intriguingly, the relative amount of P42 (or possibly intermediate degradation products since immunoreactivity was often present as a leading trail of the P47 band) increased between 4 and 16 h after TPA addition but diminished by 48 h just as P15 became apparent (Fig. 4.1.5, lanes 4-6). It is possible these products represent stepwise degradation of P47.

Although both migrated in the same region, the low M_r species present in translation lysates was not related to P15 because the latter did not react with pre-immune serum on control immunoblots of samples which contained P15 (not shown), whereas the translation species was immunoprecipitated with pre-immune serum (Figure 4.1.4 panel b, lane a). In addition the 2 species had opposite induction kinetics (compare Fig. 4.1.4 panel a with Fig. 4.1.6 panel a). Interestingly, a similar

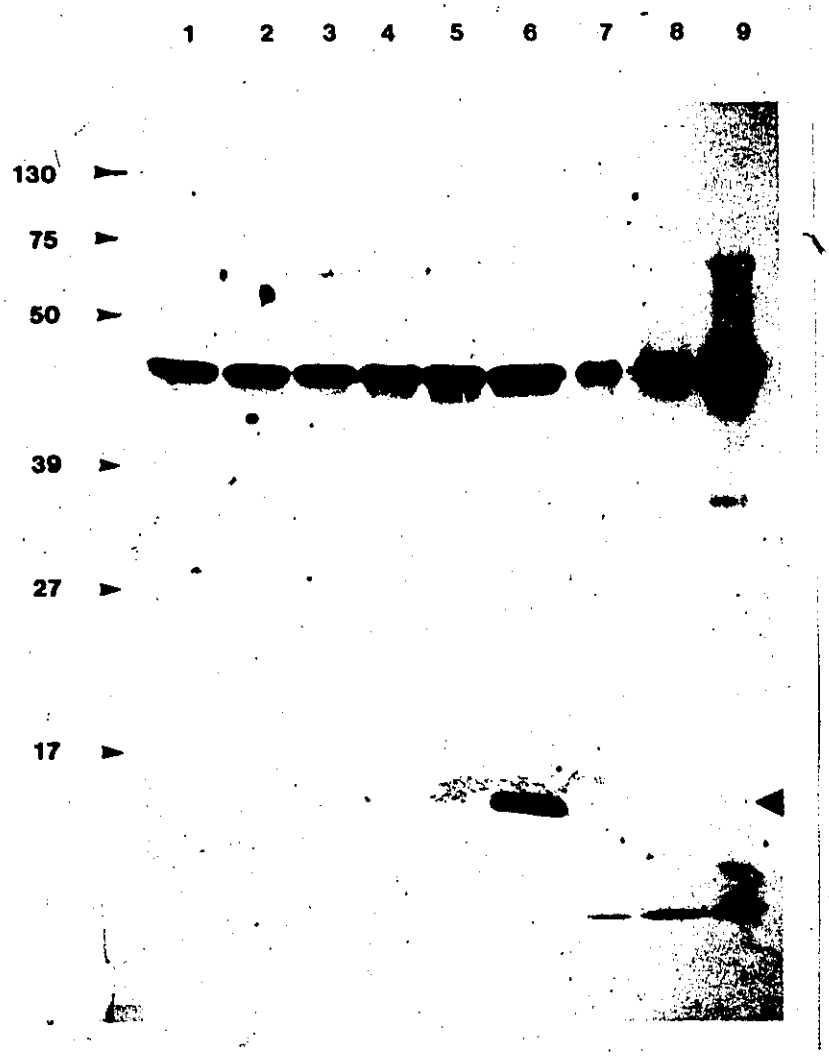


Fig. 4.1.5. Time course of P47 induction by TPA. HL-60 cells were treated with 20 nM TPA for various periods of time and subjected to immunoblot analysis: 1 h (lane 1); untreated (lane 2); 2 h (lane 3); 4 h (lane 4); 16 h (lane 5); 48 h (lane 6). P47 standards: 30, 100 and 300 ng of purified platelet P47 (lanes 7-9, respectively). Arrow indicates low M_r immunoreactive species (P15).

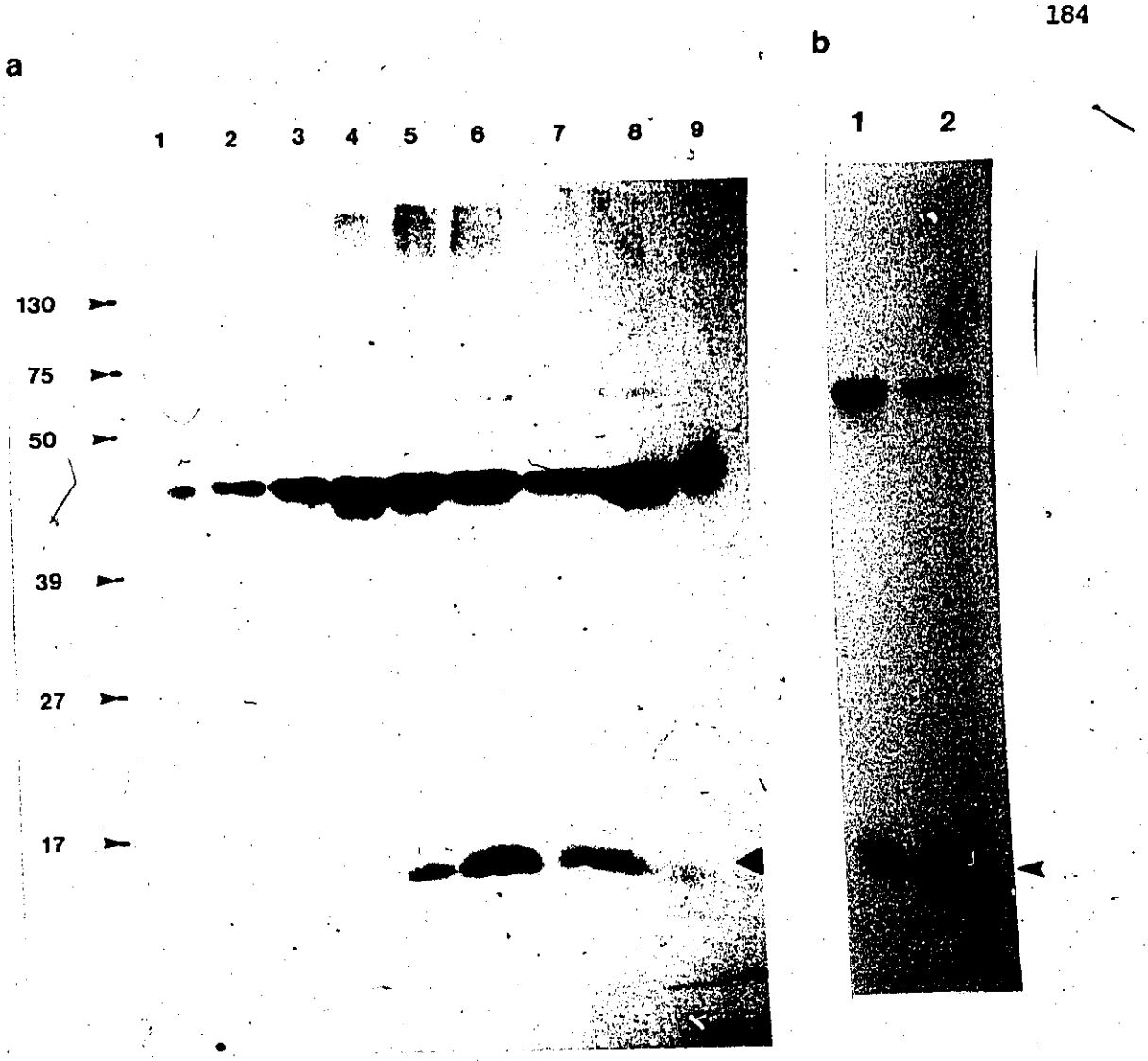


Fig. 4.1.6. Induction of P15 in RA-differentiated HL-60 cells. a, RA-differentiated cells (1 μM RA, 5 d) were treated with 20 nM TPA for the indicated periods of time and subjected to immunoblot analysis: undifferentiated, untreated (lane 1); undifferentiated, 6 h TPA (lane 2); undifferentiated, 48 h TPA (lane 3); 5 d RA, untreated (lane 4); 5 d RA, 6 h TPA (lane 5); 5 d RA, 24 h TPA (lane 6); 5 d RA, 48 h TPA (lane 7); 7 d RA, untreated (lane 8). P47 standard: 30 ng purified platelet P47 (lane 9). b, Effect of prolonged culture in 1 μM RA. RA-differentiated cells (1 μM RA, 7 d) were cultured for an additional 3 d in the absence or presence of 20 nM TPA: 10 d RA, untreated (lane 1); 10 d RA, 3 d TPA (lane 2). Arrow indicates low M_r immunoreactive species.

immunoreactive species is expressed at relatively high levels in the Raji B cell leukemia line (Stewart et al, in preparation).

To further characterize the induction of P15 by TPA, a time course was carried out with RA-differentiated HL-60 cells (Fig. 4.1.6). Strikingly, P15 became visible after only 6 h exposure to TPA. In these cells also, P42 appeared inversely correlated with P15 (Fig. 4.1.6 panel a, lane 4 versus 6). If cells were cultured for long periods in the presence of RA, the amount of P15 was increased (Fig. 4.1.6 panel b, lane 1). In one experiment, treatment of such cultures with TPA for 1 d caused an apparent loss of full length P47 and an increase in P15 immunoreactivity (Fig. 4.1.6 panel b, lane 2). This enhanced response to TPA may reflect either more complete differentiation of these cells or possibly decreased integrity of the older cultures. These results suggested proteolysis of P47 occured upon prolonged activation of PKC, especially in RA-differentiated cells. Like PKC itself (Young et al, 1988) and high M_r components of the platelet cytoskeleton (Fox et al, 1985), P47 may be subject to proteolytic regulation in HL-60 cells.

An attempt was made to test a possible precursor-product relationship between P47 and the low M_r immunoreactive species by a pulse-chase time course study on RA-differentiated HL-60 cells labeled with [^{35}S]-methionine (Fig. 4.1.7). The immunoprecipitated products corresponded not only to P47 and its associated 42 kDa band but also to P15 and a high M_r protein (Fig. 4.1.7, lanes 1 and 6). Other minor species migrating at approximately 32, 66 and > 100 kDa were also immunoprecipitated. Except for P15, these other proteins were never detected on immunoblots; this implied that they co-precipitated with

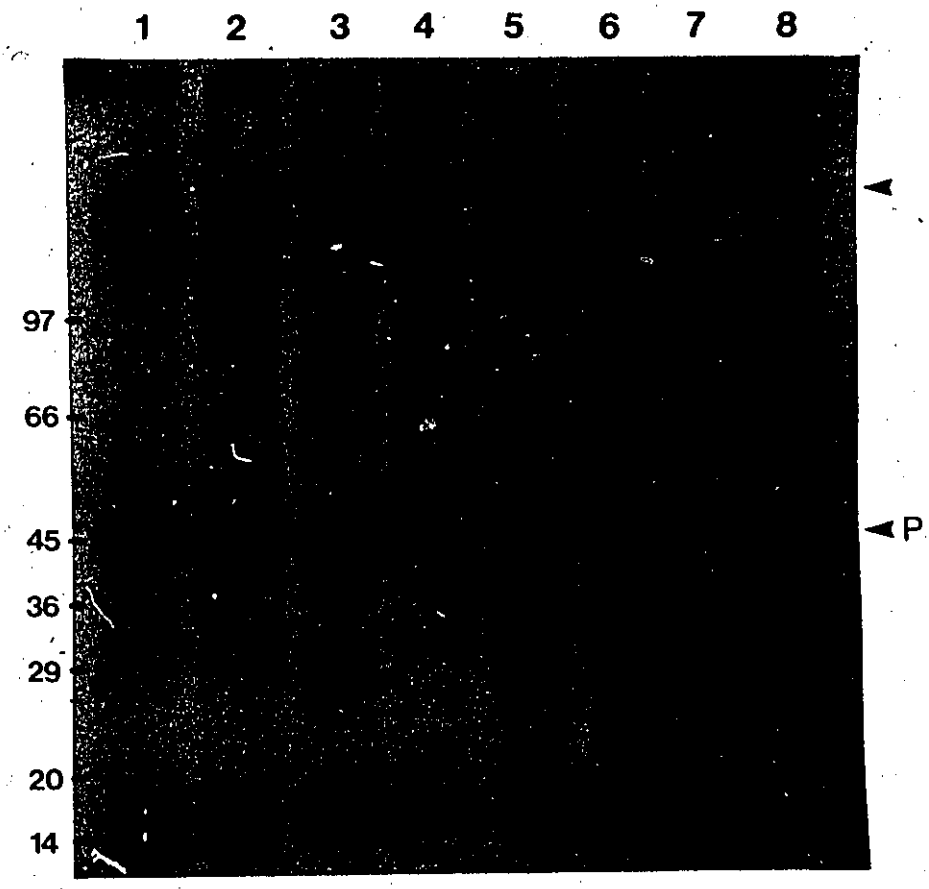


Fig. 4.1.7. Immunoprecipitation of P47-related species from HL-60 cells. RA-differentiated cells (1 μM, 7 d) were labeled with ^{35}S-methionine for 4 h, chased with 1 mM cold methionine and 20 nM TPA for various periods of time and immunoprecipitated with P47 antiserum: no chase (lane 1); 5 min chase (lane 2); 20 min chase (lane 3); 1 h chase (lane 4); 4 h chase (lane 5); 8 h labeling, no chase (lane 6). Alternatively, cells were incubated for 45 min with $^{32}PO_4^{3-}$ in phosphate-free medium in the absence or presence of 20 nM TPA for the last 30 min of incubation: untreated (lane 7); 20 nM TPA (lane 8). Products were electrophoresed on a 10 % acrylamide gel and visualized by exposure to X-ray film without fluorography. Location of P47 is indicated by P and arrow shows high M_r phosphoprotein which co-immunoprecipitated with P47.

P47, possibly because of association with P47 in the cell. After a 4 h labeling period with [^{35}S]-methionine, cold methionine and 20 nM TPA were added to cultures with the expectation that an increase in P15 would occur at the expense of P47. Although the methionine chase (1 mM) was 10 fold higher than the concentration of methionine in RPMI 1640 and 300 fold higher than in the labeling medium, HL-60 pools of [^{35}S]-methionine were not effectively diluted since incorporation of radioactive material continued throughout the 4 h chase period (Fig. 4.1.7, lanes 2-5). Thus, the intensity of P15 did not appreciably increase compared to P47 and a precusor-product relationship could not be demonstrated.

RA-differentiated HL-60 cells were also labeled with $^{32}PO_4^{3-}$ and stimulated with TPA to see if P47 could be heavily phosphorylated, as in the platelet. As shown in Fig. 4.1.7 (lanes 7 and 8), incorporation of $^{32}PO_4^{3-}$ into P47 was substantially increased after a 30 min exposure to 20 nM TPA. This effect was less dramatic than that reported in the platelet (Imaoka et al, 1983), possibly because a sub-optimal concentration of TPA was used. Several other ^{32}P-labelled species were also precipitated by the P47 antiserum, most notably the high M_r band that also labeled with [^{35}S]-methionine. P42 was also labeled slightly, in proportion to its abundance relative to P47. Incorporation of ^{32}P into P15 was barely detectable. The specificity of $^{32}PO_4^{3-}$ incorporation was shown by the fact that several bands visible in [^{35}S]-methionine labelled samples were not apparent in $^{32}PO_4^{3-}$ labelled lysates. Also TPA did not stimulate the additional phosphorylation of some phosphoproteins present in unstimulated cells.

Given the possible role of P47 in cytoskeletal reorganization (Hashimoto et al, 1987), and reports of similar M_r proteins in neutrophils which associate with the cytoskeleton after phosphorylation by PKC (Pontremoli et al, 1987), the influence of TPA on the cytoskeletal association of P47 was tested. Immunoblots of total, cytosolic and Triton X-100 insoluble HL-60 cell fractions before and after stimulation with 100 nM TPA were carried out (Fig. 4.1.8). Most of the anti-P47 immunoreactivity was localized to the cytosolic fraction, as expected from studies on the platelet (Stewart et al, in preparation). Despite marked adherence to plastic of both intact cells and isolated cytoskeletal fractions after TPA treatment, no alteration of P47 distribution was observed. The slight increase of P15 in the cytoskeletal fraction may be noteworthy. Since these experiments were carried out identically to those in which the neutrophil 44 and 48 kDa phosphoproteins associate with the cytoskeleton (Pontremoli et al, 1987), it appeared unlikely that P47 was related to these species. Unfortunately, a direct comparison between each lane in Fig. 4.1.8 cannot be made because equal cell equivalents were not loaded. Based on protein determinations, the cytoskeletal fraction contained only 1/5 the protein of the Triton X-100 soluble fraction, implying that the former contained little or no P47. The reason for the apparent decrease in total P47 immunoreactivity during fractionation is unclear (Fig. 4.1.8, compare lane 1 to lanes 3 and 5).

Finally, the influence of TPA on the subcellular distribution of P47 was assessed (Fig. 4.1.9). Most P47 was localized in the 100,000 x g cell supernatant. Treatment with TPA did not dramatically alter this

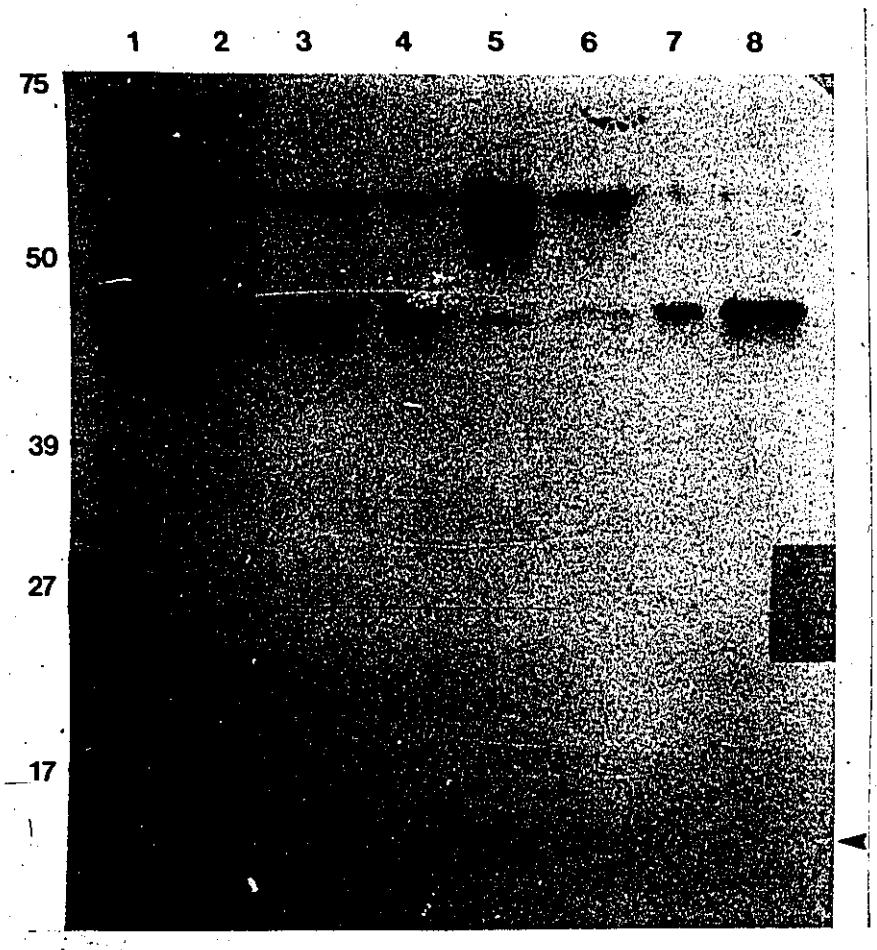


Fig. 4.1.8. Cytoskeletal distribution of P47. HL-60 cells differentiated with RA for 7 d were incubated for 10 min in the absence or presence of 100 nM TPA, separated into Triton X-100 soluble and insoluble fractions and subjected to immunoblot analysis: total cell lysate of cells incubated without or with TPA (lanes 1 and 2, respectively); Triton X-100 soluble cytosolic fraction of cells incubated without or with TPA (lanes 3 and 4, respectively); Triton X-100 insoluble cytoskeletal fraction of cells incubated without or with TPA (lanes 5 and 6, respectively). Each lane was loaded with 80 µg of protein except lane 3 (68 µg) and lane 4 (45 µg). P47 standards: 10 and 30 ng of purified platelet P47 (lanes 7 and 8, respectively). Arrow indicates low M_r immunoreactive species in the cytoskeletal fraction. Immunoblot is representative of 2 experiments.

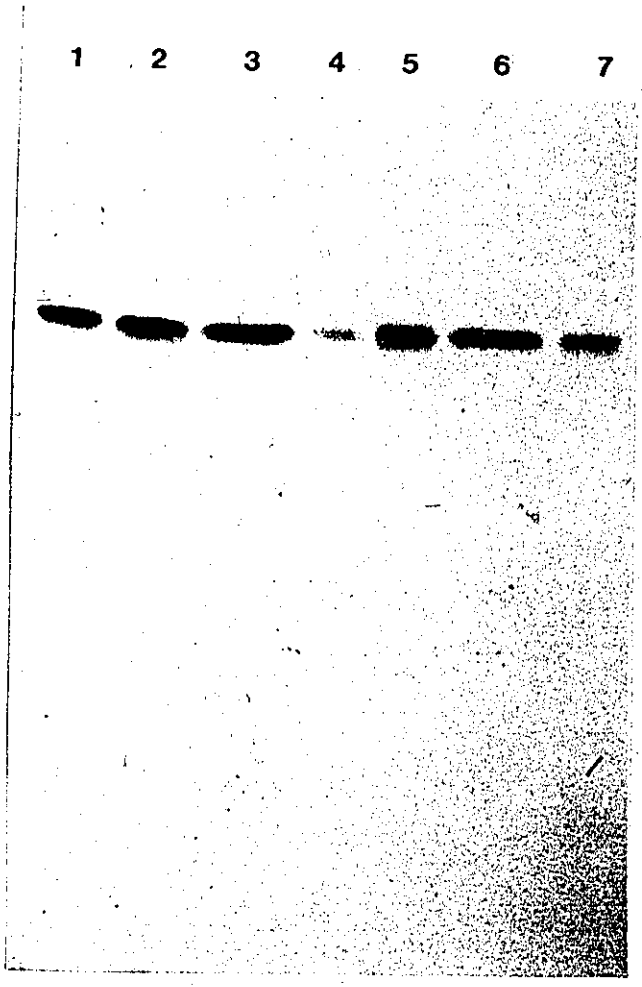


Fig. 4.1.9. Subcellular distribution of P47. HL-60 cells treated with RA for 7 d were washed and resuspended in PBS (10^7 cells/ml), incubated at 37°C in the absence and presence of TPA for 20 min and then separated into cytosolic and membrane fractions: total cell lysate, cytosolic fraction and membrane fraction after incubation without TPA (lane 2, 3 and 4); total cell lysate, cytosolic fraction and membrane fraction after incubation with TPA (lanes 5, 6 and 7). Each lane was loaded with 80 µg of protein. P47 standard: 60 ng of purified platelet P47 (lane 1). Immunoblot was a single experiment.

distribution although a slight increase of P47 in the membrane fraction was apparent (Fig. 4.1.9, lane 4 versus lane 7). Again since equal cell fractions were not loaded, the extent of this effect could not be assessed. Protein determinations indicated that the pellet fraction contained approximately twice the protein content of the 100,000 x g supernatant fraction; thus, the pellet fraction may contain substantial amounts of P47. If real, association of P47 with the membrane fraction is intriguing since P47 is one of the few cytoplasmic targets for membrane-bound PKC activity.

4.2 MOLECULAR CLONING OF THE P47 CDNA

4.2.1 Preliminary Isolation and Sequencing of P47 Clones

The strategy for cloning P47 was based upon immunological screening of expression libraries because peptide sequence from purified platelet P47 was unavailable at the time. Initial attempts to obtain enriched fractions of P47 mRNA by polysome immunoprecipitation (Kraus and Rosenberg, 1982) did not yield any mRNA detectable by translation. This approach was abandoned in favour of the lambda gt11 expression vector system (Young and Davis, 1983), which has proved reliable for immunological detection of many recombinant proteins (reviewed in Snyder et al, 1987). Since identification of positive recombinants ultimately depended upon in-frame fusion of the vector beta-galactosidase reading frame to a P47 cDNA reading frame, the classical S1 nuclease method of cDNA synthesis was chosen (Wickens et al, 1978). In this method cleavage of single stranded 5' hairpins by S1 causes a partial 5'

deletion of the cDNA, thereby minimizing the probability of 5' upstream termination codons. A slight modification of this procedure was used in that mung bean nuclease (MBN) was substituted for S1 nuclease. The former has milder endo- and exo-nucleolytic action and thus degrades DNA less than S1 nuclease.

Based on RA dose response and time course studies (see Section 4.1.1), HL-60 cells that had been treated for 5 d with 1 μM RA were used as an enriched source of P47 mRNA. Total RNA yields from RA treated cells were only $^1/_5$ of that from undifferentiated cells; thus several litres of cells were required to obtain sufficient quantities of poly(A)$^+$ RNA. For this reason, initial RNA preparations employed HL-60/MT-myc cells (see Appendix A). This subline had a doubling time of 24 h, required only 10% FCS for optimum growth and contained slightly more P47 than original passage HL-60 cells. Construction of the initial cDNA library in lambda gt11 (see Figure 4.2.1) is described in Section 2.5.1. Titration of the library on plates containing XGAL and IPTG indicated that $^2/_3$ of the plaques contained inserts and that roughly 10^6 independent clones had been obtained from approximately 0.5 μg of cDNA. Based on a P47 abundance of 0.07% in differentiated cells and a one in six chance of obtaining the correct reading frame, the expected frequency of immunoreactive plaques was estimated at 0.01%. This concurred with the observed frequency as 30 immunoreactive recombinants were obtained from about 300,000 plaques. The extent of the colour reaction catalyzed by the alkaline phosphatase conjugate varied considerably amongst the clones detected. Retrospective analysis of several clones revealed that all contained P47 sequences and that

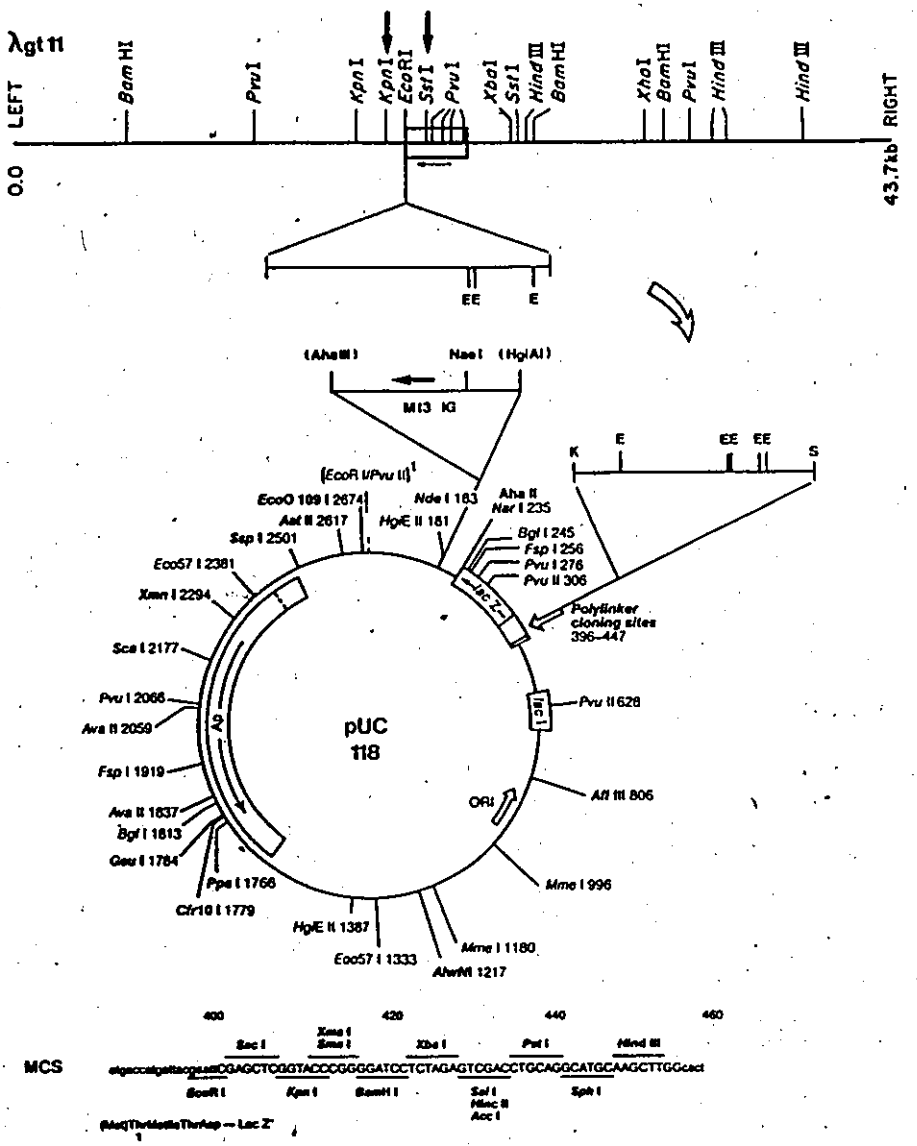


Fig. 4.2.1. Vectors used in the initial isolation and sequencing of P47 cDNA. Maps of lambda gt11 (top) and pUC118 (bottom) indicate important restriction sites and the direction of lac Z transcription. The arrow above the M13 fragment used to create pUC118 from pUC18 (Yannisch-Perron et al, 1985; Viera and Messing, 1987) indicates the orientation of the replication origin. P47 recombinants were subcloned by inserting the KpnI-SstI lambda gt11 fragment containing the EcoRI cloning site into the multiple cloning site (MCS) of pUC 118 (shown below). The universal primer is on the 3' side of the MCS (i.e. the HindIII side). The P47 insert shown represents clone 34A. Modified from Davis et al (1986) and New England Biolabs Catalogue (1988).

variable immunoreactivity probably arose from expression of different subsets of P47 epitopes. The C-terminus appeared to account for many of the immunodominant epitopes. For instance, clone 37 contained only the C-terminal $^1/_5$ of the P47 coding region yet reacted as strongly as clone 34A, which encompassed the entire coding region (Fig. 4.2.2).

Initially, the inserts from the three strongly immunoreactive clones shown in Fig. 4.2.2 were studied. Digestion of clones 34A and 34B revealed that the inserts had related structures in that a 2 kb fragment and one or two fragments of 500-600 bp were liberated. The third clone (37) released only a 700 bp EcoRI piece. The original sequencing strategy began with the 2 kb EcoRI fragment which was subcloned into pUC118. This was sequenced in its entirety using M13 universal primers, specific primers made from P47 sequence and three fragments generated by PstI digestion of the 2 kb EcoRI fragment. However, only a relatively short 5' unbounded open reading frame of 329 nucleotides was found. From this it was tentatively concluded that the remaining EcoRI fragments contained more than $^2/_3$ of the P47 reading frame. Although these fragments were subcloned into pUC118, anomalous doublet bands were visible in ethidium bromide stained gels of the 600 bp fragment. Also a fragment of less than 200 bp was detected in both 34A and 34B clones by fill-in of EcoRI cut lambda DNA (see Fig. 4.2.6). The size of this fragment relative to the lambda genome made it difficult to subclone. For these two reasons, sequencing of subcloned individual EcoRI fragments was abandoned in favour of a nested set of deletions that encompassed the entire insert (Henikoff, 1984). Inspection of the lambda gt11 restriction map revealed KpnI and SacI

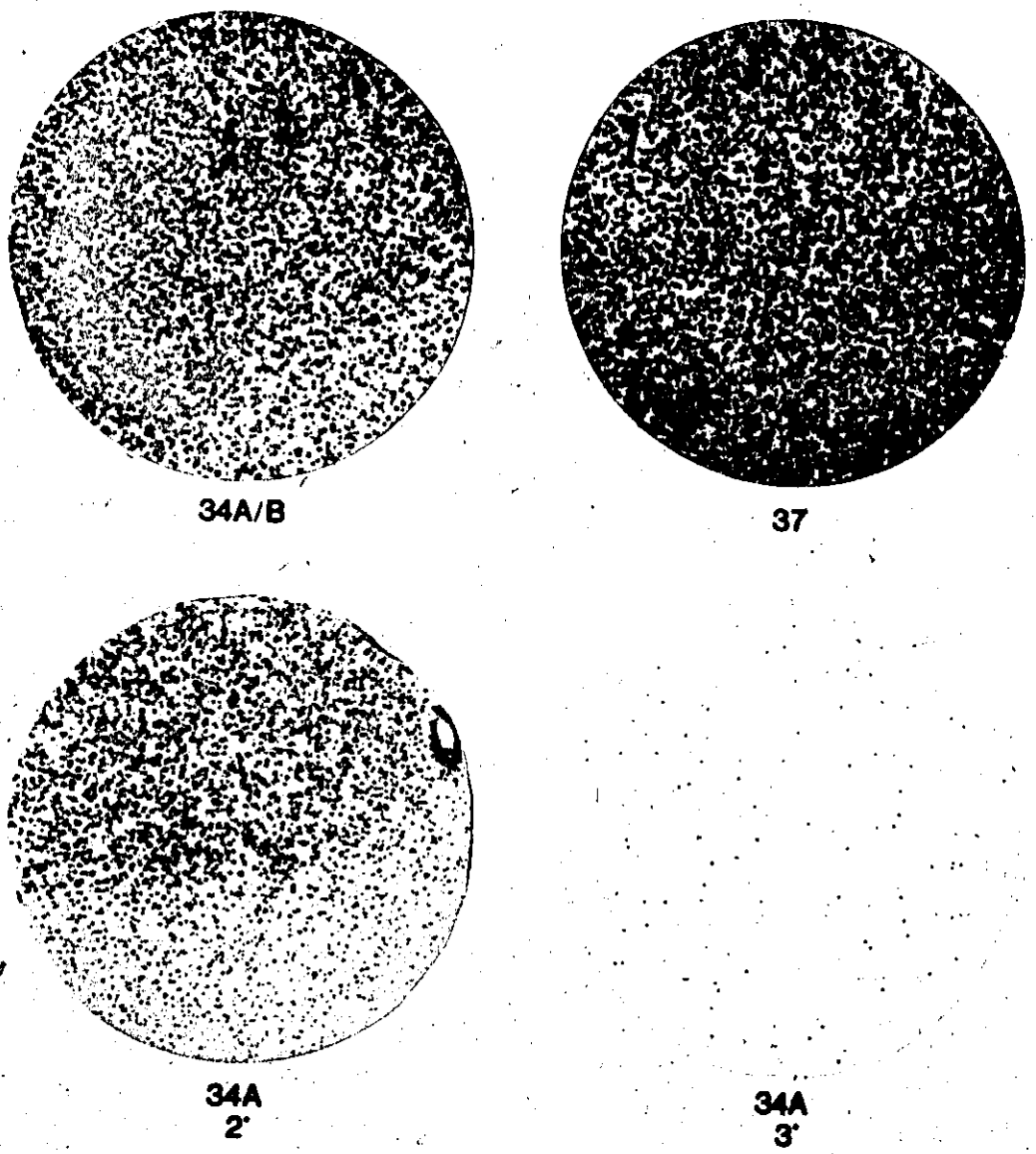


Fig. 4.2.2. Detection and purification of P47 lambda gt11 clones. Plaque lifts were reacted with a 1:500 dilution of rabbit P47 antiserum, then with goat anti-rabbit alkaline phosphatase conjugate and finally with BCIP/NBT to produce coloured plaques. The filters shown are those used to identify the three original P47 cDNA's which were sequenced in whole (34A, 34B) or in part (37). Plaque purification of clone 34A through secondary (2°) and tertiary (3°) screens is also shown.

sites within 1 kb of either side of the EcoRI site (Fig. 4.2.1). Fortuitously, there were no such sites in the P47 cDNA insert. Digestion of the lambda gt11 clones with KpnI and SacI liberated fragments of 4.7 kb for 34A and 5.2 kb for 34B; from this the insert sizes were deduced to be 2.7 kb and 3.3 kb respectively. Given the two insert sizes were centred about the transcript size of 3.0 kb (see Section 4.3.1), both clones were sequenced. Deletions of each KpnI-SacI fragment were carried out in pUC118 by cutting in the multiple cloning site with Bam HI and SphI, neither of which cut the insert. This left an Exo III sensitive 5' overhang (Bam HI site) next to the insert and an Exo III resistant 3' overhang (SphI site) on the vector side, next to the M13 universal primer binding sites. Overlapping deletion clones were identified by digestion with SacI and Hin dIII, which precisely liberated inserts from the vector (see Fig. 4.2.1). The entire sequence of the lower strand of 34A and 34B was obtained from the universal primer. The top strand of 34A was sequenced using specific primers and existing subcloned fragments. Physical maps and sequencing strategies of all the P47 cDNA clones eventually analyzed are shown in Fig. 4.2.3. A detailed restriction map of the P47 cDNA is given in Appendix B. It was fortunate that subcloning and sequencing of individual EcoRI fragments was discontinued because in addition to the 130-160 bp most 5' sequence there was an internal EcoRI fragment of only 34 bp (Fig. 4.2.3). This may have accounted for the doublets seen in some digests. Compared to 34B, the 34A sequence extended 34 nucleotides further 5' but was 21 nucleotides shorter at the 3' end. The larger size of the 34B lambda gt11 insert arose from ligation of an irrelevant 500 bp DNA

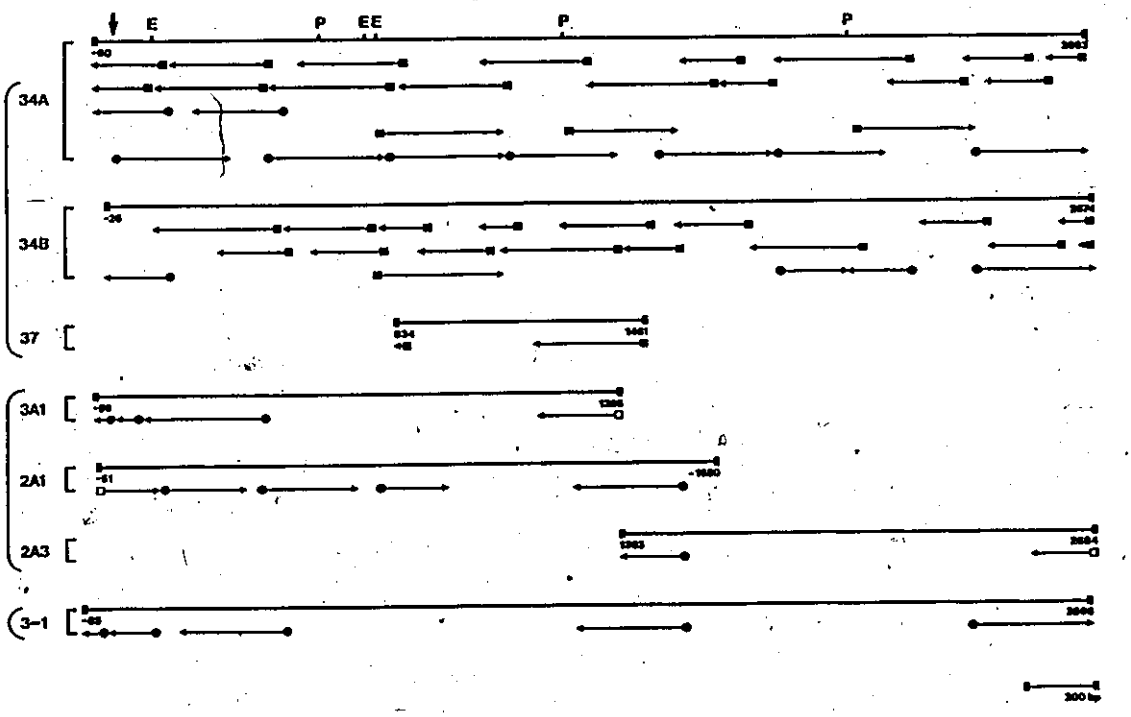


Fig. 4.2.3. Physical map and sequencing strategy for all P47 cDNA clones. Three recombinants from the original lambda gt11 library (34A, 34B and 37), three from a lambda ZAP library (3A1, 2A1 and 2A3) and one from a subsequent lambda gt11 library (3-1) were investigated in detail. The lower strands of 34A and 34B were completely sequenced from the M13 universal primer (■) by nested sets of deletions (Henikoff, 1984). All other sequence data was obtained using M13 universal primers (■, forward; ⧈, reverse) or specific P47 primers (●) on either intact inserts or subcloned fragments. Exact positions and sequences of the P47 primers are compiled in Table 2.1.1. Beginning and ending nucleotides for each clone are shown beneath each end of the heavy bars. Diagnostic EcoRI sites (E) and PstI sites (P) are indicated. The arrow shows approximately where P47 translation initiates. Clones 34A, 34B, 37 and 2A1 were oriented in the same direction as lac Z transcription in the phage vectors, whereas clones 3A1, 2A3 and 3-1 happened to insert in the opposite direction.

fragment to the P47 cDNA via a single linker. Other than this, the two sequences were identical except for a few single base discrepancies (see Section 4.2.2).

Translation of all three reading frames revealed a 5'-unbounded open reading frame (ORF) of 1,110 nucleotides that encoded a protein of M_r 42,064 (Fig. 4.2.4). This was slightly smaller than the apparent M_r of P47 (e.g. Imaoka et al, 1983). Proof of the actual size of P47 is considered below in Section 4.3.1. The putative reading frame for P47 was confirmed with peptide sequences from platelet P47. Three attempts to obtain sequence from the intact purified protein were unsuccessful, suggesting that the α-NH_2 group of P47 was blocked (R. Haslam, personal communication). However, 7 CNBr fragments and 5 tryptic fragments of purified human P47 were isolated and partially sequenced at Smith-Kline and French Laboratories (see Section 2.3.4). The peptide sequence matched the deduced sequence closely except for a few of the initial phenythiohydantoin-amino acid assignments. After re-analysis of HPLC chromatograms, only 3 residues of amino acid sequence could not be unequivocally identified and therefore not matched against the deduced amino acid sequence (Fig. 4.2.4). In all, the sequenced peptide fragments covered more than 40% of the ORF, thereby confirming that the correct reading frame was maintained throughout the coding region.

Prior to the availability of cDNA sequence, 2 degenerate oligonucleotides deduced from original peptide sequence were used to confirm the P47 cDNA. However, because of mistakes in the original phenylthiohydantoin-amino acid assignments only one of these hybridized to P47 cDNA and mRNA (not shown).

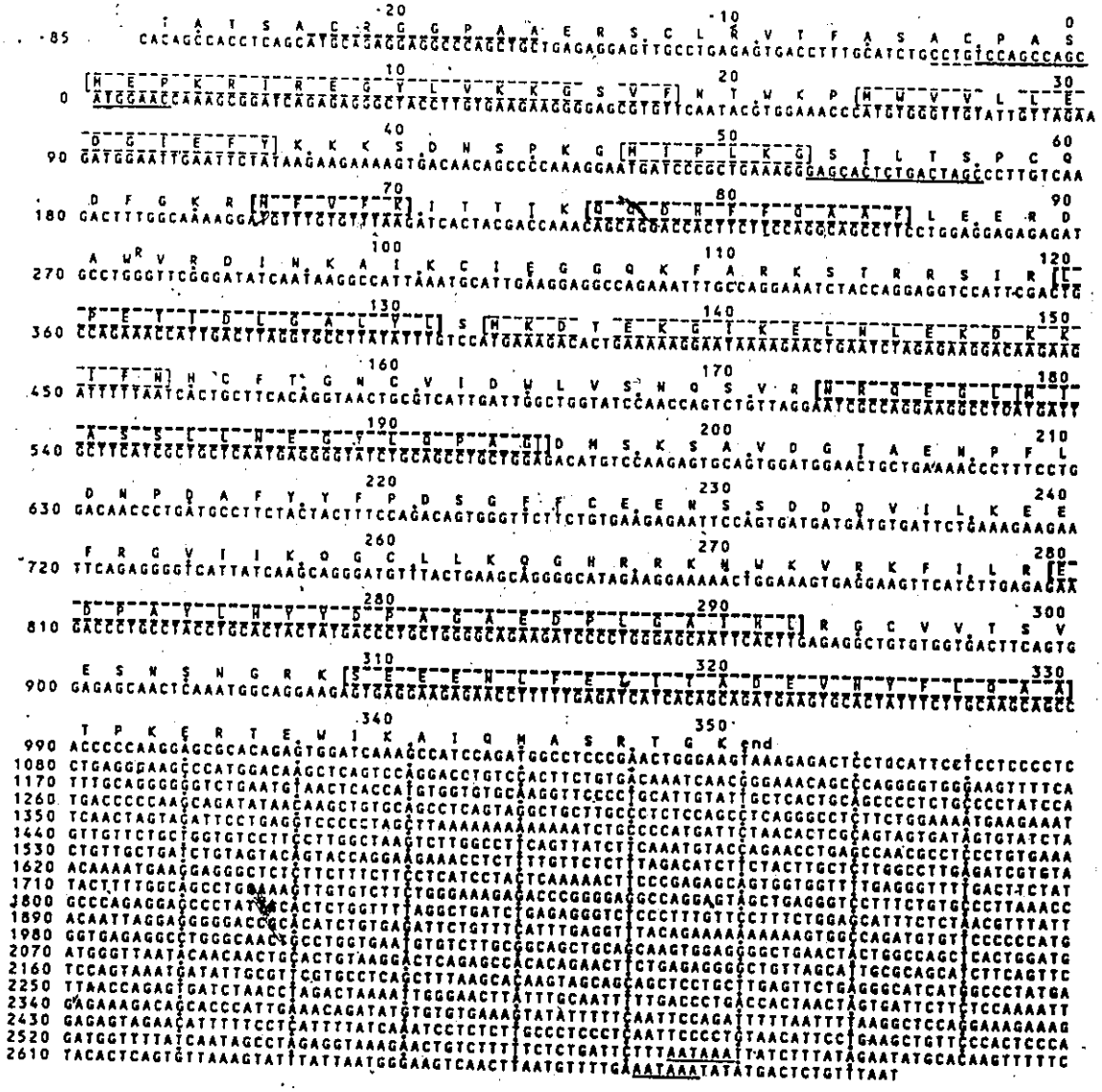


Fig. 4.2.4. Nucleotide and deduced amino acid sequence of the composite P47 cDNA. The 378 amino acids encoded by the 5' unbounded 1134 nucleotide ORF are shown above the nucleotide sequence. An out of frame ORF at the extreme 5' end is underlined by double dashes. Peptide sequences obtained from platelet P47 are boxed but interupted by gaps where residues could not be unequivocally identified. Residues determined from CNBr fragments are enclosed by single dashed lines whereas those from tryptic fragments are enclosed by double dashed lines. The oligonucleotides used for site-directed mutagenesis (AB336), screening (AB206) and primer extension (AB207) are underlined. Possible polyadenylation signals are also underlined. Two nucleotides of the sequence were ambiguous despite several sequencing attempts; the GC at position 1288 sometimes appeared as CG and the single G at position 1318 sometimes appeared as a doublet.

Several considerations made it seem likely that further 5' sequence was necessary to complete the P47 coding region. The deduced M_r of 42,064 was somewhat smaller than has been observed on several gel systems (e.g. Haslam and Lynham, 1977; Imaoka et al, 1983). In addition, the most N-terminal CNBr fragment included the most 5' methionine. Since the N-terminus of P47 was blocked, relatively few post-translational modifications could explain its sensitivity to CNBr cleavage, if this fragment was indeed contained the N-terminus. Also, the 3.0 kb P47 mRNA (see Section 4.4.1) was slighlty larger than the composite cDNA, even if a poly(A) tail of 100-200 nucleotides was assumed. For these reasons, two oligonucleotides (AB206 and AB207) were synthesized in order to determine how far the P47 mRNA extended past the 5' end of the 34A clone. Primer extension analysis of poly(A)$^+$ RNA from undifferentiated and differentiated (both RA and TPA) HL-60 cells revealed unexpected heterogeneity at the 5' end of the transcript (see Fig. 4.4.5). Four discrete transcripts, corresponding approximately to nucleotide positions -70, -100, -120, and -170, were identified. Thus, between 10 and 110 nucleotides remained to complete the 5' end of the P47 cDNA sequence.

Several attempts were made to obtain longer clones. Other P47 clones identified by immunological screening of the initial lambda gt11 library were purified and compared to 34A by fill-in of EcoRI-digested DNA. In all cases, clone lengths were less than that of 34A (not shown). The original library, which was amplified after immunoreactive clones had been isolated, was rescreened with P47 cDNA. This yielded roughly 300 new positive clones but only one of these (clone OA16)

hybridized to AB206. Apparently, clones containing 5' P47 sequence were selected against during amplification since abundance dropped from 13-40% (at minimum 4 of 30 original immunoreactive plaques; at maximum 4 of 10 original plaques purified and analyzed) to less than 0.3% (i.e. 1 of 300). Unfortunately, clone QA16 was refractory to EcoRI digestion and gave unexpected digestion patterns with SacI and KpnI. It was therefore abandoned as subsequent libraries screened in parallel had yielded positive clones by this point.

4.2.2 Isolation of Longer P47 cDNA Clones

All libraries described from this point on were constructed out of poly(A)$^+$ RNA isolated from RA-differentiated parental HL-60 cells of low passage number. This allowed confirmation of clones derived from the HL-60/MT-myc cell line, which had an unusual history (see Appendix A). A pBR322 plasmid library was constructed from cDNA that had been primed from AB207 to enrich for 5' P47 cDNA sequence. Second strand synthesis employed the method of Gubler and Hoffman (1983) in order to maximize the occurence of full length cDNA. Despite this, no positive clones were found in 4,000 colonies (not shown).

Two subsequent lambda phage libraries were made, one in lambda gt11 and one in lambda ZAP (Stratagene). The latter system was tried because subcloning, sequencing, and *in vitro* transcription could all be carried out after excision of the pBluescript plamsid from the original lambda ZAP clone (Fig. 4.2.5). RA-differentiated HL-60 cell cDNA for these libraries was made exactly according to Guebler and Hoffman (1983) using $(dT)_{18}$ to prime first strand synthesis. After methylation and

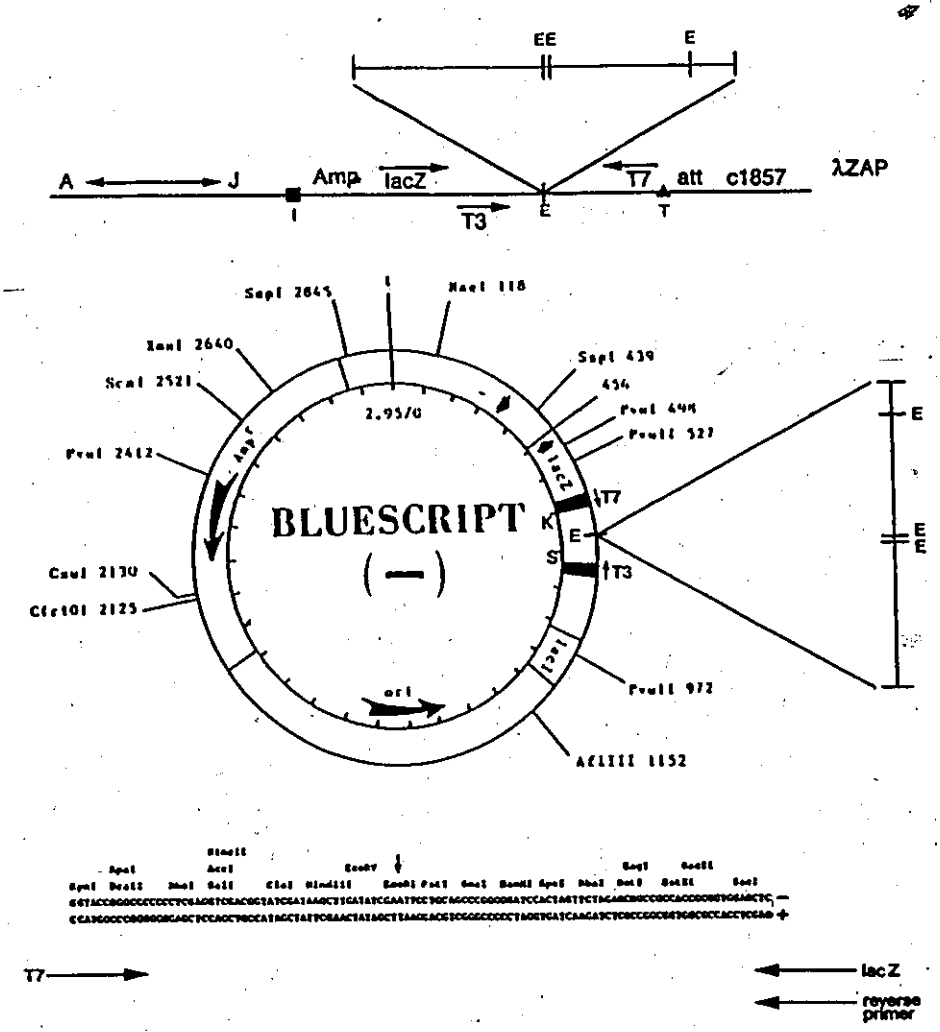


Fig. 4.2.5. Schematic of the lambda ZAP/pBluescript cloning system. The insert shown represents clone 3A1. Adapted from the Stratagene lambda ZAP manual.

linker addition, half the cDNA was ligated to lambda ZAP arms and half to lambda gt11 arms. Although both vectors were packaged in identical fashion, only 10^4 independent plaques were obtained from lambda ZAP compared to aproximately 10^6 from lambda gt11. Thus, although the lambda ZAP vector had some advantages, it apparently suffered from poor packaging and/or infection and replication.

In any event, both new unamplified lambda phage libraries were screened with AB206; this yielded 3 lambda ZAP clones and 8 lambda gt11 clones. Superinfection of lambda ZAP bearing cells with defective M13 phage allowed in vivo excision of the pBluescript plasmid, from which single stranded template DNA was made. These clones were partially sequenced with the M13 reverse primer and specific P47 primers (see Fig. 4.2.3). Strangely, the 2A3 clone, which initially hybridized to AB206, contained only 3' non-coding sequence. This clone extended the 3' end 15 nucleotides past clone 34B. Still no poly(A) tail was found (reviewed in Humphrey and Proudfoot, 1988), although a possible third polyadenylation signal began with the last 3 nucleotides (Fig 4.2.4). The 2 remaining lambda ZAP clones (2A1 and 3A1) were sequenced over most of the coding region, but neither extended the P47 ORF as both ended within 10 nucleotides of the 5' end of the 34A sequence.

Analysis of the 8 lambda gt11 clones by EcoRI digestion and fill-in showed that all extended to nearly the same position as 34A (Fig. 4.2.6). However, clone 3-1 had a 5' EcoRI fragment that appeared 20-30 nucleotides longer than 34A. Subcloning and sequencing of the KpnI-SacI fragment of this clone yielded 25 more nucleotides of 5' P47 sequence (Fig. 4.2.4). Neither an in frame stop codon nor an in frame

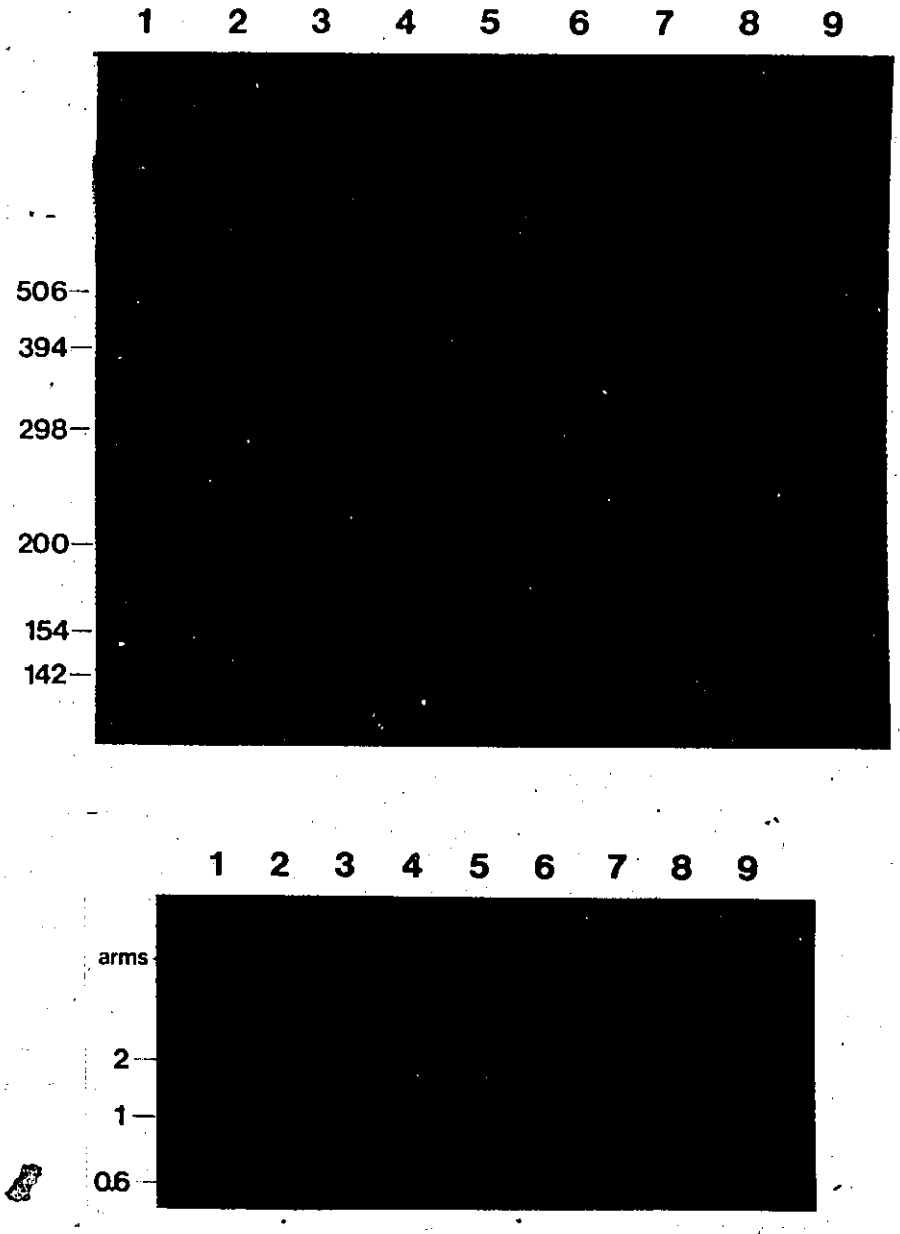


Fig. 4.2.6. Analysis of lambda gt11 P47 cDNA clones. Fill-in reactions ([^{32}P]-α-dATP) of EcoRI digested DNA were electrophoresed on 5 % acrylamide (top) and 1 % agarose (bottom). The latter were dried onto GelBond before exposure to X-ray film. An EcoRI fill-in reaction of clone 34A in pUC 118 was used as a control (lane 1). Original numbering system of the other clones was: 9-1, 8-2, 8-1, 6-4, 6-1, 5-2, 4-3 and 3-1 (lanes 2-9, respectively). Marker sizes are shown to the left in bp (top, BRL 1 kb ladder) and kbp (bottom, clone 34A). Lambda gt11 vector bands are denoted by "arms".

5' ATG was found, though an out-of-frame ATG which initiated a short ORF of only 7 amino acids was uncovered at the extreme 5' end (Fig. 4.2.4). Thus, it was still unclear if more sequence was necessary to find the true initiator ATG. It was puzzling that most clones from RA-differentiated cells ended at the same place; further screening for longer clones did not seem promising.

Before elucidation of the P47 N-terminus is described,, other aspects of the nucleotide sequence will be considered. Some heterogeneity was found amongst the 7 independent clones (from 3 different libraries) that were sequenced in whole or in part. Single base differences were scattered throughout the cDNA sequence, but were also clustered about the first of 2 poly(A) tracts (nucleotide 1383-1395) in the 3' untranslated region (Fig. 4.2.7). The mutation pattern was clearly non-random but could not be explained as allelic variation, assuming HL-60 cells are diploid for the P47 locus. Given the variable size of this poly(A) tract (11-13 nucleotides), it is tempting to speculate that this site is a recombinational hot spot. Alternatively, slippage of reverse transcriptase and decreased fidelity in this region could explain the observed heterogeneity. This appeared unlikely since the other poly(A) tract (nucleotide 1945-1954) showed no such variation. Overall, the variation in P47 cDNA sequence was approximately 15 out of 9,000 nucleotides sequenced; this is somewhat higher than anticipated, based on an AMV reverse transcriptase error rate of 1 in 17,000 nucleotides (Roberts et al, 1988).

In the coding region, 1 T to C nucleotide substitution occured in 2 of 5 clones which extended to that point (nucleotide 273). The

(a)

```
Clone                    Sequence

34A     TGAGGTCCCCCTAGCTTAAAAAAAAAAAAATCTGCCCCATGATTCT
              ╪  ╪    ╪                        ╪
34B     TGAGGTCTCCCCAGCTT·AAAAAAAAAAAATCTGCCCCGTGATTCT
              ╪  ╪                             ╪
37      TGAGGTCTCCCCAGCTTAAAAAAAAAAAAATCTGCCCCGTGATTCT
              ╪  ╪                             ╪
3-1     TGAGGTCTCCCCAGCTTAAAAAAAAAAAAATCTGCCCCGTGATTCT
              ╪  ╪    ╪╪                       ╪
2A1     TGAGGTCTCCCCAGCTT··AAAAAAAAAAATCTGCCCCGTGATTCT

3A1     TGAGGTCCCCCTAGCTTAAAA

2A3                      AAAAAAAAAAAAATCTGCCCCATGATTCT
```

(b)

Clone	Nucleotide Substitution (position)	Amino Acid Change (position)
34B	G → A (548)	Ser → Ser (183)
	G → C (1057)	—
	T → C (2017)	—
37	—	—
3-1	T → C (1463)	—
	A → G (2362)	—
2A1	T → C (273)	Trp → Arg (92)
	T → G (290)	Asn → Lys (97)
	A → G (559)	Glu → Gly (187)
	A → G (571)	Gln → Arg (191)
3A1	T → C (273)	Trp → Arg (92)
2A3	—	—

Fig. 4.2.7. Nucleotide substitutions amongst P47 cDNA clones. (a) Sequences were aligned about a 3' untranslated poly(A) tract (nucleotides 1383-1395) to show multiple single base changes in each clone. Partial clones 3A1 and 2A3 apparently initiated and terminated cDNA synthesis, respectively, at this poly(A) tract. Differences from clone 34A are shown by a cross and deleted bases are represented by dots. (b) Remaining single base differences amongst P47 cDNA clones. Substitutions are indicated with respect to the 34A sequence.

transition resulted in a W to R substitution at amino acid 92 and also generated a polymorphic site for restriction enzymes which recognize CCCGGG (e.g. SmaI, AvaI, XmaI, HpaII, MspI). This potential restriction fragment length polymorphism (RFLP) is discussed in Section 4.5.1. The only other amino acid substitutions occured in the 2A1 clone (Fig. 4.2.7) but these were not substantiated by sequencing in both directions. Despite differences amongst cDNA clones, the P47 coding sequence was not altered in HL-60/MT-myc cells, as identical nucleic acid sequence was found in a clone (3-1) derived from early passage ATCC HL-60 cells. More mundane details of the P47 cDNA sequence are presented in Appendix B.

4.3 DETERMINATION AND ANALYSIS OF THE P47 CODING REGION

4.3.1 Expression of P47 Sequences In Vitro and in E. coli

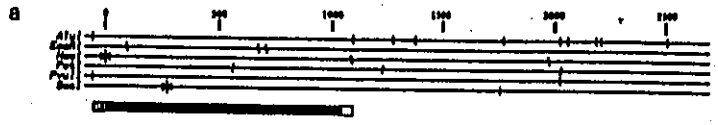
Inspection of the sequence flanking the most 5' in frame ATG revealed a close match (CCAGCATGG to CCACCATGG) to the consensus for initiation of translation (Kozak, 1987a). Presence of the purines at -3 and +4 in particular suggested that this sequence could initiate translation of P47 (Kozak, 1986a). By contrast, the ATG of the short upstream ORF had a worse consensus sequence (TCAGCATGC); however, it did have a dominant A residue at position -3 which should allow it to function *in vivo* (Kozak, 1986a). Two parallel approaches were used to determine the P47 coding region. *In vitro* transcription and translation of P47 templates was used to test the ability of the 5' most ATG in the long ORF to initiate translation and to compare the size of the product

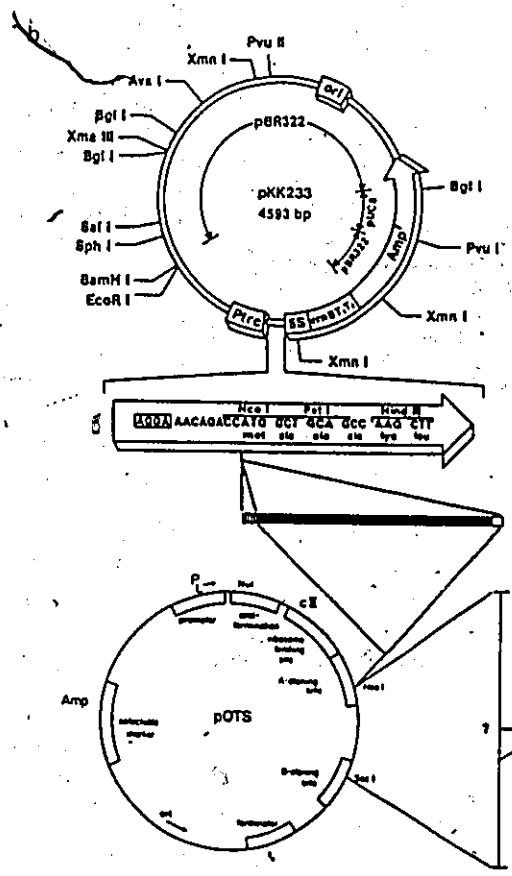
to authentic P47. To rule out fortuitous initiation in vitro and possible difficulty in resolving a partial P47 product from platelet P47, the precise P47 coding region and the 5' extended ORF were expressed in E. coli. The P47 sequences and the various vectors utilized are shown in Fig. 4.3.1.

Since 2 isoforms of the p47 ORF were cloned (Trp^{92} and Arg^{92}), in vitro transcription and translation of both sequences (from clones 34A and 3A1, respectively) was carried out. The 3A1 clone in pBluescript (Fig. 4.2.5) was used directly, whereas the PvuII fragment (nucleotide -53 to 2024) of 34A was subcloned into pGEM3Z (Fig. 4.3.1). Both constructs generated products which co-migrated with purified platelet P47 (see Fig. 4.3.3, lanes 5 and 6). This tentatively confirmed the P47 ORF.

In order to precisely express the putative P47 coding region in E. coli without any upstream P47 sequence, an NcoI site was created at the putative start codon by mutating the G at position -2 to a C. The resultant NcoI fragment (nucleotide -1 to 1091) was cloned into the NcoI site of pKK-322 (Fig. 4.3.1). Selection of mutant colonies was complicated by equal hybridization of the mutant oligonuleotide to the wild type and mutant sequences. The G:G mismatch between the oligonucleotide and wild type sequence was stable enough that washing at increasing stringencies did not identify mutant clones (not shown). This was overcome by competing out mutant oligonucleotide with excess wild type oligonucleotide (Miyada and Wallace 1987; see Fig. 4.2.4 for oligonucleotide overlap). Under conditions of equilibrium binding, mutant colonies were easily detected upon washing at increasing

Fig. 4.3.1. a, Simplified restriction map of P47 cDNA showing sites used in cloning and subsequent manipulations. The NcoI site created by site directed mutagenesis in order to clone the complete coding region, and the polymorphic SmaI site are indicated by *. The bar represents cDNA fragments (PvuII - NcoI, or NcoI* - NcoI) expressed in E. coli (solid area, P47 coding region; hatched extension, 5' extended ORF; open extension, 3' untranslated region). b, Map of pKK-233 and pOTS Nco12 indicating which P47 fragments were inserted into the NcoI start sites. The fragments expressed in pKK-233 were from clone 34A, while the NcoI* - NcoI fragments of both 34A and 3A1 were expressed in pOTS Nco12. The vertical insert of pOTS Nco12 is an unknown cDNA fragment that was present in the vector as it was supplied. It was removed by partial NcoI digestion and re-circularization of SacI digested P47 constructs. c, Map of the pGEM-3Z vector used to express P47 in vitro. The PvuII fragment of clone 34A was inserted into the SmaI site; transcription with T7 RNA polymerase was carried out on BamHI linearized plasmid. In vitro transcription of clone 3A1 was from the T7 promoter of BamHI linearized pBluescript plasmid (Fig. 4.2.5). Adapted from: a, Pharmacia product data sheet (pKK-233); b, Rosenberg and Schatzman (1987); c, Promega Biotech 1987 Catalogue (pGEM-3Z).




Pvu II
Xmn I
Ava I
Bgl I
Xma III
Sal I
Sph I
BamH I
EcoR I
pBR322
pKK233
4593 bp
Amp
Bgl I
Pvu I
Xmn I
Ptrc
SS
Xmn I
AGGA
Nco I
Pst I
Hind III
met
ala
ala
ala
lys
leu
cII
Amp
pOTS
P
E

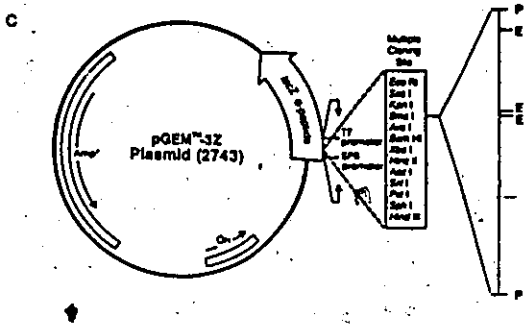
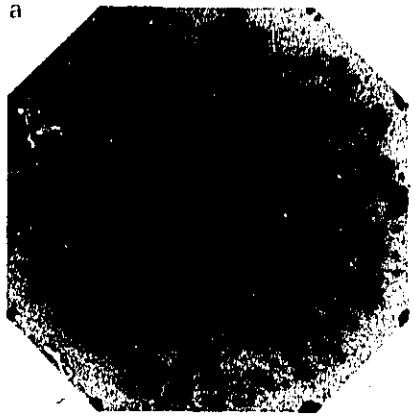
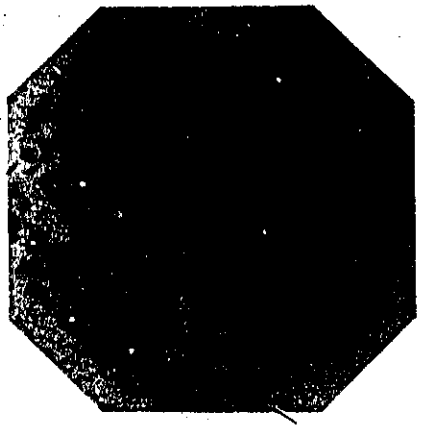

pGEM™-3Z
Plasmid (2743)
P
E
P

Fig. 4.3.2. Detection of P47 site directed mutants. a, Mutant colonies revealed by increasing stringency of washing after hybridization of colony lift on nitrocellulose with a 10:1 ratio of cold competing oligonucleotide (AB206) to end labeled mutagenic oligonucleotide (AB336). All autoradiographic exposures were for 2-3 h at -70 °C in the presence of an intensifying screen. b, As for above except that overgrown randomly picked colonies were hybridized to equilibrium (18 h). All washes were in 6 X SSC for the indicated temperatures and times.

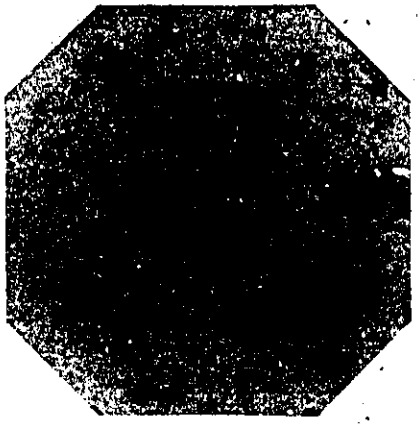
a



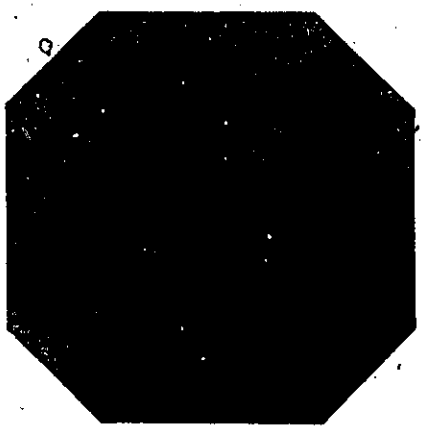
22°, 3×10 min



48°, 3×5 min

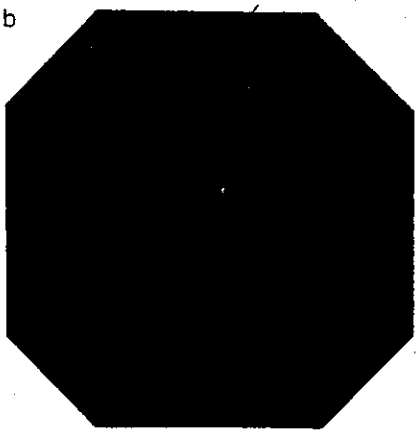


58°, 5 min

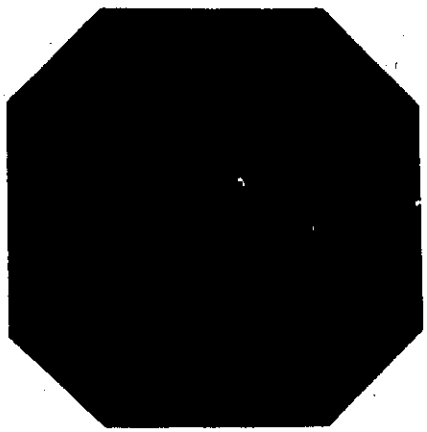


62°, 5 min

b



22°, 3×10 min



48°, 2×5 min

stringencies (Fig. 4.3.2, a). If hybridization of the mutant oligonucleotide was driven to completion by equilibrium binding to large amounts of target plasmid, mutant colonies could be identified after a single low stringency wash (Fig. 4.3.2, b). Digestion of mutant plasmid with NcoI liberated the expected 1.1 kb fragment in less than half the positive clones, probably because of incomplete synthesis from the primer (Zoller and Smith, 1984; 1987). In any event, the NcoI fragment containing the putative P47 coding region was obtained for both 34A and 3A1 clones. A second fragment based on the entire ORF of the cDNA was made by ligating in-frame NcoI linkers to the 2 kb PvuII fragment (nucleotide -53 to 2024) and digesting to completion with NcoI. Both fragments were subcloned into the NcoI site of the pKK-233 expression vector (Aman and Brosius, 1985), as indicated in Fig. 4.3.1. Bacteria transformed with each construct contained an additional band that was visible in stained gels of bacterial lysates (Fig. 4.3.4). Cells harboring the PvuII-NcoI construct expressed low levels of a protein that migrated above purified P47, whereas cells expressing the shorter ORF contained a protein that co-migrated with platelet P47 (Fig. 4.3.4). The relative migration and specificity of recombinant P47 was confirmed by immunoblot analysis of bacterial lysates (Fig. 4.3.3). Occasionally recombinant P47 migrated just below purified platelet P47; this may have been due to removal of the initiator methionine in the bacterial cells (Ben-Basset and Bauer, 1987). In spite of this, there was no doubt that P47 was encoded by nucleotides 0 to 1049 and thus had a predicted M_r of 40,087 (Trp^{92} variant) or 40,057 (Arg^{92} variant).

The true P47 coding region was expressed at high levels, up to

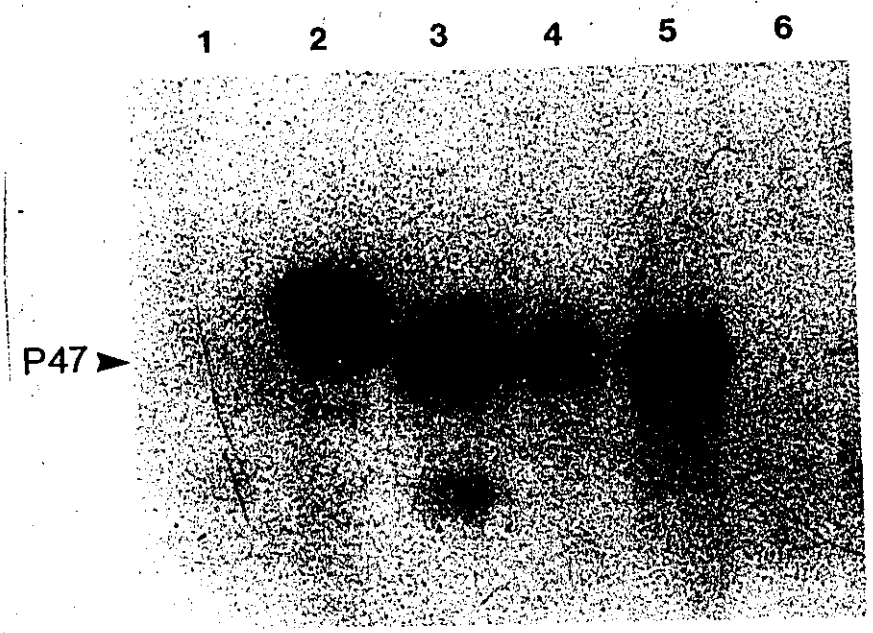


Fig. 4.3.3. Expression of the P47 coding region in E. coli and in vitro. Lysates from E. coli JM109 cells transformed with pKK-233 bearing inserts from clone 34A were probed with P47 antiserum on an immunoblot: no insert (lane 1), nucleotide -53 to 1091 corresponding to M_r 42,112, including 2 amino acids contributed by linker sequence (lane 2), nucleotide -1 to 1091 corresponding to M_r 40,087 (lane 3), 100 ng purified platelet P47 (lane 4). The remaining 2 lanes show products from in vitro transcription and translation of clone 34A in pGEM-3Z: nucleotide -53 to 1091 (lane 5); no insert control (lane 6). Translations (1 µl of each, corresponding to 3 X 10^5 cpm from the P47 template) were electrophoresed on the same gel as immunoblot samples; since approximately 3 ng of in vitro synthesized P47 was loaded, the autoradiographic signal arises from [^{35}S]-methionine, not [^{125}I]-labeled protein A. Position of P47 is indicated on the left.

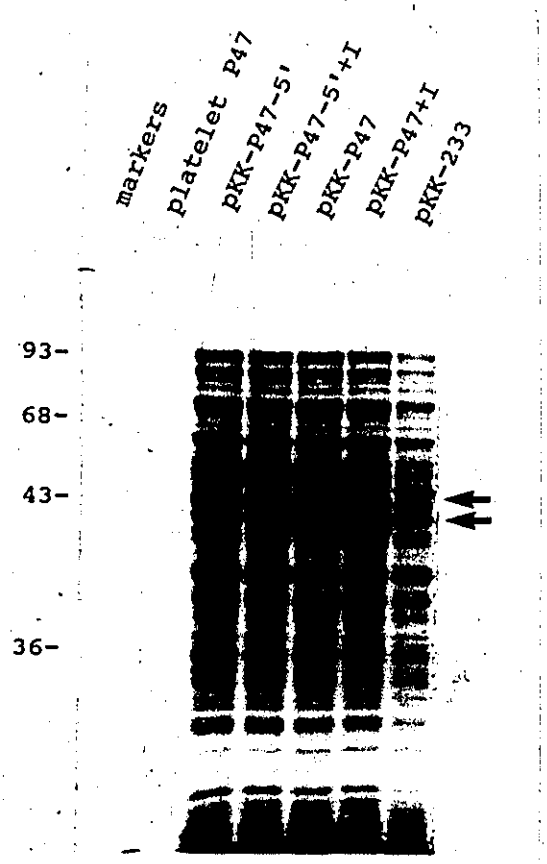


Fig. 4.3.4. Expression of recombinant P47 in E. coli. P47 ORF's were inserted in the pKK-233 prokaryotic expression vector and lysates from transformed E. coli JM109 cells analysed by SDS-PAGE and Coomassie blue staining: markers (lane 1), 30 ng purified platelet P47 (lane 2), nucleotides -53 to 1091 corresponding to M_r 42,112 (lane 3 and 4, without and with IPTG respectively), nucleotides -1 to 1091 corresponding to M_r 40,087 (lane 5 and 6, without and with IPTG respectively), and control pKK-233 lysate (lane 8). Arrows indicate the position of recombinant protein. Marker sizes in kDa are indicated on the left.

10% of bacterial protein as estimated by visual comparison to known amounts of platelet P47. In contrast, the larger protein was barely visible on stained gels (Fig. 4.3.4). Poor bacterial codon usage in the extended reading frame could in part account for decreased accumulation of the larger protein; however, the true coding region appeared to have equally poor codon usage. Alternatively, protein folding may not occur correctly in N-terminally extended P47, perhaps targeting it for degradation by bacterial proteases. There were no obvious P47 proteolytic fragments in bacterial lysates (Fig. 4.3.3) but long exposures revealed many products that specifically reacted with P47 antiserum. Finally, despite expression in a lac I^q background, IPTG had no effect on the expression of either construct (Fig. 4.3.4). Absence of induction could be due to titration of the lac repressor by high plasmid copy number (Amann et al, 1988).

Preliminary efforts at isolating soluble P47 from E. coli were hampered by extreme insolubility of the bacterial inclusion bodies that contained the recombinant protein (see Schoemaker et al, 1985). Initially, it was difficult to obtain consistent high level expression of P47 in JM109 cells. It appeared that restreaked cells from saturated cultures or cells kept on plates for prolonged periods of time expressed much less recombinant protein than freshly transformed cells. Plasmid loss or selection for cells able to repress the lac-trp promoter on pKK-233 may have occured. Routine isolation of recombinant P47 was therefore carried out with freshly transformed cells kept on plates for no longer than a month. Most bacterial P47 was recovered from cell lysates as a Triton X-100 insoluble fraction that could be separated on

a small scale from other bacterial material as the flow-through fraction of a Sepharose CL-4B column (not shown). Attempts to solubilize P47 inclusion bodies caused severe proteolysis of the recombinant protein (not shown). Protease inhibitors such as PMSF, antipain, leupeptin, and pepstatin did not prevent degradation.

A second bacterial expression vector, the pOTS Nco12 variant of the P_L vector series (Rosenberg and Schatzman, 1987), was tried in the hope that rapid induction of P47 by temperature upshift would yield more soluble protein. Inserts from 3A1 and 34A site-directed mutants were cloned into the NcoI site of pOTS 12 (Fig. 4.3.1). Both isoforms (from 34A and 3A1 coding fragments) of P47 were used in anticipation of possible functional studies on the recombinant protein. As provided, the vector had an unknown insert downstream of the NcoI site, but this could not be easily removed without destroying available cloning sites. Deletion of this insert in subsequent constructs showed that it did not interfere with the production of recombinant P47, although induction of the 3A1 coding region was improved slightly (Fig. 4.3.5). Immunoblot analysis of bacterial lysates indicated that P47 was produced in N5151 cells upon heat inactivation (at 42°C) of the temperature sensitive cI repressor (Fig. 4.3.5). Despite fairly sharp temperature induction, P47 comprised only a small fraction of bacterial protein; the 3A1 construct in particular expressed very small amounts of recombinant protein (Fig. 4.3.5). The reason for this is unclear since other proteins expressed from the P_L promoter accumulate to high levels (see Rosenberg and Schatzman, 1987). Transformation of AR68 cells, which are temperature sensitive for some proteolytic pathways and therefore less likely to

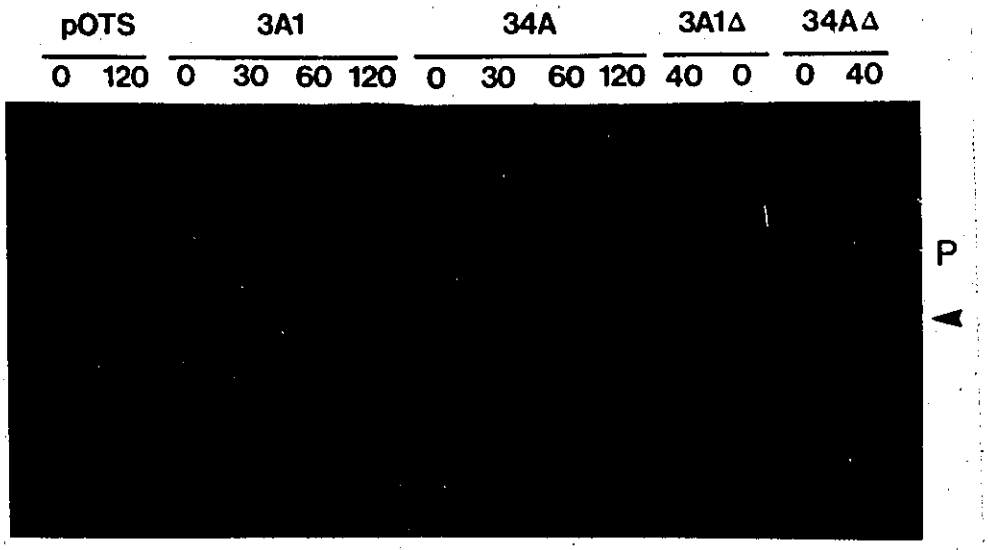


Fig. 4.3.5. Expression of recombinant P47 from the P_L promoter. Transformed N5151 cells were grown to saturation at 32°C then shifted to 42°C for the indicated number of min. Cells were lysed directly in sample buffer and subjected to immunoblot analysis. pOTS indicates vector with no P47 insert. 3A1 and 34A indicate vector containing the 1.1 kb NcoI fragment of clones 3A1 and 34A, respectively. 3A1Δ and 34AΔ indicate constructs from which the unknown insert in pOTS Nco12 was removed (Fig. 4.3.1). P shows the position of P47 and the arrow points to a bacterial protein which reacted with P47 antiserum.

degrade induced recombinant protein, also did not yield high level expression (Fig. 4.3.5). At this point, eukaryotic expression vectors appeared more promising for obtaining native recombinant P47 and work with prokaryotic recombinant protein was discontinued.

4.3.2 Analysis of the Primary Structure of P47

This section contains only a limited analysis of the predicted P47 protein sequence. More speculative and/or mundane aspects are documented for the computer enthusiast in Appendix B. The final compilation of P47 cDNA sequence and deduced amino acid sequence is shown in Fig. 4.3.6. In accord with recent database guidelines, sequence enumeration begins with the A of the initator ATG, assigned as nucleotide 0, and the initiator methionine, assigned as residue 1.

Since the most N-terminal CNBr fragment of P47 contained the initiator methionine, it is this residue which is blocked in the mature protein. As many eukaryotic proteins are acetylated (Augen and Wold, 1986), this is the most likely modification of P47. Acetylation of the initiator methionine occurs for only 5% of all acetylated proteins (Augen and Wold, 1986), so P47 may fall into a slightly unusual category. An analysis of post-translational processing has revealed that proteins which retain their initiator methionine exclusively contain a penultimate residue with a large side chain (Boissel et al, 1988). The second amino acid of P47 is a glutamate; this has a radius of gyration of 1.75 Å, well above the empirically determined cut-off value of 1.3 Å (Boissel et al, 1988). The N-terminus of P47 thus conforms to the deduced modification rules.

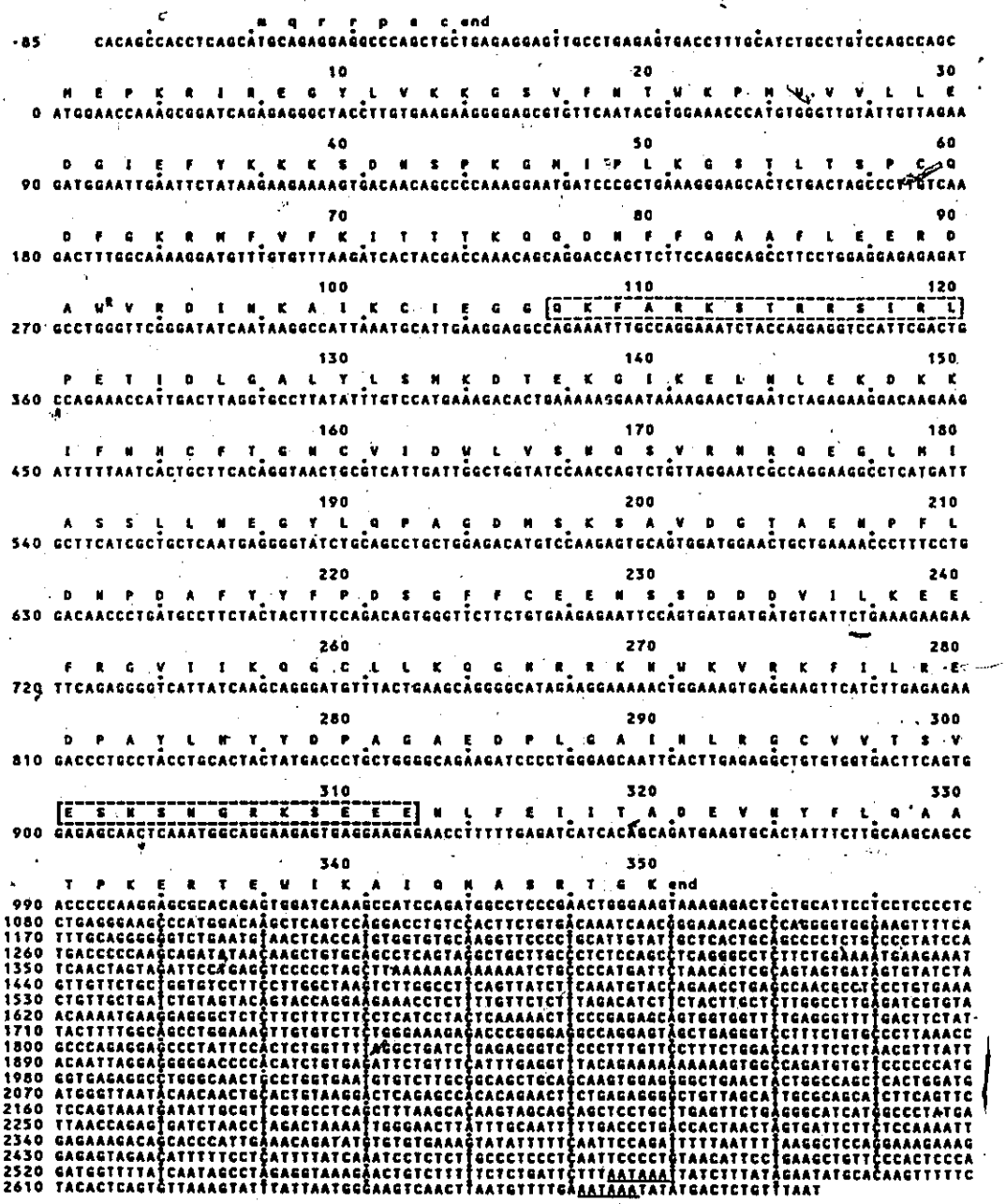


Fig. 4.3.6. Final nucleotide and deduced amino acid sequence of P47 cDNA. The 350 amino acids of P47 encoded by the 1050 nucleotide ORF are shown in upper case 1 letter code. The nucleic acid sequence is numbered relative to the first base of the coding region, which is assigned coordinate 0. The short ORF upstream of the P47 coding region is translated into lower case 1 letter code. Possible polyadenylation signals at the 3' end of the sequence are underlined. The best candidate PKC phosphorylation site (single dashed line) and the putative EF hand Ca^{2+}-binding loop (double dashed line) are boxed. The W to R substitution caused by a T → C transistion at nucleotide 273 in two clones (2A1 and 3A1) is shown by superscript R at residue number 92.

The amino acid composition of P47 was unremarkable except for a preponderance of charged residues (Table 4.3.1). The charge profile of P47 revealed a contiguous stretch of 39 residues (202 to 240) containing 12 acidic side chains immediately followed by a stretch of 28 residues (242 to 279) containing 10 basic residues (Fig. 4.3.7). Overall, basic amino acids slightly out-numbered acidic residues; the calculated pI of P47 was 7.9 for the Trp^{92} isoform and 8.2 for the Arg^{92} isoform. This difference in pI is consistent with multiple unphosphorlyated isoforms of P47 observed on 2-D gels, although the predicted pI values are 1-1.5 pH units higher than experimentally determined values (Imaoka et al, 1983).

The hydropathy profile of P47 did not reveal any potential membrane spanning regions, as expected for a highly charged, cytosolic protein (Fig. 4.3.7). Three basic regions, indicated by arrows in Fig. 4.3.7, may be sensitive sites for proteolysis. In particular, either the N- or C-terminal peaks could be the points at which proteolysis generates P42 and/or P15 (See Section 4.1.1). Since both P15 and the fusion protein encoded by lambda gt11 clone 37 were very immunoreactive, P15 may derive from the C-terminus of P47. Given the possible inverse relationship between P42 and P15 abundance, P42 could arise from N-terminal cleavage of P47. Finally, the predicted secondary structure of P47 suggested a relatively high α-helical content (Fig. 4.3.7).

Alignment of the P47 sequence against itself revealed a significant internal homology. This was visible by dot matrix analysis and by alignment of residues 1-102 and 241-348 (Fig. 4.3.8). P47 may have evolved by ancestral duplication. When P47 sequences were searched

Table 4.3.1. Amino acid composition of P47.

Ala	20	(5.7)	Leu	27	(7.7)
Arg	20/21	(5.7)	Lys	33	(9.4)
Asn	16	(4.6)	Met	8	(2.3)
Asp	22	(6.3)	Phe	20	(5.7)
Cys	7	(2.0)	Pro	14	(4.0)
Gln	12	(3.4)	Ser	22	(6.3)
Glu	28	(8.0)	Thr	16	(4.6)
Gly	24	(6.9)	Trp	6/5	(1.7)
His	6	(1.7)	Tyr	10	(2.9)
Ile	22	(6.3)	Val	17	(4.9)

Acidic	(Asp + Glu)	50	(14.3)
Basic	(Arg + Lys)	53/54	(15.1)
Aromatic	(Phe + Trp + Tyr)	36/35	(10.3)
Hydrophobic	(Aromatic + Ile + Leu + Met + Val)	110/109	(31.4)

M_r = 40,087

Residues frequencies were calculated with Beckman Microgenie software. The values separated by a slash correspond to the Arg^{92} variant, which has a M_r of 40,057.

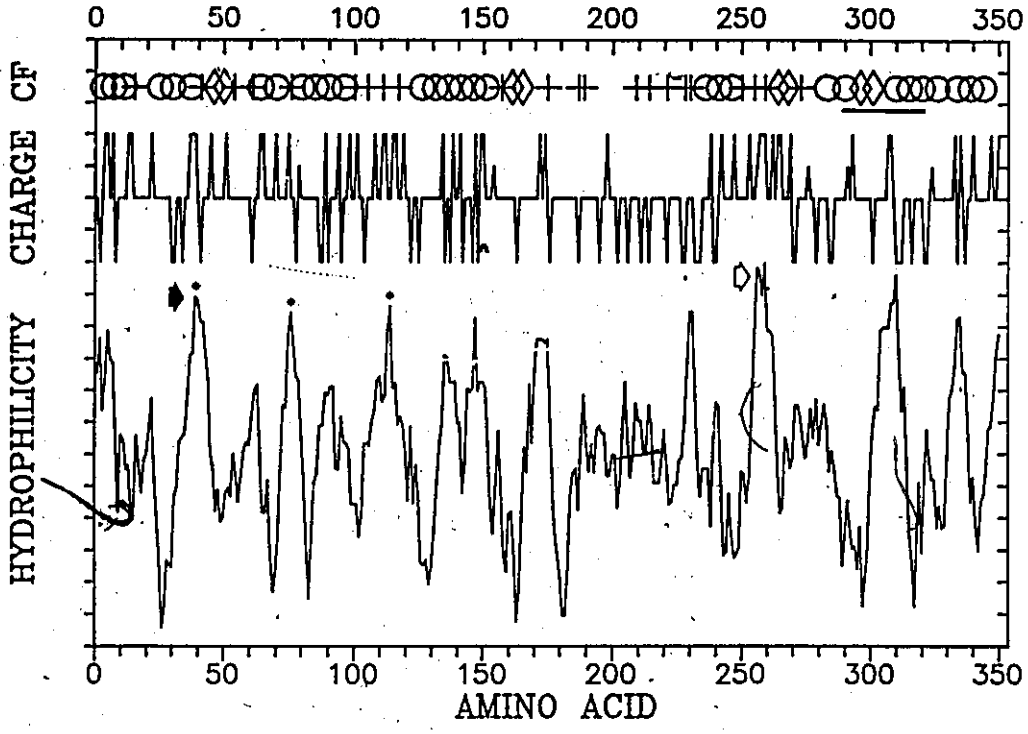


Fig. 4.3.7. Predicted secondary structure, charge profile and hydrophilicity plot of P47 protein. Secondary structure and hydrophilicity were determined with standard parameters on the University of Wisconsin Genetic Computer Group software using the methods of Garnier et al (1978) and Kyte and Doolittle (1982), respectively. Secondary structure symbols represent α-helix (○), β-sheet (◇), and turn (+). The charge profile was plotted by assigning +1 to R or K residues, +0.5 to H, and -1 to E or D residues. Potential proteolytic sites that could yield P42 (see text) are indicated by arrows. Predicted PKC phosphorylation sites are indicated by * and the putative Ca^{2+}-binding EF-hand is represented as a bar.

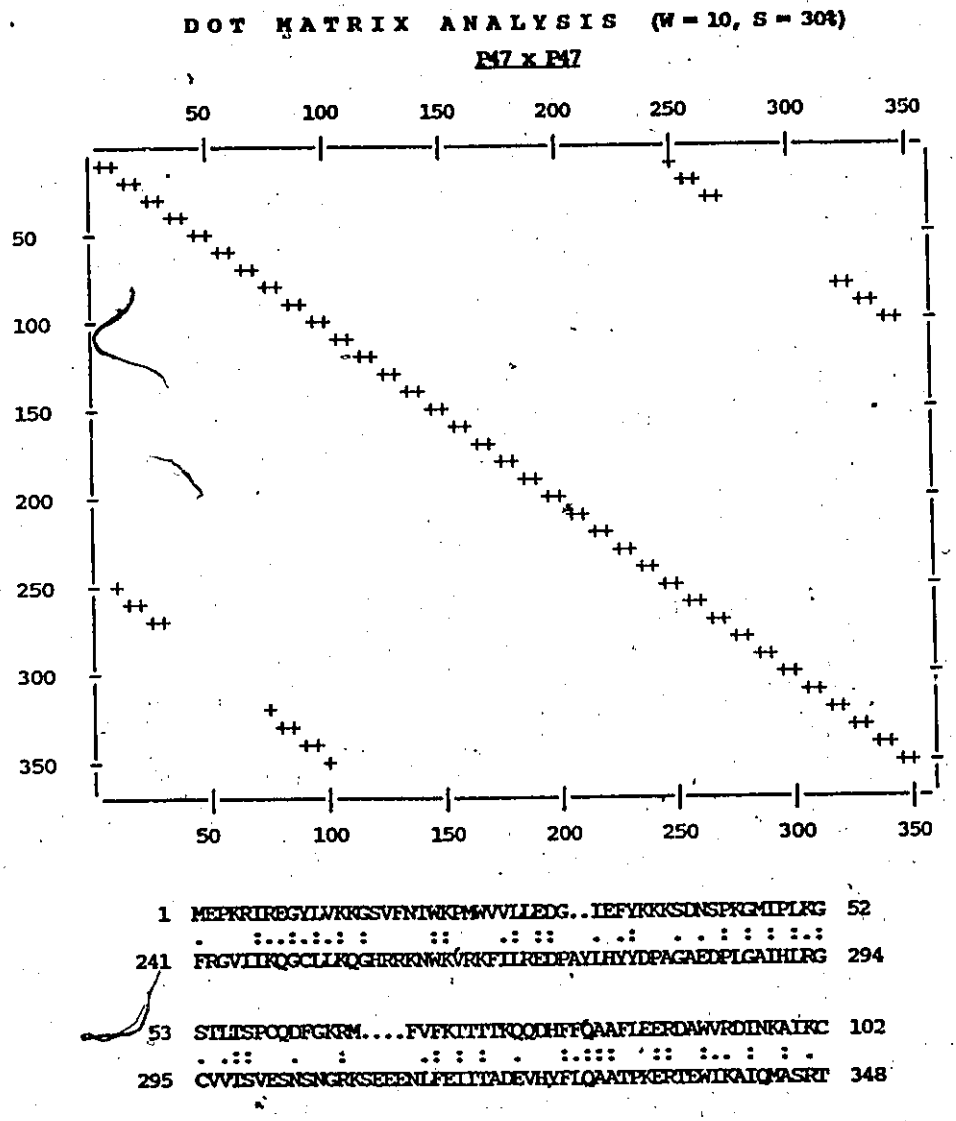


Fig. 4.2.8. Duplicated region in P47 found by alignment of P47 against itself. Dot matrix analysis was performed with Beckman Microgenie software. Region 1-102 and 241-348 were aligned; 2 dots indicate amino acid identity and 1 dot indicates similar amino acids. The alignment score of these regions was 8 standard deviations greater than the mean alignment score of corresponding random P47 subsequences. Above 4-6 standard deviations is considered to suggest biological significance.

against current protein and nucleic acid databases using the FASTN, FASTP and FASTA algorithms no significant similarities were found (Pearson and Lipman, 1988). The best 3 alignments between P47 and the NBRF protein database indicate the random nature of the matches:

protein	% similarity	amino acid overlap
E6 protein (cottontail rabbit)	33	36
repressible acid phosphatase	33	45
rabbit IgG C region	23	43

Surprisingly the protein which exhibited the strongest similarity to P47 of any known sequence was the putative Ras effector, called GTPase-activating-protein or GAP, recently been cloned by Vogel et al (1988). Under standard alignment conditions, GAP and P47 are 37% similar over the entire length of the P47 sequence; a less stringent alignment is given in Appendix B. Compared to randomly shuffled amino acids of the same composition the similarity between GAP and P47 is approximately 10 SD above the mean score. Like the internal duplication in P47, this too borders on biological significance. However, GAP is more similar (> 20 SD above random) to several other proteins involved in signal transduction, namely the cytosolic protein tyrosine kinases (e.g. yes, fgr, tck), the crk oncogene and PLC-148 (Vogel et al, 1988; Mayer et al, 1988). Unlike P47, the regions of similarity between GAP and these proteins maps in part to the so-called SH_2 regulatory domain (Sadowski et al, 1986). The significance of the similarity between P47 and GAP is therefore uncertain at best.

Two intriguing consensus sequences were noticed in P47. A

potential Ca^{2+}-binding loop bounded by two predicted α-helical regions suggested that the C-terminus of P47 may contain an EF-hand domain (Kretsinger, 1980). Comparison to known Ca^{2+}-binding proteins by multiple sequence alignment (Bacon and Anderson, 1986) revealed that the P47 EF-hand scored worse than other EF-hands, including some that do not bind Ca^{2+} (Fig. 4.3.9). Criteria based on crystallographic data (Kretsinger, 1980) state that of 16 conserved residues required for Ca^{2+}-binding, a minimum of 12 are required for a functional EF-hand. The putative P47 EF-hand contains only 11 of these. However, two proteins which may not bind Ca+, namely p11 (Gerke and Weber, 1985) and α-actinin (Baron et al, 1987), have lost essential Ca^{2+} liganding residues whereas P47 is predicted to have all 6 oxygens which chelate Ca^{2+}. Two defects in the P47 EF-hand are obvious: 1) a highly conserved hydrophobic residue in the middle of the Ca^{2+}-binding loop has been replaced by a lysine (indicated by the arrow in Fig. 4.3.9); 2) the flanking segments lack several conserved residues and may be only weakly α-helical. Speculation as to the structure and function of the P47 EF-hand are presented in Appendix B.

Given the extent to which P47 can be phosphorylated, several potential PKC phosphorylation sites were anticipated. P47 isolated from stimulated platelets consists of 9-11 pI isoforms, possibly generated by 4 or 5 independent phosphorylation sites (Imaoka et al, 1983). Prediction of which serine or threonine residues may be targets for phosphorylation was hampered by the lack of a precise consensus sequence for PKC-mediated phosphorylation. This was overcome by multiple sequence alignment to define a priori residues that are conserved

i)	1	2	3	4	5	6	7	8	9	0	1	2	3	4	5	6	7	8	9	0	1	2	3	4	5	6	7	8	9	SCORE	
ii)	E	h			h	h				h	d		d		d	G		i	d			E	h			h	h			h	KI	AIN
iii)											X		Y		Z	-Y		-X			-Z										
PLE	L	R	G	C	V	V	T	S	V	E	S	N	S	N	G	R	K	S	E	E	E	N	L	F	E	I	I	T	A	11	1607
CaM	E	F	K	E	A	F	S	L	F	D	K	D	G	N	G	T	I	T	T	L	E	L	G	T	V	M	R	S	L	16	1781
S100	V	V	D	K	V	M	E	T	L	D	S	D	G	D	G	E	C	D	F	Q	E	F	M	A	F	V	A	M	I	14	1720
p11	A	V	D	K	I	M	K	D	L	D	Q	C	R	D	G	K	V	G	F	Q	S	F	F	S	L	I	A	G	L	12	1685
αACT	E	F	A	R	I	M	S	I	V	D	P	N	R	M	G	V	V	T	F	Q	A	F	I	D	F	M	S	R	E	13	1693
FIB	H	N	M	G	Q	F	S	T	W	D	N	D	N	D	K	F	E	G	N	C	A	E	Q	D	G	S	G	W	W	6	1542

Fig. 4.3.9. Comparison of predicted P47 EF-hand to similar structures in known Ca^{2+}-binding proteins: i) numbering system proposed by Kretsinger (1980) ii) consensus sequence (h = A,F,I,L,M,V or Y; d = D,E,N,Q,S, or T; i = I or V); iii) octahedral vertices formed by liganding oxygens; P47 292-320 (this work); CaM, calmodulin I 19-37 (Babu et al, 1985); S100 β52-80 (Gerke and Weber, 1985); p11 50-78 (Gerke and Weber, 1985); αACT, chicken fibroblast α-actinin 786-814 (Baron et al, 1987); FIB, fibrinogen τ307-336 (Dang et al, 1985). The degree of similarity, based on the criteria of Kretsinger (KI) and a modified multiple sequence alignment program (AIN) is shown by scores on the right. The core set for alignment employed 4 proteins for which crystallographic data has unambiguously assigned EF-hand residues: calmodulin (Babu et al, 1985); intestinal Ca^{2+}-binding protein (Szebenyi et al, 1981); troponin C (Collins et al, 1977); parvalbumin (Kretsinger et al, 1973). The ALIGN program was run with a window size of 30 in which only the resides identified by Kretsinger (1980) contributed to the final score; in addition, residues in the Ca^{2+}-binding loop were weighted twice those in the flanking helices.

SUBSTRATE	SEQUENCE	SCORE
IL-2-R (241-250)	Q R R Q R K S R R T	450
SM-MLC (3-12)	K R A K A K T T K K	450
PKCPS (19-28)	R F A R K G S L R Q	450
RS6 (230-239)	K R R R L S S L R A	450
EGFR (648-657)	H I V R K R T L R R	441
P60 (5-14)	S K P K D P S Q R R	431
MBP (104-113)	G K G R G L S L S R	408
P36 (19-28)	P P S A Y G S V K A	392
PLE (108-117)	K F A R K S T R R S	470
(107-116)	Q K F A R K S T R R	455
(111-120)	R K S T R R S I R L	450
(37-46)	K K K S D N S P K G	433
(34-43)	E F Y K K K S D N S	427
(67-76)	F V F K I T T T K Q	416

Fig. 4.3.10. Comparison of known PKC phosphorylation sites to potential sites in P47. Sequences of 8 PKC substrates were aligned 4 at a time using a window size of 10 residues and a matrix which favoured alignment of basic residues and alignment of serine or threonine residues. The best alignment of these (IL-2-R, interleukin-2 receptor (Woodget et al, 1986); SM-MLC, smooth muscle myosin light chain (Ikebe et al, 1987); PKCPS, PKC pseudosubstrate (House and Kemp, 1987); RS6, ribosomal S6 protein (House et al, 1987)) was used as a core set for comparison to other substrate sequences (MBP, myelin basic protein (Turner et al, 1985); EGFR, epidermal growth factor receptor (Woodget et al, 1986); P60, $p60^{src}$ (Woodget et al, 1986); and P36, p36 (Woodget et al, 1986)) and the entire P47 protein sequence. Align scores for each sequence are indicated. Phosphorylated sites in known substrates and putative sites in P47 are boxed, while basic residues are underlined.

amongst known PKC sites. In general, a requirement for basic residues on the C and/or N terminal sides of the target residue has been found (see references in legend to Fig. 4.3.10). Three possible alignments of the P47 region 107-120 scored higher than the adjusted core site value, and considerably higher than other known PKC sites (Fig. 4.3.10). The P47 region was remarkably similar to the pseudosubstrate site of PKC, which is the most potent peptide inhibitor of PKC yet synthesized (House and Kemp, 1987). Other lower scoring potential PKC sites in P47 are also indicated in Fig. 4.3.1. One weaker site not defined in Fig. 4.3.10 may be of interest because of its location in the putative Ca^{2+}-binding loop (Arg^{307}-Lys-Ser^{309}). In contrast to the candidate regions for phosphorylation by PKC, no consensus sequence for cAMP dependent kinase phosphorylation was found in P47 nor was any other known consensus sequence.

4.4 ANALYSIS OF P47 mRNA

4.4.1 Regulation and Distribution of P47 mRNA

The P47 cDNA hybridized to a 3.0 kb transcript that was up-regulated during RA-induced HL-60 cell differentiation (Fig. 4.4.1). The degree of mRNA induction was estimated at 10 fold by densitometric scanning; this was slightly greater than the 4 fold increase in P47 protein (see Section 4.1.1). Induction of P47 by RA was thus entirely accounted for at the mRNA level; this comprised part of the initial evidence that the correct cDNA had been cloned. P47 mRNA was present in the poly(A)$^+$ RNA population and migrated at the same size as in total

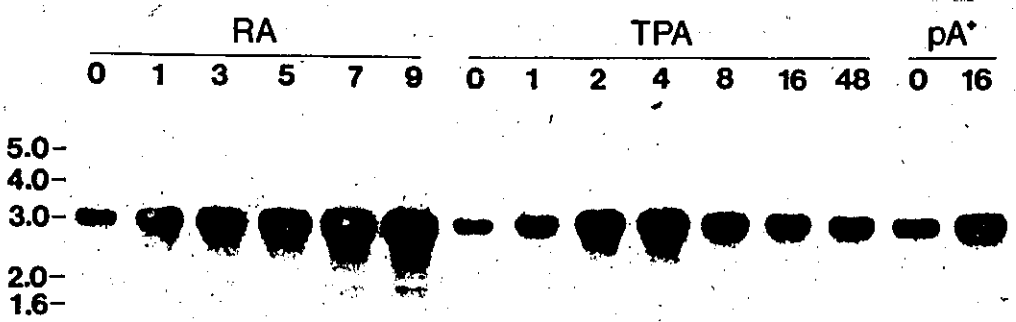


Fig. 4.4.1. Regulation of P47 mRNA during HL-60 cell differentiation. Total RNA (15 µg) from cells treated with 1 µM RA for the indicated number of days or 20 nM TPA for the indicated number of hours was analyzed by Northern transfer using the 2 kb P47 cDNA PvuII fragment. Poly $(A)^+$ RNA (0.5 µg) from cells treated with TPA for 0 and 16 hours is also shown. Marker sizes in kb are indicated to the left.

RNA samples (Fig. 4.4.1).

Since P47 is a target for PKC, and since TPA had unusual effects on immunoreactive species (see Section 4.1.2), the regulation of P47 mRNA by TPA was investigated. Undifferentiated HL-60 cells responded to 20 nM TPA with a marked induction of P47 mRNA that peaked at 2-4 h and declined to near basal levels by 48 h (Fig. 4.4.1). This was confirmed in studies on synergistic HL-60 differentiation, which also incidently showed that P47 was not induced by Ca^{2+} (see Section 3.2.2). However, in RA-differentiated cells the 3.0 kb P47 mRNA was not induced by TPA treatment (data not shown). The transient elevation of P47 mRNA during TPA induced differentiation may in fact be a component of the new program of gene expression, rather than a specific response to PKC activation. On this basis, various phorbol ester responsive elements may not be found in the P47 promoter. Finally, it is not clear why a only a slight increase in P47 protein synthesis occured in parallel with induction of the mRNA (Fig. 4.1.5); P47 may be subject to translational regulation (see Section 4.4.2).

In addition to RA and TPA, other agents caused induction of P47 transcripts (Fig. 4.4.2). Aside from RA, the strongest inducer was 1,25-$(OH)_2D_3$ which, like RA, caused a parallel increase in P47 protein (Fig. 4.1.1). As indicated above, macrophage-like differentiation in response to either TPA or TPA/A23187 only slightly increased P47 mRNA content. Monocytic maturation induced by dibutyryl cAMP or cytosine arabinoside (data not shown) was not accompanied by an increase in P47 transcripts. P47 mRNA abundance also did not increase upon differentiation of U937 cells with TPA (data not shown).

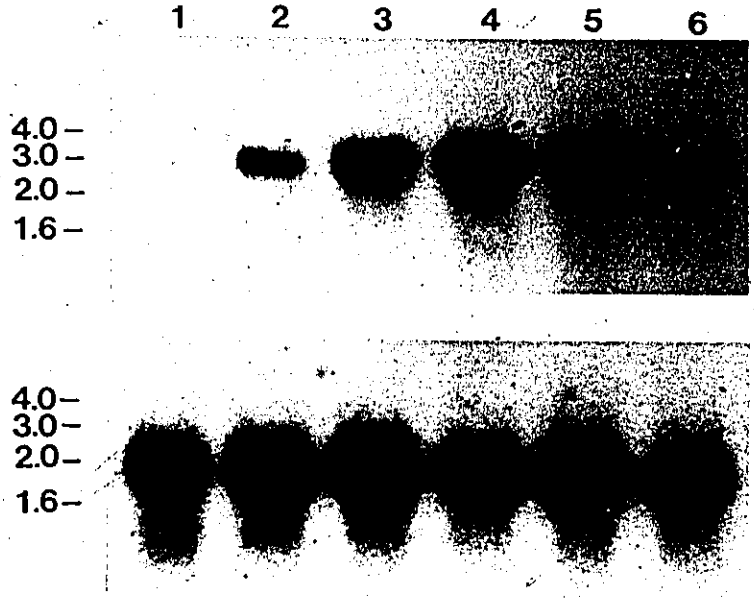


Fig. 4.4.2. Abundance of P47 mRNA in variously differentiated HL-60 cells. Total RNA was probed with the 2 kb P47 PvuII cDNA fragment: untreated (lane 1); 20 nM TPA, 2 d (lane 2); 1 nM TPA and 400 nM A23187, 2 d (lane 3); 1μM RA, 7 d (lane 4); 100 nM $1,25(OH)_2D_3$, 7 d; (lane 5); 500 μM dibutyryl cAMP, 3 d (lane 6). Lower panel shows the same filter reprobed with actin oligonucleotide. P47 (P) and actin (A) transcripts are indicated to the left.

The expression of P47 in monocytic and granulocytic forms of HL-60 cells was confirmed in normal human peripheral blood mononuclear cells. A mixed population of lymphoctyes and monocytes contained the identical 3.0 kb transcript (Fig. 4.4.3, lane 1). In addition, P47-related transcripts were identified in mouse spleen tissue (Fig. 4.4.3, lane 5). Surprisingly, the mouse mRNA was even larger than the human homolog. A more thorough investigation of the tissue distribution was carried out with various human cell lines (Fig. 4.4.4). P47 mRNA was found in white cells of both lymphoid and myeloid lineages but not in the more primitive K562 line (Clarke et al, 1987). Several cell lines expressed more P47 mRNA than undifferentiated HL-60 cells. The richest source was the Raji B cell lymphoma line which contained roughly the same amount of P47 mRNA as RA-differentiated HL-60 cells. High levels also occured in the monocytic lines U937 and THP-1. This suggested that macrophage-like differentiation of HL-60 cells may be incomplete with respect to P47 expression. No transcripts were detectable in even very long exposures of samples corresponding to liver, fibroblast, skin, lens, and neuronal cells. Finally, induction of P47 was not generally associated with differentiation or cell cycle exit since SH neuroblastoma cells treated with NGF did not contain P47 mRNA (Fig. 4.4.4, lane 13).

P47 expression thus correlated strongly with differentiated hemopoeitic cells. This provided some clue as to P47 function (see Section 4.6). Induction of P47 mRNA in HL-60 cells depended on the inducing agent; in particular, monocytic differentiation was accompanied by quite variable increases, ranging from near maximal with 1,25-$(OH)_2D_3$

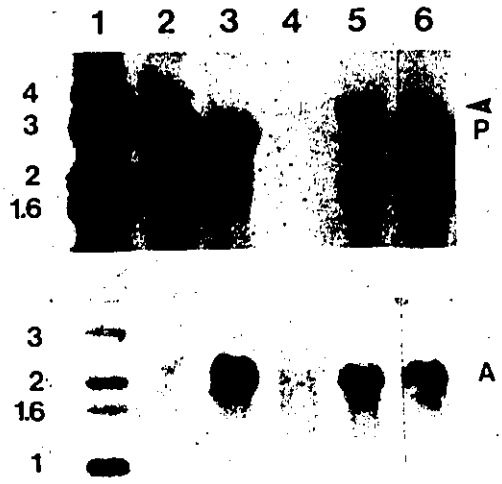


Fig. 4.4.3. Analysis of P47 mRNA in normal tissue. Total RNA (15 µg) from the indicated sources was probed with the 2 kb PvuII P47 cDNA fragment: kinased BRL 1 kb ladder (lane 1); human mononuclear blood cells (lane 2); undifferentiated HL-60 cells (lane 3); mouse spleen (lane 4); mouse spleen (lane 5); undifferentiated HL-60 cells (lane 6). The same filter probed with actin oligonucleotide is shown below. P47 (P) and actin (A) transcripts are indicated to the right and marker sizes in kb to the left. The arrow shows the larger sized P47 related transcript in mouse lymphoid tissue. Lane 4 showed little hybridization because the sample leaked from the well during loading. The reason for reduced actin hybridization in lane 2 is unclear.

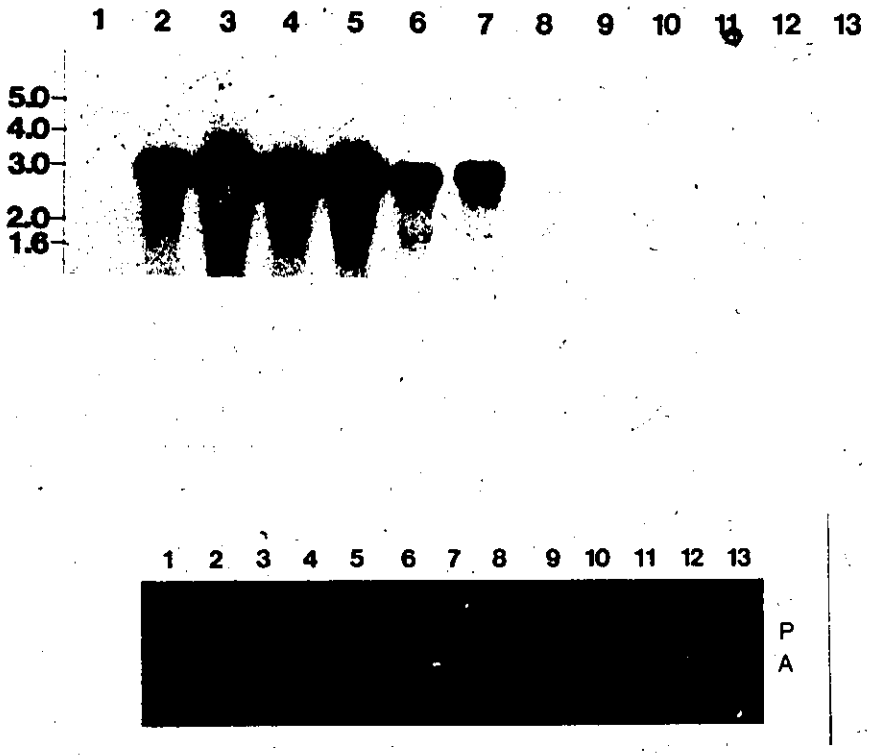


Fig. 4.4.4. Tissue distribution of P47 mRNA. Human cell lines were used as sources of tissue specific RNA which were probed with the 2 kb P47 cDNA PvuII fragment: K562 undifferentiated myeloblastic leukemia (lane 1), HL-60 promyelocytic leukemia (lane 2), U937 histiocytic leukemia (lane 3), THP-1 monocytic leukemia (lane 4), Raji Burkitts lymphoma (lane 5), JO5 EBV transformed B cell (lane 6), HUT 78 T cell (lane 7), HepG2 hepatoma (lane 8), MJ fibroblast (lane 9), COLO 16 keratinocyte (lane 10), HEC lens epithelial (lane 11), SH neuroblastoma (lane 12), SH neuroblastoma differentiated with 10 ng/ml NGF for 48 h (lane 13). Marker sizes in kb are indicated to the left. The same filter re-probed with actin oligonucleotide before stripping off P47 probe is shown below. The position of P47 (P) and actin (A) transcripts are indicated.

to not at all with dbcAMP and cytosine arabinoside. This probably reflects defective or incomplete maturation of HL-60 cells (Ross et al, 1986) rather than any physiologically significant variation.

4.4.2 Structure of P47 mRNA

As mentioned in Section 4.2.2, the P47 mRNA appeared to have a complex 5' end. Primer extension analysis revealed 4 distinct transcripts that corresponded approximately to nucleotide positions -70, -100, -120 and -170 (Fig. 4.4.5). The heterogeneous mRNAs were not resolved by Northern analysis since the total variation in transcript sizes was no more than 100 bases. Upon differentiation the relative abundance of each mRNA was altered such that an increase in the smallest and largest mRNA species occured at the expense of the middle 2 transcripts (Fig. 4.4.5). This result should be confirmed by S1 mapping studies. If the alternate P47 transcripts have different translational efficiencies, this could provide a basis for the discrepancy between the induction of P47 mRNA and protein in TPA-treated HL-60 cells. The complex nature of the 5' region of the P47 gene may underlie differential RNA processing or multiple cap sites that presumably generate the 4 P47 transcripts (see Section 4.5). Primer extension analysis has revealed similar 5' heterogeneity in the mouse c-myb mRNA (Watson et al, 1987) and Torpedo acetylcholinesterase mRNA (Sikorav et al, 1987) but in neither instance has the basis or function of the heterogenous transcripts been elucidated.

Sequence analysis of the longest P47 cDNA (clone 3-1) revealed an upstream ATG that initiated a short ORF of 7 amino acids (see Fig.

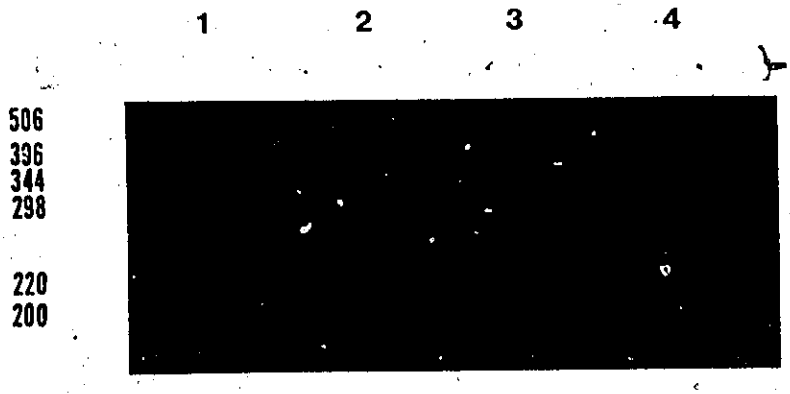


Fig. 4.4.5. Analysis of the 5' end of P47 mRNA and regulation during HL-60 differentiation. Poly(A)$^+$ RNA was reverse transcribed from a ^{32}P-end-labeled primer: kinased BRL 1 kb ladder (lane 1); undifferentiated cells (lane 2); 20 nM TPA, 16 h (lane 3); 1 µM RA, 5 d (lane 4) . The primer used (AB207) began 256 nucleotides from the 5' end of the cDNA. The predominant bands thus correspond approximately to nucleotide positions -70, -100, -120 and -170. Marker sizes in bp are indicated on the left. The faint bands in between lanes 1 and 2 is spill over from the marker lane. The autoradiogram is representative of 3 independent experiments.

4.3.6). This is in contrast to 90% of vertebrate mRNA in which the first ATG begins the true ORF (Kozak, 1986b). While by no means optimal, the P47 upstream ATG does contain a dominant purine at the -3 position and hence may be utilized *in vivo*. Importantly, the first reading frame terminates before the authentic P47 start site. Still, this would require re-initiation at the true ATG, a phenomenon which apparently depends on the length of the intercistronic region (Kozak, 1987b). For P47, this distance is only 46 nucleotides, just on the borderline value for re-initiation of the preproinsulin mRNA (Kozak, 1987b). Aside from oncogenes, for which re-initiation after a short upstream ORF is relatively common (Kozak, 1986b), HL-60 cells contain 2 known differentiation-regulated mRNAs with similar characteristics. Myeloperoxidase has 1 upstream ORF (Morishita *et al*, 1987) and α-tubulin has 4 such reading frames (Salser *et al*, 1985). In contrast to P47, both these mRNAs decrease in abundance during differentiation, although α-tubulin expression is maintained by increased translational efficiency after differentiation.

Although excessive transcript sizes are by no means uncommon (Kozak, 1987a), the P47 mRNA was about twice as large as was expected to code for a 40 kDa protein. One possible explanation for this is that a second mRNA became fused to an original P47 mRNA. Subsequent mutations could have disrupted the ORF of the downstream mRNA. Inspection of the region immediately 5' to the first poly(A) stretch revealed two cryptic polyadenylation signals, AATGAA (nucleotide 1339) and AAGAAA (nucleotide 1343), both within 45 nucleotides of the first poly(A) tract in the P47 3' untranslated region (UTR). However, codon usage patterns in all

reading frames of the 3' UTR showed no evidence of an ancient coding region.

Several possible regulatory functions of the P47 3' UTR can be imagined. P47 translation could be controlled by mRNA secondary structure. Simple duplex stability analysis of mRNA stem-loop structures revealed many potential duplexes throughout P47 mRNA (Table 4.4.1). Most intriguing was a potential pairing between nucleotides 17-40 and 1423-1400; this is obviously in a position to disrupt translation. An RNA hairpin structure (a 12 bp stem containing 34 bonds with a calculated ΔG of -30 kcal/mol) significantly impairs translation under some conditions of cellular stress (Kozak, 1988). Although the stem structure at the 5' end of the P47 mRNA also contained a uninterupted 12 bp stretch with 32 bonds, the large loop size mitigates against the stability of this structure. In a more straightforward mechanism, the long 3' UTR may simply protect against exonuclease activity. It is perhaps significant that P47 mRNA abundance increases dramatically during RA-induced differentiation whereas the total RNA content of the cell drops approximately 5 fold. The sequence diversity in the HL-60 mRNA population decreases approximately 3 fold after differentiation with RA (Torelli et al, 1984). In any event, conservation of the P47 mRNA size in mouse suggests the 3' UTR has a functional signficance.

Table 4.4.1 Stem-loop structures in the P47 mRNA

STEM	LOOP	MATCHES	BONDS
17 tgcagaggaggCCCAGCTGCTGAG 1423 AGGTCTTCTCCGGGACTCCGACCT	1359	17/24	48
1854 GTAGCTGAGGGTCCTTTCTGTGC 2197 CACCGAGACTCAGGAATGTCACG	298	17/23	46
1621 GCTGATCTGTAGTACAGTACC 2145 CGACCGGTCATCAAGTCGGGG	483	15/21	42
398 GGAGGCCAGAAATTTGCCAGG 1567 CTTCCGGTTCTGAATCGGTTC	1128	17/21	42
34 TGCTGAGAGGAGTTGCCTGAG 1612 GTGTCCCTCCGCAACCGAGTC	1537	14/21	39
2100 TCTTGCGGCAGCTGCAGCAA 2309 AGTTCGTCCTCGACGACGAT	170	14/21	39
839 CTGAAGCAGGGGCATAGAAG 932 GGGGTCGTCCCAGTATCATC	54	15/21	40
329 CAGGCAGCCTTCCTGGAGG 622 GTACTCCGGAAGGACCGCT	256	14/19	39

RNA stem-loop structures were calculated with the STEMLOOP routine of the UWGCG software package. Minimum stem length was set to 15 bases; minimum bonds/stem was set to 30; maximum loop size was set to 1970 bases; nibbling threshold was set to 0.5. The top 8 of 101 stems found under these conditions are shown.

4.5 CHARACTERIZATION OF THE P47 GENE

4.5.1 Analysis of the P47 locus

Southern analysis of the P47 locus was carried out to determine the size, evolutionary conservation, and complexity of the human gene. Human genomic DNA digested with EcoRI hybridized to the P47 cDNA at sizes of 9.2, 7.8, 7.0, 5.5 and 1.2 kbp (Fig. 4.5.1, lanes 1 and 2). HL-60 DNA and normal human lymphocyte DNA yielded identical restriction patterns, suggesting that the P47 locus has not been grossly rearranged in the HL-60 line. Although the P47 cDNA contained 3 EcoRI sites, 2 of these were only 34 bp apart, such that the fragment liberated would be far too small to either bind to nitrocellulose or hybridize to probe sequences. Thus 2 additional sites within introns were required to explain the 5 EcoRI genomic fragments. Obviously, small fragments and fragments within introns would not be detected. Southern analysis after digestion with PstI, Hin dIII, and MspI confirmed the size of the P47 gene (data not shown).

The multiple EcoRI genomic fragments had various hybridization intensities which could be explained by either the presence of related sequences in the human genome or different target sizes within each fragment. Southern transfers probed with specific EcoRI cDNA fragments indicated the latter (Fig. 4.5.2). The two weak signals at 5.5 kbp and 9.2 kbp arose from hybridization to the 5' 160 bp fragment of the P47 cDNA. This suggests that the 5' end of the P47 gene is spread over 14 kbp, although it is also possible that this region contained a polymorphic EcoRI site in both the HL-60 and normal human DNA samples

Fig. 4.5.1. Southern analysis of the P47 gene. DNA from various organisms was analyzed: human (HL-60 cells, lane 1; normal leukocytes, lane 2); mouse (lane 3); chicken (lane 4); rainbow trout (lane 5); D. melanogaster (lane 6); S. cerevisiae (lane 7). DNA (10 µg) from each species was digested with EcoRI then electrophoresed on 0.8 % agarose, denatured, transferred to nitrocellulose and probed with the entire P47 cDNA. Hybridization was in 30 % formamide and the high temperature wash was in 0.5 X SSC. Marker sizes in kbp are indicated on the left.

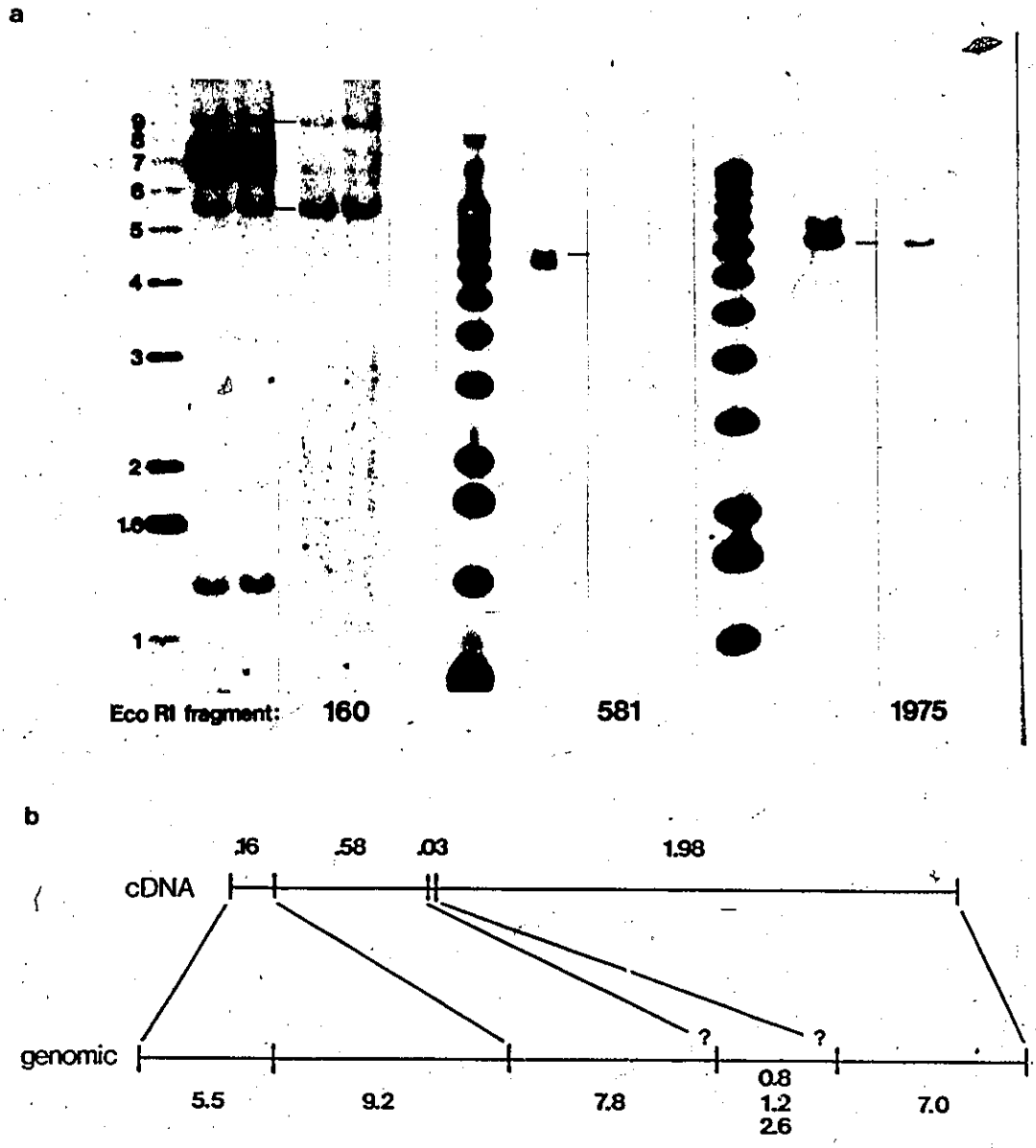


Fig. 4.5.2. a, Correspondence between P47 cDNA and genomic DNA EcoRI fragments. Three independent Southern blots were stripped of the initial P47 probe and re-probed with specific EcoRI cDNA fragments, as indicated. These were aligned next to the original autoradiograms to determine which genomic band corresponded to each cDNA fragment. The EcoRI fragments used as probes are shown in linear order below their respective blots. Weak hybridization of the 1975 bp probe to the 1.2 kb fragment cannot be seen in this exposure. Marker sizes in kbp are labeled on the first blot only. b, Line map indicating deduced correspondence between cDNA and genomic DNA EcoRI fragments. Additional information from genomic clones is included (see Fig. 4.5.6).

used in Fig. 4.5.1. The presence of additional sequences in the human genome related to just the 5' end of the P47 gene was even more unlikely. The 1975 and 581 bp EcoRI cDNA fragments hybridized to 7.0/1.2 and 7.8 kbp genomic fragments, respectively. The deduced linear order of human P47 EcoRI exon fragments is also shown in Fig. 4.5.2.

Sequence comparison of P47 cDNA clones indicated a possible RFLP at nucleotide 273 (see Fig. 4.3.6). Confirmation of this polymorphism in the human population was attempted by Southern analysis of SmaI digested normal human DNA. Unfortunately, the DNA was refractory to cleavage by SmaI and also to AvaII, an isoschizomer of SmaI. For this reason the DNA was re-digested with MspI. Although this enzyme recognizes CCGG, the depletion of CpG dinucleotides in vertebrate DNA causes an expected average size of at least 1 kbp that is still amenable to Southern analysis. To reduce the number of bands visualized, the transfer was probed with the 581 bp cDNA fragment containing the polymorphic site. Two different polymorphisms were detected in a sample of seven unrelated human DNA (Fig. 4.5.3). Comparison of band intensities in homozygous and heterozygous individuals suggested that the 4.0/4.4 kbp bands constitute one polymorphism and the 2.0/2.6 (3.0) kbp bands the other. Since HL-60 DNA contains only the latter polymorphism (refered to as P47-273 for the site of mutation), this must correspond to the sequence difference detected in 2 of the cDNA clones. Hybridization with oligonucleotides close to the polymorphic site would confirm this. The 2.6 kbp fragment appeared to migrate at 3.0 kbp in HL-60 cells, suggesting either another polymorpism or a small rearrangement. Since parental HL-60 and HL-60/MT-myc cells both contained the P47-273

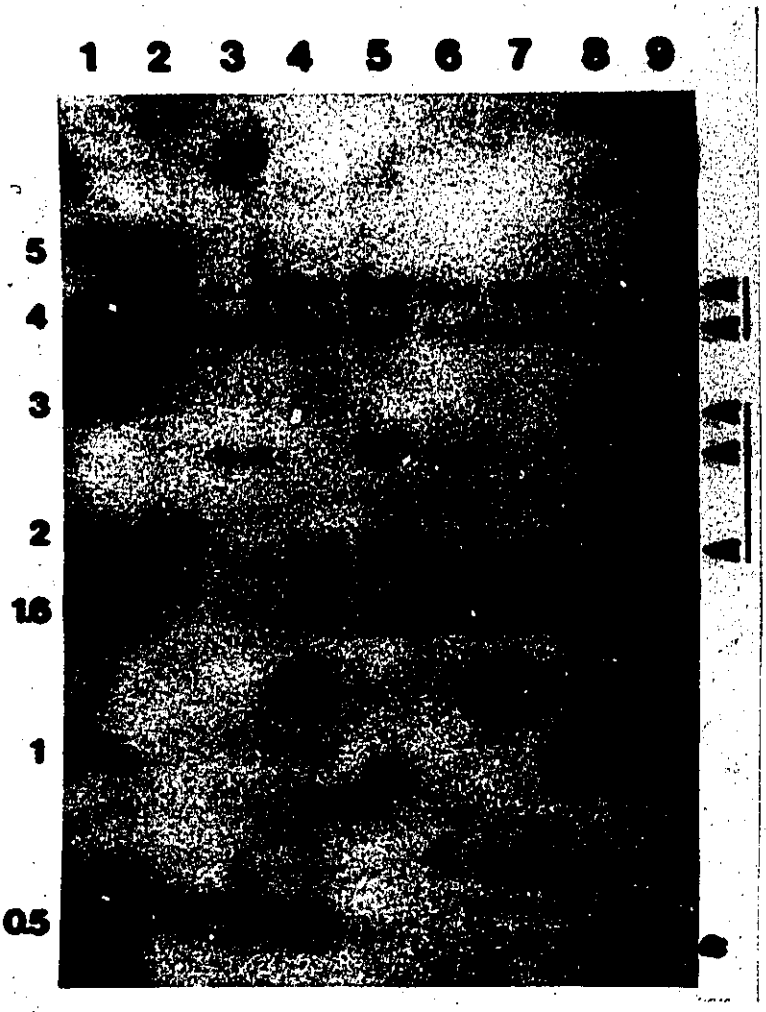


Fig. 4.5.3. Restriction fragment length polymorphisms in the human P47 gene. DNA (20 µg) from 7 unrelated donors was digested to completion with Msp I and probed with the 581 bp P47 cDNA EcoRI fragment: kinased BRL 1 kb ladder (lane 1); RAR (lane 2); DKS (lane 3); KL (lane 4); SS (lane 5); TVA (lane 6); CBH (lane 7); parental HL-60 (lane 8); HL-60/MT-myc (lane 9). Arrows indicate sets of bands likely to arise from polymorphic variation. Marker sizes in kbp are indicated to the left.

polymorphism, it obviously did not arise during genesis of the latter subline. Additional MspI polymorphisms at 0.6, 3.1, 3.5, 4.5, 5.0 and 5.2 kbp were detected by hybridization to the entire 34A cDNA (data not shown). This relatively high degree of allelic variation in P47 contrasted to the c-myc locus which in the same individuals had a single 1.7/2.1 kbp polymorphic site when probed with a 1.5 kbp ClaI-EcoRI fragment covering the third exon (data not shown). Given its multiple polymorphisms, the P47 gene may have useful applications in forensic and paternity analysis. It is merely a matter of increasing the sample size to establish accurate P47 MspI RFLP frequencies in the human population. Finally, assignment of the P47 chromosomal location will allow the cDNA to be used in linkage analysis of the human genome.

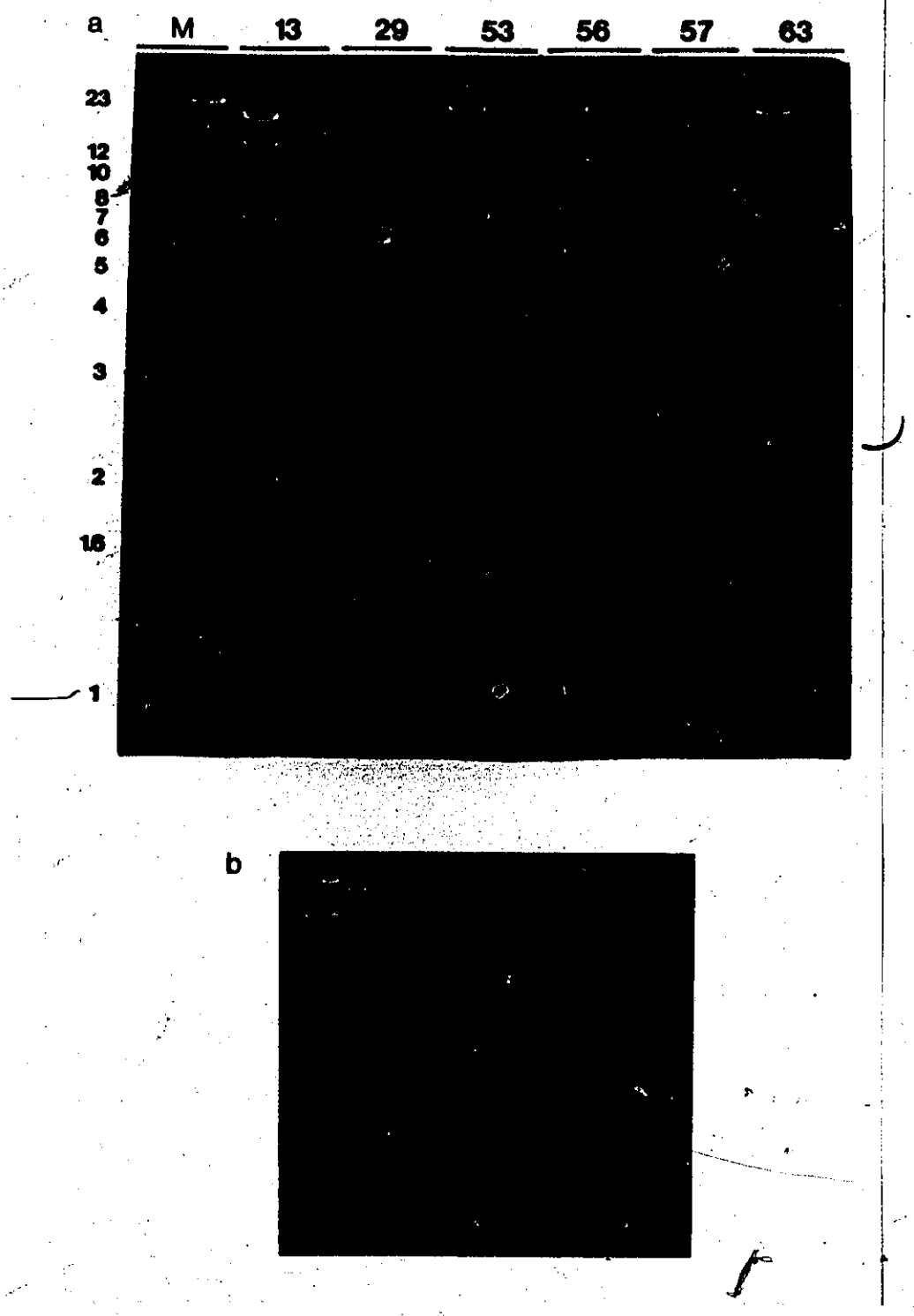
Analysis of DNA from other species, also digested with EcoRI, indicated that P47-related sequences were present in mouse, chicken, and fish but not in insects or yeast (Fig. 4.5.1, lanes 3-7). This was corroborated by the 3.3 kbp P47-related mRNA detected in mouse spleen (see Section 4.4.1). The gene size in all these vertebrate species appeared somewhat smaller than the human homolog, although weak hybridization may not have been detected. Interestingly, hybridzation to specific cDNA fragments indicated that the extreme 5' region of the mouse P47 gene resided on the 2.8 kbp EcoRI fragment (not shown). When compared to c-myc hybridization intensity, P47 appeared to be a single copy number gene that had been conserved equally well through evolution (not shown). Whatever its function, P47 appears to correlate with the complex hemopoietic system of vertebrate species.

4.5.2 Isolation of P47 Genomic Clones.

The genomic sequence of P47 was of interest with respect to promoter structure, multiple transcription start sites and/or 5' mini-exons, and sequence hetergeneity found in cDNA clones. Originally, genomic clones were sought to complete the P47 coding sequence. Two human leukocyte EMBL-3 clones were isolated from approximately 10 genome equivalents of an amplified commercial library (clone 13 and 29) but these hybridized to only the 3' end of P47 cDNA. In conjunction with the depletion of 5' sequences from the original amplified cDNA library, this suggested that selection against the P47 5' region may be a general phenomenon in lambda vectors. Subsequently, the 5' end of the P47 gene was sought from an unamplified normal human leukocyte EMBL-3 library. In this case 4 positive plaques (clone 53, 56, 57 and 63) were obtained from a theoretical equivalent of only 1 genome. This result was not unique to P47, since another single copy gene (anti-thrombin IIIA) cloned from identical plates was also found in 4 independent clones (F. Fernandez-Rachubinski, personal communication).

Restriction enzyme analysis of each clone yielded the patterns shown in Fig. 4.5.4. Several of the bands were of the same size as bands detected by Southern analysis of genomic DNA. Hybridization to specific EcoRI cDNA fragments (Fig. 4.5.5) permitted assignment of most of fragments from the genomic clones to the physical map indicated in Fig. 4.5.6. However, some ambiguities could not be resolved. Three clones (53, 56 and 57) contained EcoRI-SalI fragments which hybridized to the 160 bp cDNA fragment; the largest of these was 7.5 kbp (clone 53), implying that the 9.2 kbp genomic fragment was 3' to the 5.5 kbp

Fig. 4.5.4. Analysis of P47 genomic clones. DNA from 6 independent EMBL 3 recombinant phage was digested with with either EcoRI (left lane) or EcoRI and SalI (right lane) and electrophoresed on 1% agarose at 175 V for 12 h. Current was switched with a field inversion apparatus (Bio-Rad Pulsewave 760) set to a forward time of 0.7 s and a forward to reverse ratio of 3:1. DNA was visualized with ethidium bromide. Markers (M) were the BRL 1 kb ladder (left lane) and Hind III digested lambda DNA; sizes in kbp are indicated to the left. None of the clones released inserts when digested with SalI alone. a, Composite picture; b, single picture of the same gel.


a
M
13
29
53
56
57
63
23
12
10
8
7
6
5
4
3
2
1.6
1
b

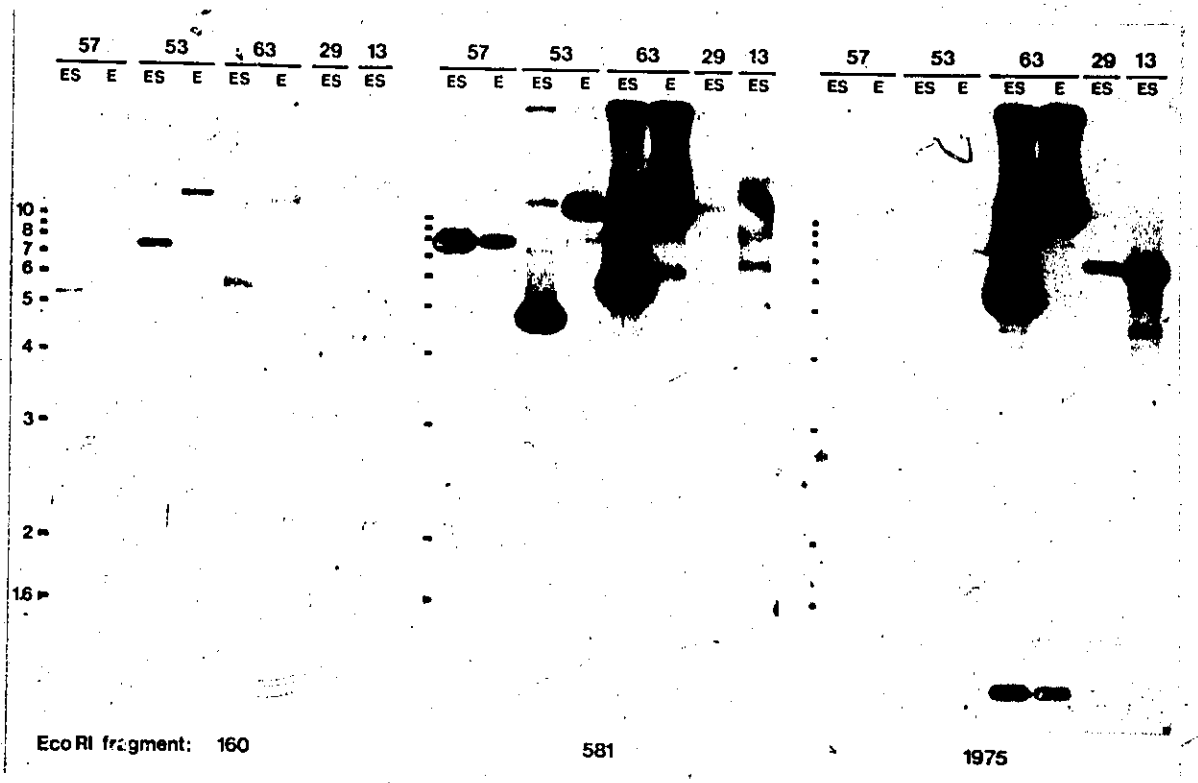
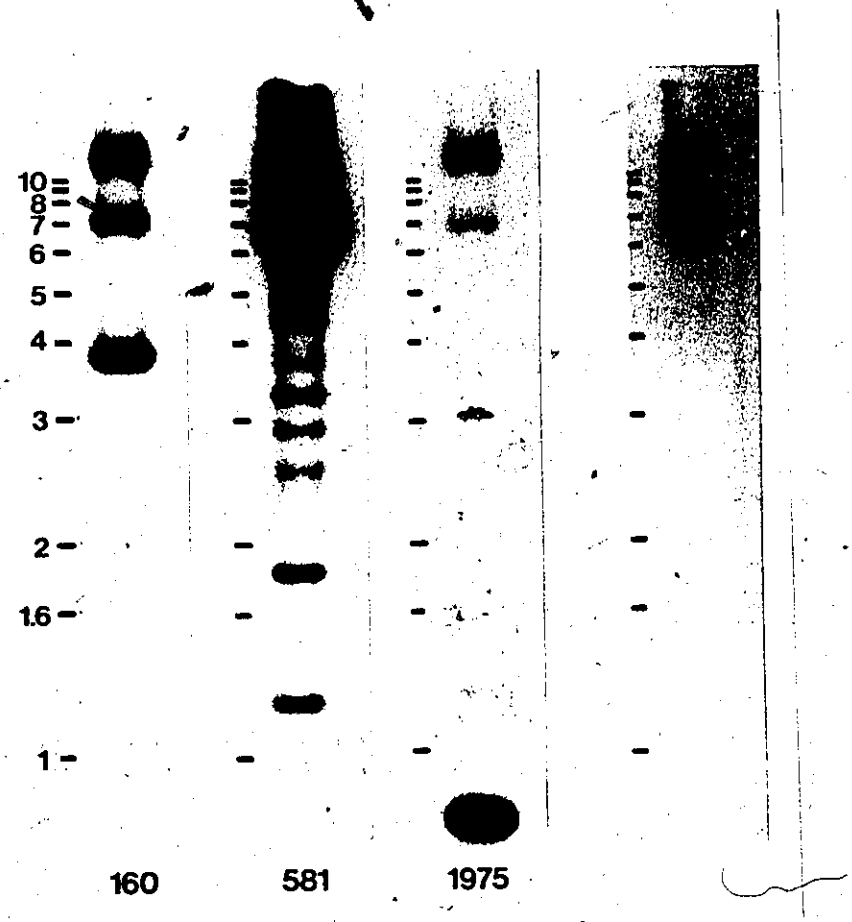


Fig. 4.5.5. Southern analysis of P47 genomic clones. DNA was digested with either EcoRI (E) or EcoRI and SalI (ES) and electrophoresed on 0.8 % agarose at 30 V for 24 h. After transfer to nitrocellulose DNA was sucessively probed with the indicated EcoRI P47 cDNA fragments. Shorter exposures revealed the 5.6 kbp fragment and 5.5 kbp fragment in clone 63 corresponded to the 581 and 1975 bp cDNA fragment, respectively. Marker sizes in kbp are indicated on the left of each exposure. Clone 56 was analyzed independently (overleaf); in this case, DNA was digested with EcoRI and SalI and run in triplicate so that all three probes could be used simultaneously. The extreme right panel is a shorter exposure of the filter probed with the 581 bp EcoRI fragment.


10
8
7
6
5
4
3
2
1.6
1
160
581
1975

fragment. None of the clones extended into the 5.5 kbp fragment although the 7.8 kbp band in clone 56 strongly cross-hybridized to both the 160 and 581 bp cDNA fragments (Fig. 4.5.5). It was unclear if this was an artifact. On the one hand the ethidium bromide stained fragment was twice as intense as neighbouring bands (Fig. 4.5.4), suggesting an overlapping doublet derived from two separate parts of the gene. However; if this were true, the insert size of clone 56 would exceed the maximum packaging size for EMBL 3 (Frischauf et al, 1983; 1987); moreover, the EcoRI restriction pattern of clone 56 would not match that of genomic P47. This matter remains unresovled. For illustrative purposes clone 56 is shown with the single 7.8 kbp fragment corresponding to the 581 bp cDNA fragment. Unfortunately, this dilemma also makes it unclear if the 3.8 kbp SalI-EcoRI fragment of clone 56 completes the 5' end of the P47 gene.

Further ambiguity arose in the 3' end of P47 clones in that the order of 3 small fragments (0.8, 1.2 and 2.6 kbp) could not be assigned. Although the 2.6 kbp band never hybridized to any part of the cDNA, the 0.8 and 1.2 kbp bands hybridized to both the 581 and 1975 bp cDNA fragments but never on the same filter (Fig. 4.5.5 and data not shown). Furthermore, the 1.2 kbp fragment in clone 57 is visible on EtBr-stained gels, but could not be detected by Southern analysis. This could have been due to variable contamination of gel purified probe fragments with the 34 bp EcoRI cDNA fragment, possibly due to incomplete digestion. The problem could also have arose from insufficient wash stringencies. Based on the absence of the 0.8 kbp band in clone 57, it was placed 3' to the other two fragments in Fig. 4.5.6.

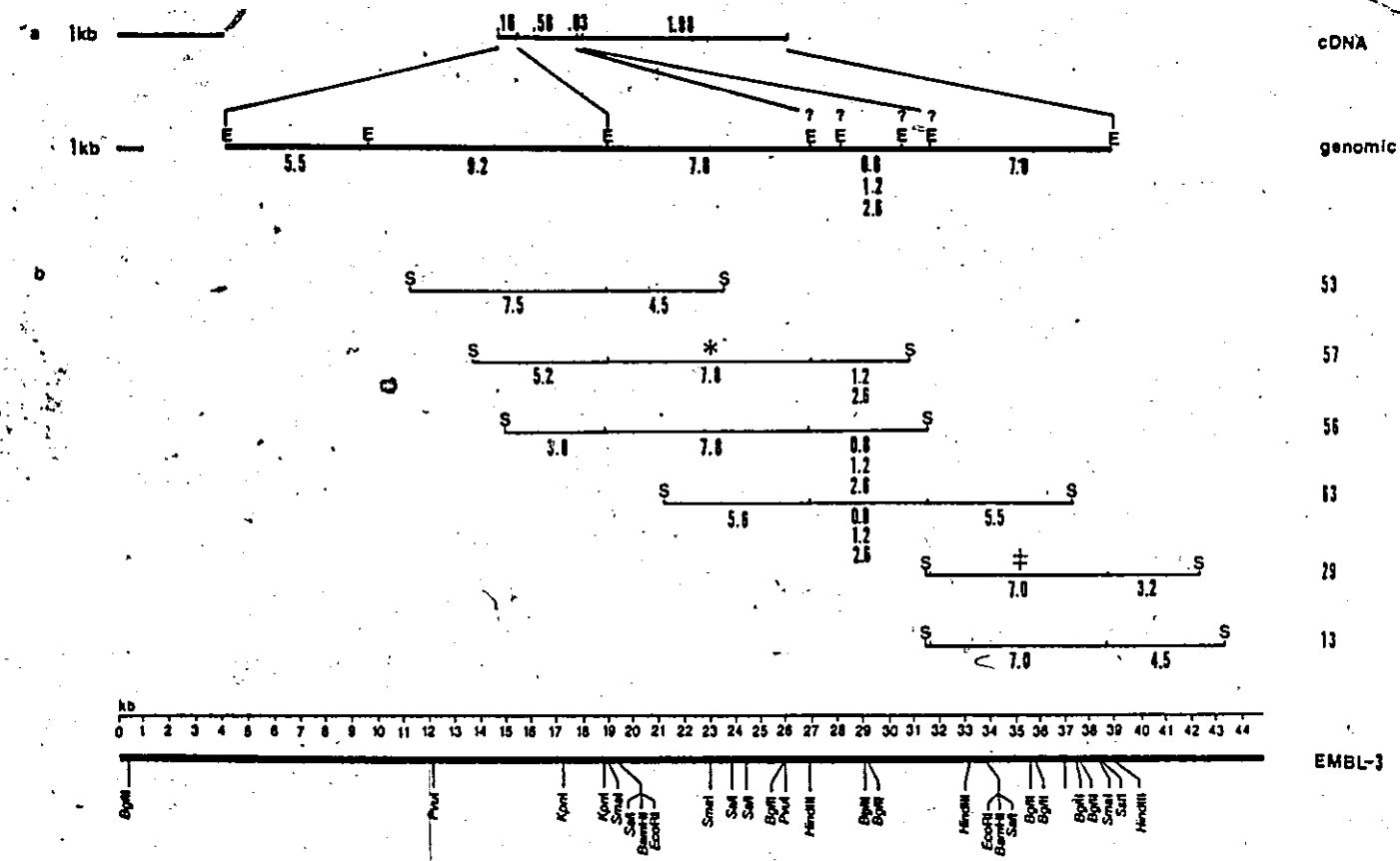


Fig. 4.5.6. a, Structure of the human P47 gene. Correspondence between the cDNA and the gene is based on Fig. 4.5.2 and the fragments liberated from P47 genomic clones. Ambiguities in the map are discussed in the text. b, Structure of human P47 EMBL-3 genomic clones. The order of fragment sizes is based on their equivalence to fragments identified on genomic Southerns and hybridization to specific cDNA fragments. A map of EMBL-3 is shown below (taken from Ausubel et al, 1987). Restriction sites: B, BamHI; E, EcoRI; S, SalI. * indicates cross hybridization to 581 bp and 160 bp EcoRI fragments; ‡ indicates weak hybridization to 1975 bp EcoRI fragment.

Finally, clone 29 was found hybridize only weakly to the most 3' cDNA probe, apparently because the 7.2 kbp fragment was present in quantities that were undetectable by ethidium bromide staining (Fig. 4.5.4 and 4.5.5). This was in spite of an easily visible 3.2 kbp fragment (Fig. 4.5.4). The assignment of this clone to the 3' end is thus also tentative. In any event, the sequences in clone 29 are a subset of clone 13 and thus not of crucial importance.

Given the ambiguities about the 5' region of the P47 gene and the large size of the locus, the clones were not sequenced. However, they will provide valuable probes for analyzing the 5' end of this complex locus.

4.6 P47 - CONCLUSIONS AND PERSPECTIVES

4.6.1 Implications for P47 Function

With the possible exception of GAP, for which slight similarity centered over a 60 amino acid stretch was found, P47 was unique among sequences in current databases. The sequence relationship between P47 and GAP is not nearly as strong as the tenuous link between PLC and GAP, nor is it in the same region (Vogel et al, 1988). Despite this, the comparison between the putative RAS effector in mammalian cells and P47 is intriguing. Recognition domains of various components of different signal transduction pathways may be more similar than imagined, and may provide a structural basis for interaction between different signalling routes in the cell. Since P47 is an immediate downstream component of PKC it could well interact with other components

of the platelet signalling network. As new domains are recognized, it will certainly be worthwhile comparing them to the P47 sequence.

The possible functions of P47 were narrowed somewhat as sequence non-identities directly ruled out 2 candidate functions. Assignment of P47 as the α-PDH subunit is now completely untenable since the human α-PDH cDNA sequence has recently been reported (Koike et al, 1988). Although P47 and α-PDH have a nearly identical deduced M_r (40,087 versus 40,334), their mRNA size (3.0 kb versus 1.8 kb) and tissue distribution are incompatible. Little sequence identity at either the amino acid level or the nucleotide level was evident. Only a single stretch of 4 residues ($E^{310}EEN^{313}$ in P47 and $E^{354}EEN^{357}$ in α-PDH) were identical but it is interesting that these occurred in the immunodominant C-terminus of P47. Whether this is sufficient for the supposed immunological cross-reactivity between the two proteins is unclear (Chiang et al, 1987). An analysis of rhodopsin peptides has suggested that as little as 4 amino acids can account for most of the immunoreactivity in a polyclonal serum (Hodges et al, 1988). P47 is also clearly unrelated to the annexin family of PLA_2 inhibitors (reviewed in Geisow et al, 1987). This class of proteins has distinct sequence and domain characteristics, none of which were found in the P47 sequence. The most recently identified annexin, endonexin II (Kaplan et al, 1988) or lipocortin V (Pepinsky et al, 1988), does share isolated non-contiguous identity with P47 at the amino acid and nucleotide level (see Appendix B for alignments). This too may be sufficient for partial immuno-crossreactivity. Interestingly, a distantly related PLA_2 inhibitor, uteroglobin, also shares isolated similarity with both P47 and lipocortins I and II (Miele

et al, 1988). Although P47 may yet have an anti-PLA_2 effect unrelated to that of the lipocortins, granulocytic differentiation of HL-60 cells is accompanied by an increase in PLA_2 activity that is apparently due to unmasking of a latent activity in undifferentiated cells (Billah et al, 1986). On a superficial level at least, this is not compatible with an inhibitory role for P47.

Unfortunately, the P47 sequence provided no direct clue about its possible relationship to the soluble platelet IP_3ase (Connolly et al, 1986). A similarity noted between the active sites of bacterial and human alkaline phosphatase (Knoll et al, 1988) was not present in the P47 sequence. Other circumstantial evidence also argues against the IP_3ase hypothesis. Northern analysis of P47 expression in cell lines of various orig[redacted], as well as immunological screening of rat tissues (Stewart et al, in preparation), indicated that P47 was restricted to differentiated white cells and platelets. This contradicts the presence of soluble IP_3ase activity in brain and smooth muscle (Hansen et al, 1987; Sasaguri et al, 1985). A soluble IP_3ase activity has been detected in rabbit neutrophils, but unlike that reported for platelets, this is not increased by stimulation of PKC (Kennedy et al, 1988). Platelet P47 can be partially separated from IP_3ase activity, such that less than 5% of P47 is associated with enzymatic activity (Stewart et al, in preparation). On evolutionary grounds as well, it is unlikely that P47 constitutes nearly 1% of platelet protein only to degrade a transient intracellular messenger. It remains possible that P47 may have a multifunctional role in the platelet and that a small fraction of appropriately modified P47 hydrolyzes IP_3. Within the context of the

IP_3ase role for P47, it is intriguing that P47 may contain a Ca^{2+}-binding EF-hand.

Thus far, the only role for P47 to escape direct criticism is the regulation of actin polymerization in vitro (Hashimoto et al, 1987). Presumably this reflects the situation during platelet and white cell activation. This hypothesis is at least consistent with the abundance of P47 and the molar ratio of unphosphorylated P47 needed to inhibit actin polymerization. The inhibitory effect of P47 on actin polymerization and the relief of this inhibition by phosphorylation coincides with, and may in fact mediate, changes in actin dynamics during secretion (reviewed in Majerus, 1987). Further, phorbol esters have long been known to have dramatic effects on the cytoskeleton (for a recent discussion Sobue et al, 1988; Burn et al, 1988).

Actin regulatory proteins have been well studied in hemopoietic cells; some traits correlate partially with known characteristics of P47. Regulation of actin pools by gelsolin and profilin may occur in a PIP_2-dependent manner (Lind et al, 1987). A weak similarity between the actin-severing domain of severin (from Dictyostelium discoideum) and vertebrate gelsolin could be partially matched to the P47 sequence (see Appendix B); the DESG consensus between gelsolin and p36 was not found in P47 (Burgoyne, 1987). Intriguingly, severin and gelsolin carry out some of their functions in a Ca^{2+}-dependent manner (reviewed in Pollard, 1986). Gelsolin and actin-binding protein both increase in abundance during macrophage-like HL-60 differentiation (Kwiatkowski, 1988; however, unlike P47, gelsolin has a wide tissue distribution (see Pollard, 1986).

It is worth comparing P47 to other known cytoskeletal components in hemopoietic cells. P47 shares some characteristics with a 41 kDa macrophage protein which exerts a Ca^{2+}-dependent capping without severing actin filaments (Southwick and Wang, 1986). Although the 2 proteins have similar Stokes radii (3.0 versus 3.3 nm) and similar isoelectric points, the abundance of the 41 kDa protein in macrophages is 1-2%; moreover, it clearly migrates below actin (Southwick and Wang, 1986). Human neutrophils contain a 48 kDa protein that dissociates from the Triton X-100 insoluble cytoskeleton when phosphorylated by PKM (Pontremoli et al, 1987). Generation of PKM in neutrophils appears to be tightly linked to the phosphorylation of cytoskeletal proteins and secretion (Pontremoli et al, 1987), whereas maintenance of PKC activity prolongs the respiratory burst (Pontremoli et al, 1988). Although similar to P47 in some characteristics, the 48 kDa protein clearly differs in its cytoskeletal association. Since P47 is clearly a PKC substrate (Tsukuda et al, 1988), it may have a role in activating membrane-associated processes rather than degranulation. Finally, the possible association of P47 with high M_r proteins in RA-differentiated HL-60 cells is intriguing given the role of high M_r species in organization of the platelet cytoskeleton (O'Halloran et al, 1985). In particular, platelet talin (P235) is subject to proteolytic regulation (O'Halloran et al, 1985) and phosphorylation by PKC (Litchfield and Ball, 1986).

Finally, the possible role of P47 in the oxidative burst and/or CGD deserves comment. Several components of the respiratory burst network are up-regulated during granulocytic HL-60 differentiation,

including the cytochrome b heavy and light chains (Newberger et al, 1988) and a cytosolic factor required for activation of the burst in vitro (Seifert and Schultz, 1987). The functional component of the latter could be P47. Phosphorylation of a Mr 47,000 protein has been shown to coincide closely with both DAG and O_2^- production in human neutrophils (Reibman et al, 1988). It is interesting that P47 may regulate actin and that reorganization of the cytoskeletal network may be involved in the oxidative burst; the cytochalasins potentiate O_2^- generation in neutrophils (e.g., Jesaitis et al, 1986). Although P47 and cytochalasin D may impinge on the same target, their actions are antagonistic in that phosphorylation of P47 permits actin polymerization, not the converse. The tissue distribution of P47 is partly at odds with a role in the oxidative burst, mainly because lymphocytes contain moderate levels of P47, yet cannot provide the cytosolic component necessary for reconstitution of the respiratory burst in vitro (Parkinson et al, 1987). Preliminary analysis with the P47 antiserum (Dr. R.J. Haslam, personal communication) also does not confirm identity with the 47 kDa CGD phosphoprotein isolated by Kramer et al (1988).

Any function eventually assigned to P47 must take into account its relatively narrow tissue distribution and conservation through vertebrate evolution. One characteristic of hemopoietic cell secretion which differs from cells with fixed polarity is that chemotaxis and release of granules are directed toward the site of stimulation. A mechanism must exist which couples the direction of cytoskeletal reorganization to the region of stimulation. It is possible that

phosphorlyation of P47 by PKC, an event necessarily localized to the point of stimulus, regulates actin polymerization in the region where secretion is required. PKC itself is apparently locally activated in platelets (Crouch and Lapetina, 1988). This feature would be shared by all secretory cells which exist in suspension, that is all differentiated hemopoietic cells.

4.6.2 Future Directions

Several experiments are immediately suggested by some of the preliminary studies on P47 protein in HL-60 cells. Manipulation of HL-60 cell labeling conditions should permit identification of a precursor-product relationship between P47 and P15. Alternatively, this could be tested in the Raji B cell line. Phosphorylation of P47 in HL-60 cells could be optimized, possibly by cotreatment with TPA and A23187, and characterized with respect to the degree of cell activation (e.g. superoxide anion production and secretion). The resultant P47 species could be compared to platelet P47 with regard to phosphorylation state and unphosphorylated isoforms. Bacterially-expressed P47 isoforms could also be compared to platelet P47. The phosphorylation-dependent association of P47 with the membrane fraction of HL-60 cells should be accurately quantitated. Recently, it has been noted that platelets contain a delicate cytoskeletal network that is disrupted by classical Triton X-100 detergent extraction (Fox et al., 1988); association of P47 with this structure could be tested. These studies may be facilitated by the generation of an antibody to recombinant P47.

The presumed transcriptional induction of P47 during HL-60

differentiation must be confirmed by nuclear run-on analysis (e.g. Bentley and Groudine, 1986). Once this is established, the developmental regulation of the P47 promoter should be defined more precisely to see if it is highly correlated with differentiated hemopoietic cells. The K562 cell line apparently differentiates into megakaryoblast-like cells upon TPA treatment (Tetteroo et al, 1984); since the undifferentiated cells do not express P47, this system may provide tight regulation of the P47 promoter. Isolation of clones containing the P47 promoter would permit analysis of its regulation during differentiation as well as clarifying the 5' heterogeneity noted in the P47 mRNA.

Many experimental avenues have been opened by the cloning of P47. These will be discussed briefly. An obvious experimental system in which to analyze P47 structure and function is the HL-60 line itself. Numerous variations exist on the theme of re-introduction of modified P47 sequences into HL-60 cells. Efficient transfection methods have recently been developed for HL-60 cells (e.g. Iguchi-Ariga et al, 1987) that should permit the following types of experiments:

i) Establishment of HL-60 sublines over-expressing P47. Both the differentiated and undifferentiated HL-60 cell phenotype could be assessed, particularly with regard to actin structure and cell activation. Immunocytochemistry of the actin network may be possible in HL-60 cells.

ii) Establishment of a null P47 phenotype by expression of antisense P47 RNA from a strong constitutive promoter. This would be of particular interest with regard to differentiated cell function in that

the ability of cells to generate a superoxide burst and/or secrete various granule contents can be easily measured.

iii) Re-introduction of mutated P47 sequences. Engineering of negative dominant mutations is now a general approach to analysis of protein function (Herskowitz, 1987). P47 should be amenable to this, in particular with regard to phosphorylation site and/or EF-hand mutants. In the absence of a defined in vitro assay for P47 function, this is one way to test the effect of site-directed mutations. From a regulatory standpoint, the role of the long 3' UTR region in P47 mRNA stability could be examined with a truncated P47 construct.

Obviously, there are many variations of such ideas; the essential component to all of them is establishment of a reliable transfection protocol for HL-60 cells.

From an alternate viewpoint, the P47 promoter region could be used to identify factors which initiate the new program of gene expression that leads to terminal differentiation. P47 is one the few gene products yet identified which is up-regulated early in granulocytic HL-60 cell differentiation. Cloning of the 5' end of the P47 gene would not only permit known regulatory elements to be identified but could also be used to first delineate sequences conferring differentiation inducibility and then to identify factors which bind such regions and initiate transcription. Also, transfection of constructs containing the P47 promoter fused to other genes regulated in differentiation could be used to test their role in differentiation. Proper regulation of re-introduced genes during HL-60 differentiation has been demonstrated (Salzer et al, 1985). Abrogation of c-myc repression in this manner

would be a prime candidate for such a study. The tight restriction of P47 expression to differentiated hemopoietic cells could also be exploited in a transgenic mouse model to investigate hemopoietic regeneration, for instance. Given the large size of the human 5' P47 genomic region and the apparent complexity of the P47 mRNA 5' end, isolation of the promoter appears far from trivial. Cloning the mouse P47 gene may be a more feasible alternative.

Purification of biologically active recombinant P47 will allow definitive tests of its activity *in vitro*. If bacterial P47 can be obtained in a soluble native state, it should be sufficient to test the actin-regulatory hypothesis since the unphosphorylated form is expected to inhibit actin polymerization. Phosphorylation by PKC *in vitro* to alleviate the putative inhibitory effect may be more difficult. The great advantage of bacterial P47 is that, unlike recombinant protein obtained from eukaryotic cells, it should not have any contaminating activities which would specifically interfere with a ubiquitous eukaryotic process like actin polymerization. Expression of P47 in a eukaryotic system does have several advantages, however. Foremost, native protein could easily be obtained in both the unphosphorylated and, after treatment of the host cells with TPA, phosphorylated form. Further, recombinant baculovirus infected *Spodoptera* cells (Dr P.J. Parker, personal communication) and COS cells (e.g. Knopf *et al*, 1986) have been used to express various PKC isoforms; co-expression of P47 and PKC in the same cell would allow the structural requirements of phosphorylation and PKC subtype specificty to be tested *in vivo*. Purification of recombinant P47 from eukaryotic cells could easily be

based on one of several methods already worked out for the platelet (e.g. Imaoka et al, 1983; Tsukuda et al, 1988). Finally, gratuitous effects of high level P47 expression in various eukaryotic cells may also give a clue to its function.

Once the activity of recombinant P47 can be defined either in vivo or in vitro, functional components of the protein may be dissected by site-directed mutagenesis. P47 mutants generated with oligonucleotides in the EF-hand and phosphorlyation site regions would directly test the role of Ca^{2+}-binding and phosphorylation in P47 function. The structural dependence of PKC phosphorylation and Ca^{2+}-binding can be tested immediately in vitro without the need for a functional assay.

Lastly, since P47 is one of the best physiological substrates for PKC it may be expected that the kinase has very high affinity for the substrate site(s). To date, the best peptide substrate and best corresponding alanine-substituted competitive inhibitor of PKC are based on the PKC pseudosubstrate site (House and Kemp, 1987). Given the close match between P47(107-120) and this sequence, a P47 synthetic peptide should be phosphorylated very efficiently in vitro. Moreover, the alanine substituted analogue(s) may be potent inhibitors of PKC and thus prove useful in elucidating the role of this kinase in various systems (reviewed in Hardie, 1988). To end on a whimsical note, the putative phosphorylation site sequence (KFARKSIRRSIRL) and the platelet/leukocyte distribution of P47 provide a new name for this protein, pleckstrin. Note also that pleckstrin is a C kinase substrate.

OVERVIEW

Two aspects of HL-60 cell differentiation have been studied. On the one hand, the signals which initiate differentiation were investigated. Ca^{2+} and PKC signalling, as mimicked by ionophores and phorbol esters, were able to cooperate in a synergistic manner and evoke full macrophage-like differentiation of HL-60 cells. Analysis of various mRNA species suggested that interactions between the two pathways lead to a slightly different phenotype than that induced by phorbol esters alone. The HL-60 cell line exemplifies the enormous variety of transcription states which must underlie tissue and stimulus-specific responses.

From an alternate perspective, the P47 substrate of PKC was cloned from HL-60 cells as a differentiation-specific cDNA. Although by no means a "master" gene, P47 was induced relatively early in the RA-differentiation program. Manipulation of the P47 sequence will provide insight into both short term aspects of signal transduction and into regulatory mechanisms of gene expression during HL-60 differentiation.

Obviously, the molecular nature of aging has not been addressed directly by this work. However, characterization of differentiating cell systems is necessary to gain insight into the mechanisms which control the self-renewal/differentiation decision. Information gleaned from simpler *in vitro* model systems may thus be applied to stem cells, which are arguably at the crux of the aging problem.

APPENDICES

Appendix A: Isolation of a TPA-Resistant HL-60 Subline

Downregulation of c-myc is a common early event in the differentiation program of many cell types (reviewed in Freytag, 1987). It was thus of interest to know if enforced expression of a deregulated c-myc mRNA could abrogate HL-60 differentiation. Although this project was pre-empted by the publication of similar work on other cell lines (Coppola and Cole, 1986; Prochownik and Kukowska, 1986; Freytag, 1987) and is incomplete as it stands, the salient features of a TPA-resistant HL-60 subline will be described. In addition, cells from this subline were used to isolate the original P47 cDNA clones.

The c-myc gene encodes a highly conserved non-translated first exon which has been implicated in translational control and mRNA stability *in vivo* and *in vitro* (reviewed in Piechaczyk *et al*, 1987). In order to constitutively express c-myc independently of HL-60 differentiation and to obviate any translational control mechanisms dependent on the first exon, the 5' untranslated region of the human $MTII_A$ ($hMTII_A$) promoter was fused to the second and third exons of human c-myc just upstream of the initiator ATG. The plasmid, designated pMT-hmyc, was confirmed by digestion with PstI (Fig. A.1).

HL-60 cells (Golde subline) were transfected according to the

electroporation method of Potter et al (1984). Since difficulty arose in growing cells in either soft agar or methylcellulose (see Begley et al, 1987), the entire electroporated cell population was transfered to G418 selective medium (see legend to Fig. A.2). G418-resistant populations were recovered from all transfections, albeit with different lag periods (pSVneo/pMT-hmyc cells in 4 weeks; pSVneo/hc-myc cells in 7 weeks; pSVneo cells in 9 weeks; see Figure A.2 legend). In contrast, untransfected populations ceased to divide.

Exposure of all G418-resistant populations to 50 nM TPA caused the usual phenotypic response of cell attachment and growth arrest. However, whereas control transfected cells became non-viable after several days, cells transfected with pMT-hmyc remained attached and viable for 3-6 months. During this period, many cells acquired a giant multi-nucleated morphology (Fig. A.2) and stained as macrophages with May-Grunwald-Geimsa (not shown). Other cells took on a foam cell-like morphology, while a very few cells remained loosely attached and appeared more or less like parental HL-60 cells. If the cell population (hereafter called HL-60/MT-myc) was released from 50 nM TPA after only 48 h, a dividing culture was recovered after 1 month in TPA-free medium. Re-exposure of these cells to TPA also resulted in attachment to substratum but in a more rapidly reversible manner. After several rounds of exposure and release from TPA, the entire resistant population was able to transiently growth arrest and adhere, then immediately resume proliferation once TPA was removed.

Southern analysis of HL-60/MT-myc DNA after 6 rounds of TPA treatment revealed only the endogenous $hMTII_A$ and c-myc fragments (Fig.

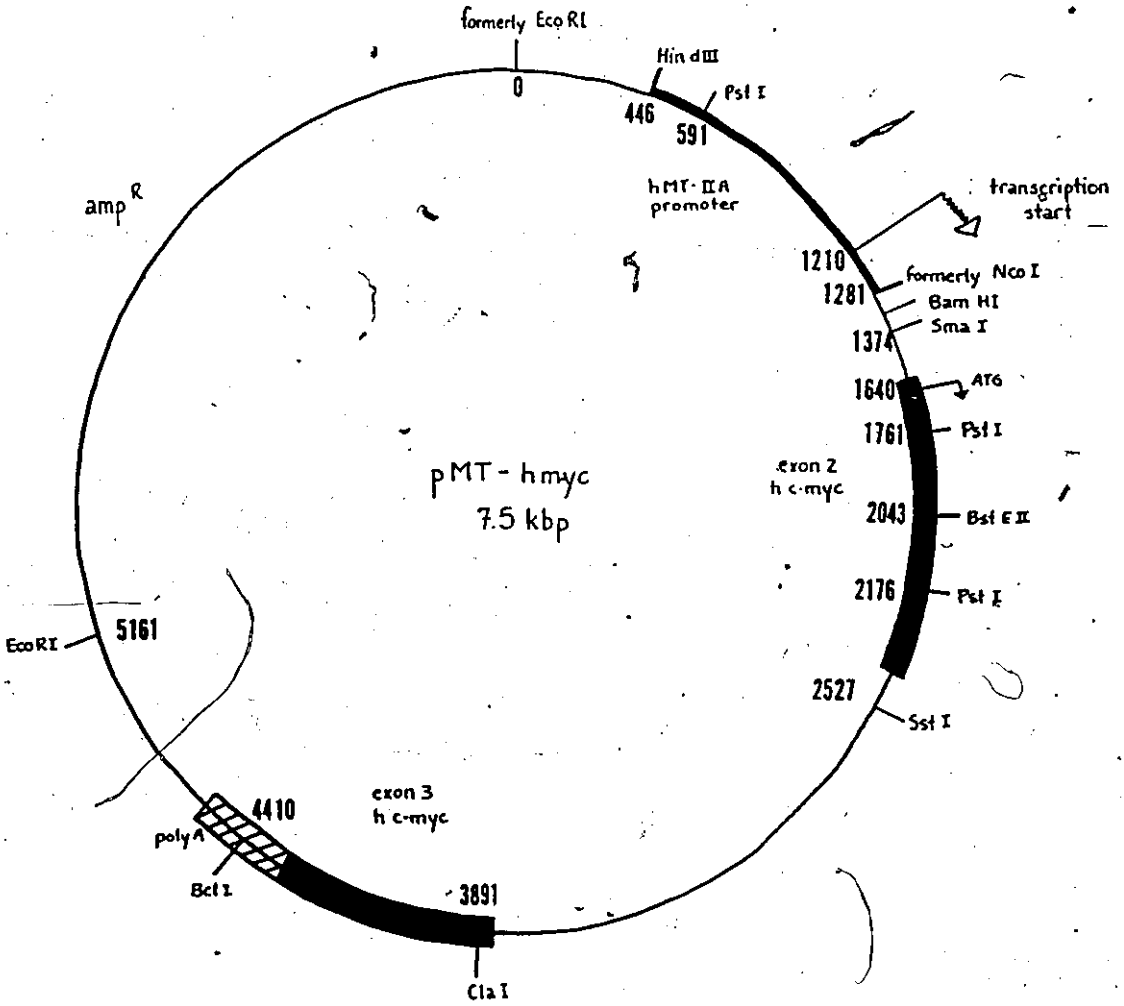


Fig. A.1. Map of pMT-hmyc. The plasmid is based on pHS-1 which contained the human $MTII_A$ promoter region (nucleotides -764 to +71) cloned into the HindIII-HincII sites of the polylinker. The SmaI-EcoRI fragment of pHSR-1 (nucleotides 2582 to 6369) containing the human c-myc coding region was cloned into the corresponding multipurpose cloning sites of pHS-1.

Fig. A.2 Photomicrograph of differentiated MT-myc cells. HL-60 cells (Golde subline) resulting from transfection/selection protocol were maintained in 50-100 nM TPA for 3 months in the same flask. Note fibroblastic and foam cell morphologies. Final magnification is approximately 150 x. Methods: Plasmid DNA (Pst I linearized pSVneo (5 µg), pSVneo + Eco RI linearized human c-hmyc (10 µg) or pSVneo + Eco RI linearized pMT-hmyc (10 µg)) was added to 10^7 cells in 1 ml PBS in a sterile cuvette and left on ice for 5 min. A single 4 kV pulse from a sequencing power supply set for maximum current (90 mA) was applied through platinum wire electrodes (gap width = 0.5 cm) mounted in a teflon cuvette cover. After a further 15 min incubation on ice cells were diluted into 20 ml RPMI 1640 and plated in 2 X 100 mm dishes. Cells were grown for 48 h before transfer to selective media containing 800 µg/ml G418 sulfate at a density of 5 X 10^5 cells/ml. Cultures were split 1:2 into selective medium after 3 days. Resistant populations were frozen once a sufficiently large healthy culture was obtained (pSVneo cells - P8; pSVneo/hc-myc - P13; pSVneo/MT-myc - P4, B10, P12). Treatment of HL-60/MT-myc cells with 50 nM TPA for 2 d caused nearly complete attachment to plastic that was reversible only after a 1-month recovery period in TPA-free medium. Thereafter, recovery from 2 d exposures to 20-100 nM TPA ocurred within 2-3 d; cells were frozen after every 2 or 3 rounds of TPA treatment (including 9^{th} and final round of TPA treatment).

A.3). There was no evidence of the unique 1.1 kbp PstI plasmid fragment in relation to either the single copy $hMTII_A$ and/or amplified c-myc sequences. Interestingly, the degree of c-myc amplification appeared to decrease from the parental (ATCC) HL-60 line to the Golde subline to the HL-60/MT-myc subline (Fig. A.3).

As expected from Southern analysis, a 1.9 kb c-myc transcript arising from the $hMTII_A$ promoter was not present in either differentiated or undifferentiated cells (Fig. A.4). Although TPA caused a decrease in the endogenous 2.4 kb c-myc transcript, distinct hybridization was still visible after 48 h. Significantly, TPA treatment also caused the appearance of c-fms transcripts (Fig. A.4, lane 5). Removal of TPA from the culture medium fully reversed the effects on c-myc and c-fms (lanes 6 and 7). Treatment of HL-60/MT-myc cells with 10 μM Cd^{2+} for 24 h influenced neither the pattern of c-myc transcripts nor the differentiation response itself (not shown). It is unclear how these cells acquired resistance to G418 and TPA.

Several reports by other workers have conclusively demonstrated that constitutive c-myc expression blocks differentiation of MEL cells (see Section 1.3.1). Hence, detailed analysis of the HL-60/MT-myc subline and reproduction of the initial transfections were not pursued. The differentiation-resistant phenotype was nonetheless of interest in its own right, particularly as it differed from most other TPA-resistant HL-60 cell sublines reported in the literature (see Section 1.5.1). However, a similar TPA-resistant phenotype has been noted for the HL-60-1E3 subline (Leftwich et al, 1987; Ely et al, 1987). In these cells, PDBu causes attachment and expression of some monocytic markers,

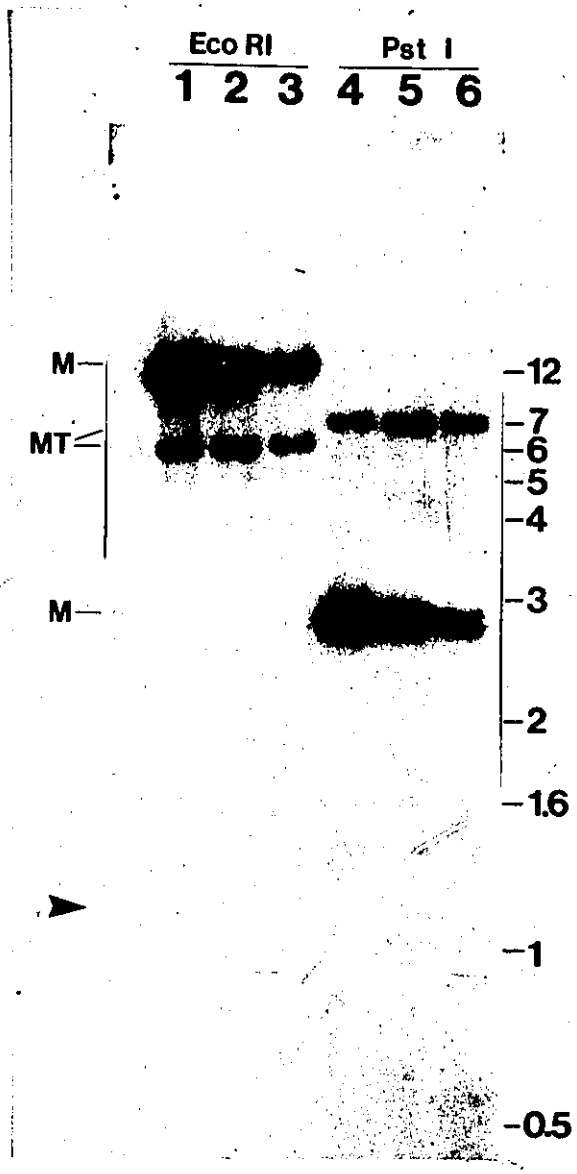


Fig. A.3. Southern analysis of HL-60/MT-myc cells. 10 μg of genomic DNA digested with either Eco RI or Pst I was electrophoresed on a 1% agarose gel, transferred to nitrocellulose and probed with the 1.1 kbp PstI fragment of pMT-hmyc: parental (ATCC) HL-60 cells (lane 1 and 4); G418-resistant HL-60/MT-myc cells before TPA selection (lane 2 and 5); HL-60/MT-myc cells after 8 rounds of TPA (lane 3 and 6). The expected Eco RI and PstI fragments derived from endogenous c-myc (M) and $MTIII_A$ (MT) loci are indicated. The position of a 1.1 kbp fragment that would arise from PstI digestion of pMT-hmyc plasmid is shown by the arrow. Marker sizes in kbp are indicated to the right.

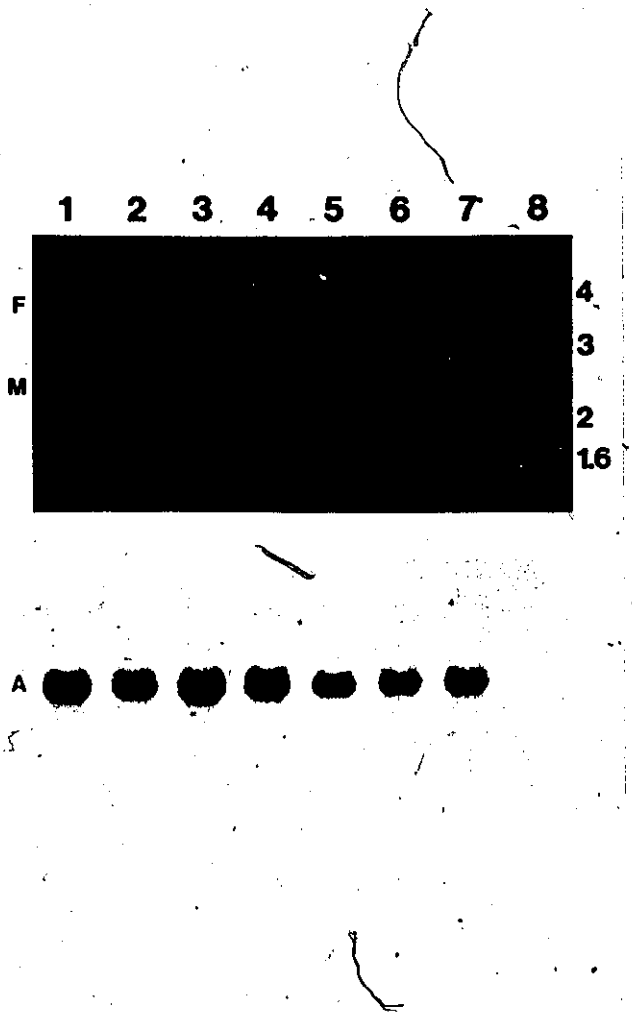


Fig. A.4. Northern analysis of RNA from HL-60/MT-myc cells. Total RNA was isolated from cells after the indicated treatments and probed simultaneously with c-fms (F) and c-myc (M) oligonucleotides and subsequently with actin oligonucleotide (A): parental (ATCC) HL-60 (lane 1); HUT 78 T cell (lane 2); HL-60/MT-myc after G418 selection (lane 3); HL-60/MT-myc after 6 rounds of selection in 50-100 nM TPA (lane 4); HL-60/MT-myc 48 h after 7th round of TPA treatment (lane 5); HL-60/MT-myc after 8 rounds of TPA (lane 6); MT-myc HL-60 after 9 rounds of TPA (lane 7); kinased BRL 1 kb ladder (lane 8). Marker sizes in kb are indicated to the right. The band apparent at 2 kb probably arises from rRNA compression of the c-myc hybridization trail.

including c-fms, but is unable to induce a terminal phenotype (Ely et al, 1987). Although c-myc mRNA in HL-60-1E3 cells is transiently repressed by phorbol ester treatment, it is re-expressed if the drug is removed after 24 h (Ely et al, 1987). Thus, the HL-60/MT-myc line is very similar to this previously reported subline. The long term effects of phorbol esters on HL-60-IE3 cells have not been examined.

Despite TPA-dependent acquisition of many differentiated characteristics, it appeared that the HL-60/MT-myc subline could not commit to differentiation. The nature of this defect is unknown. Based on the presence of detectable c-myc hybridization after TPA treatment (Fig. A.4) it could be argued that c-myc regulation has been altered and that c-myc does not fall below a critical threshold required for permanent cell cycle exit. Alternatively, the apparent reduction in c-myc amplification may play a role in differentiation resistance, despite the absence of a precedent in other sublines (Graham et al, 1985; Szabo et al, 1988). Another obvious mechanism could involve activation of other oncogenes which overide the differentiation program. Avian myelocytes transformed with a temperature sensitive v-myb gene cease dividing and acquire a macrophage-like morphology when shifted to the non-permissive temperature (Beug et al, 1987). Upon re-culturing at the lower temperature the cells revert to growth in suspension as undifferentiated cells; this effect became less pronounced as cells were maintained at 42°C for increasing periods of time (Beug et al, 1987). Reversible differentiation of HL-60/MT-myc cells could thus result from superposition of TPA-induced differentiation on a more transformed cell type. Finally, and perhaps most interestingly, the commitment mechanism

itself could be altered in HL-60/MT-myc cells (see Weir and Scott, 1986).

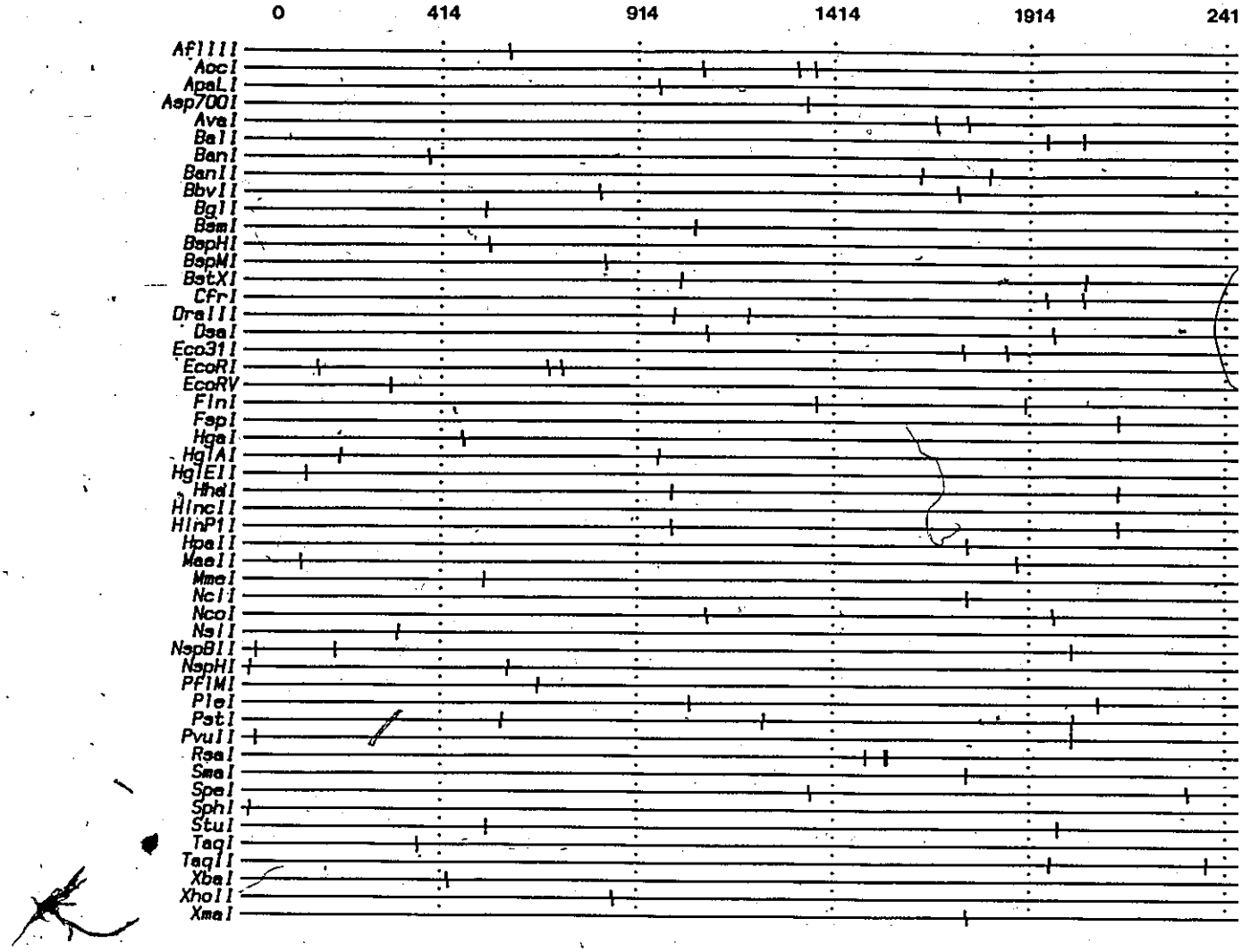
A more thorough analysis of the HL-60/MT-myc subline is required on several grounds. The basis of G418 resistance should itself be tested by probing HL-60/MT-myc DNA with the neomycin gene. It should be established that PKC activation is not altered, for example by examining phorbol ester binding or protein phosphorylation in response to TPA. Decreased expression of c-myc in the presence of TPA should be established rigorously with poly(A)$^+$ RNA. A detailed study of the ability of cells to recover after various periods of exposure to TPA would allow a comparison with the avian system described above. With regard to the original idea behind this project, it is still worth obtaining pMT-hmyc transfected cells to see if enforced c-myc expression blocks HL-60 differentiation.

Appendix B: Further Analysis of the P47 Sequence

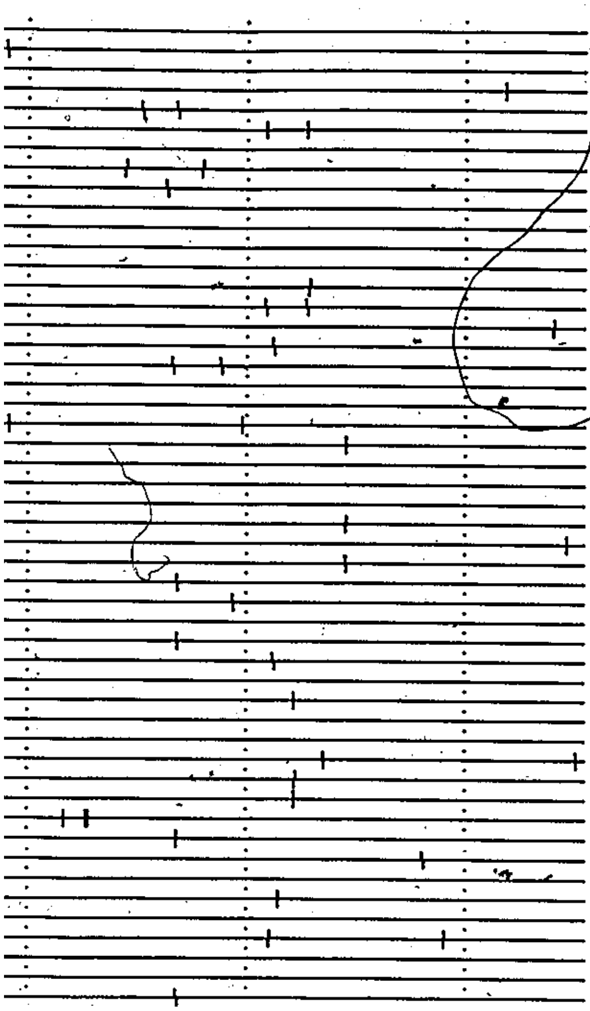
This appendix contains ancillary computer predictions, details of the P47 sequence and some speculation on the structure of P47. A thorough restriction map of the P47 cDNA is shown in Fig. B.1. Table B.1 shows the nucleotide and di-nucleotide composition of the P47 cDNA sequence. The slight A+T richness and strong suppression of CpG dinucleotides are typical for mammalian coding regions (reviewed in Max, 1984). Direct analysis of the cDNA is completed by the codon usage data given in Table B.2; the P47 sequence closely resembles other mammalian genes in this parameter (Aota et al, 1988).

The following more detailed structural analyses were generated with the University of Wisconsin Genetics Computer Group software package (Devereux et al, 1984). As shown in Fig. B.2, the Chou and Fasman (1978) and Garnier et al (1978) secondary structure predictions differ considerably in some regions. Empirical determination of P47 secondary structure content by circular dichroism would help narrow these predictions. The numerous predicted surface regions in P47 (Fig. B.2) are not unexpected given the highly charged nature of the protein. Not surprisingly the best potential PKC site in P47 is predicted to lie on the protein surface by all 4 criteria shown at the top of Fig. B.2. It probably constitutes a β-turn region of the protein as well (Fig. B.2). P47 contains two potential O-linked glycosylation sites (Fig. B.2); although there is precedent for glycosylation of cytosolic proteins (Holt and Hart, 1986), in this case it is highly unlikely since bacterially-expressed P47 co-migrates with platelet P47. A stylized 2-

Fig. B.1. Detailed restriction map of the composite P47 cDNA sequence. The numbering system has assigned the first nucleotide as 1 and is thus shifted forward by 85 bp compared to other figures. The figure was generated with the MAPPLOT routine of the University of Wisconsin Genetics Computer Group (UWGCG) software package.


0
414
914
1414
1914
241
AflIII
AocI
ApaLI
Asp700I
AvaI
BalI
BanI
BanII
BbvII
BglI
BsmI
BspHI
BspMI
BstXI
CfrI
DraIII
DsaI
Eco31I
EcoRI
EcoRV
FlnI
FspI
HgaI
HgiAI
HgiEII
HhaI
HincII
HinP1I
HpaII
MaeII
MmeI
NciI
NcoI
NsiI
NspBII
NspHI
PflMI
PleI
PstI
PvuII
RsaI
SmaI
SpeI
SphI
StuI
TaqI
TaqII
XbaI
XhoII
XmaI

1414 1914 2414



1 A'CryG_T
3 CC'TnA_GG
1 G'TGCA_C
2 GAAnn'nnTTC
2 C'yCGr_G
2 TGG'CCA
1 G'GyrC_C
2 G_rGCy'C
2 GAAGACnn'nnnn_
1 GCCn_nnn'nGGC
1 GAATG_Cn'
1 T'CATG_A
1 ACCTGCnnnn'nnnn_
2 CCAn_nnnn'nTGG
2 y'GGCC_r
3 CAC_nnn'GTG
2 C'CryG_G
2 GGTCTCn'nnnn_
3 G'AATT_C
1 GAT'ATC
2 GTCCC
1 TGC'GCA
1 GACGCnnnnn'nnnnn_
2 G_wGCw'C
1 ACCnnnnnnGGT
2 G_CG'C
1 GTy'rAC
2 G'CG_C
1 C'CG_G
2 A'CG_T
1 TCCrACnnnnnnnnnnn...
2 CC<s_GG
2 C'CATG_G
1 A_TGCA'T
3 CmG'CkG
2 r_CATG'y
1 CCAn_nnn'nTGG
3 GAGTCnnnn'n_
3 C_TGCA'G
2 CAG'CTG
3 GT'AC
1 CCC'GGG
2 A'CTAG_T
1 G_CATG'C
2 AGG'CCT
1 T'CG_A
2 gACCgAnnnnnnnnn_n...
1 T'CTAG_A
1 r'GATC_y
1 C'CCGG_G

Enzymes that do not cut:

AatII	AccI	AflII	AhaII	ApaI	Asp718I	AsuII	AvrII	BamHI	BbeI	BclI	BglII
BspMII	BssHII	BstEII	Cfr10I	ClaI	DraI	Eco47III	Eco78I	EcoNI	EspI	GdiII	HaeII
HindIII	HpaI	KpnI	MluI	NaeI	NarI	NdeI	NheI	NotI	NruI	PmaCI	PvuI
RsrII	SacI	SacII	SalI	ScaI	SciI	SfiI	SnaBI	SplI	SapI	ThaI	Tth111I
				XcaI	XhoI	XmaIII					

Enzymes excluded; MinCuts: 1 MaxCuts: 3

AluI	AlwI	AlwNI	AvaII	BbvI	Bsp1286	BsrI	BstNI	CviJI	DdeI	DpnI	DraIII
Eco57I	EcoRII	Fnu4HI	FokI	GsuI	HaeI	HaeIII	HinfI	HphI	Ksp632I	MaeI	MaeIII
MboII	MnlI	NlaIII	NlaIV	PpuMI	PssI	Sau3AI	Sau96I	ScrFI	SecI	SfaNI	SsoII
					StyI	Tth111II					

Table B.1. Nucleotide frequencies in the P47 cDNA sequence

A	754	(27.2)			
C	623	(22.5)	A + T	1487	(53.7)
G	660	(23.8)	C + G	1283	(46.3)
T	733	(26.5)			

AA	234	(8.5)	CA	189	(6.8)	GA	217	(7.8)	TA	114	(4.1)
AC	124	(4.5)	CC	187	(6.8)	GC	141	(5.1)	TC	170	(6.1)
AG	239	(8.6)	CG	25	(0.9)	GG	168	(6.1)	TG	228	(8.2)
AT	157	(5.7)	CT	222	(8.0)	GT	134	(4.8)	TT	220	(7.9)

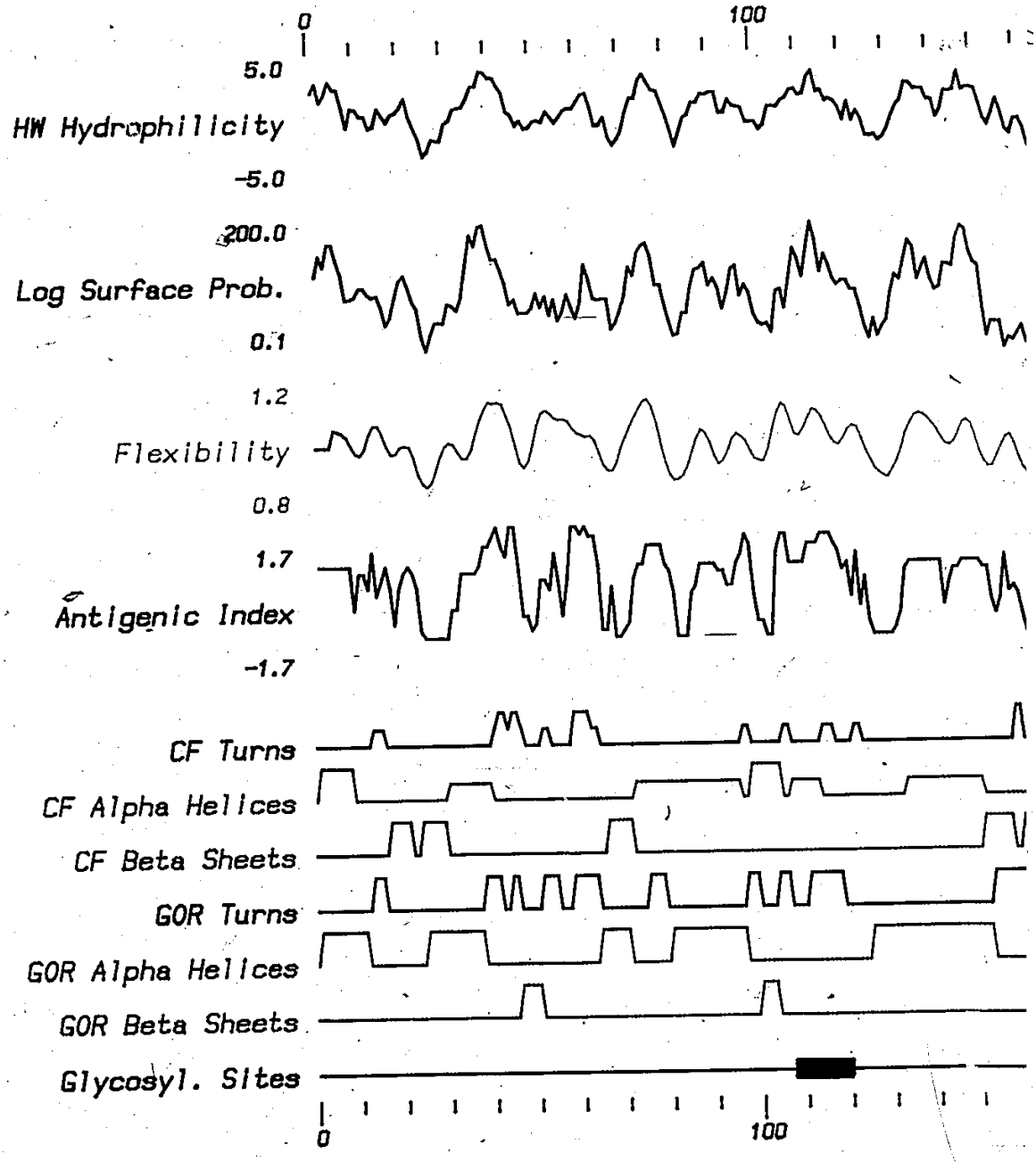
Compositions were calculated with Beckman Microgenie software. The sequence analyzed was based on the clone 34A sequence with 5' and 3' sequence addended from clones 3-1 and 2A3, respectively.

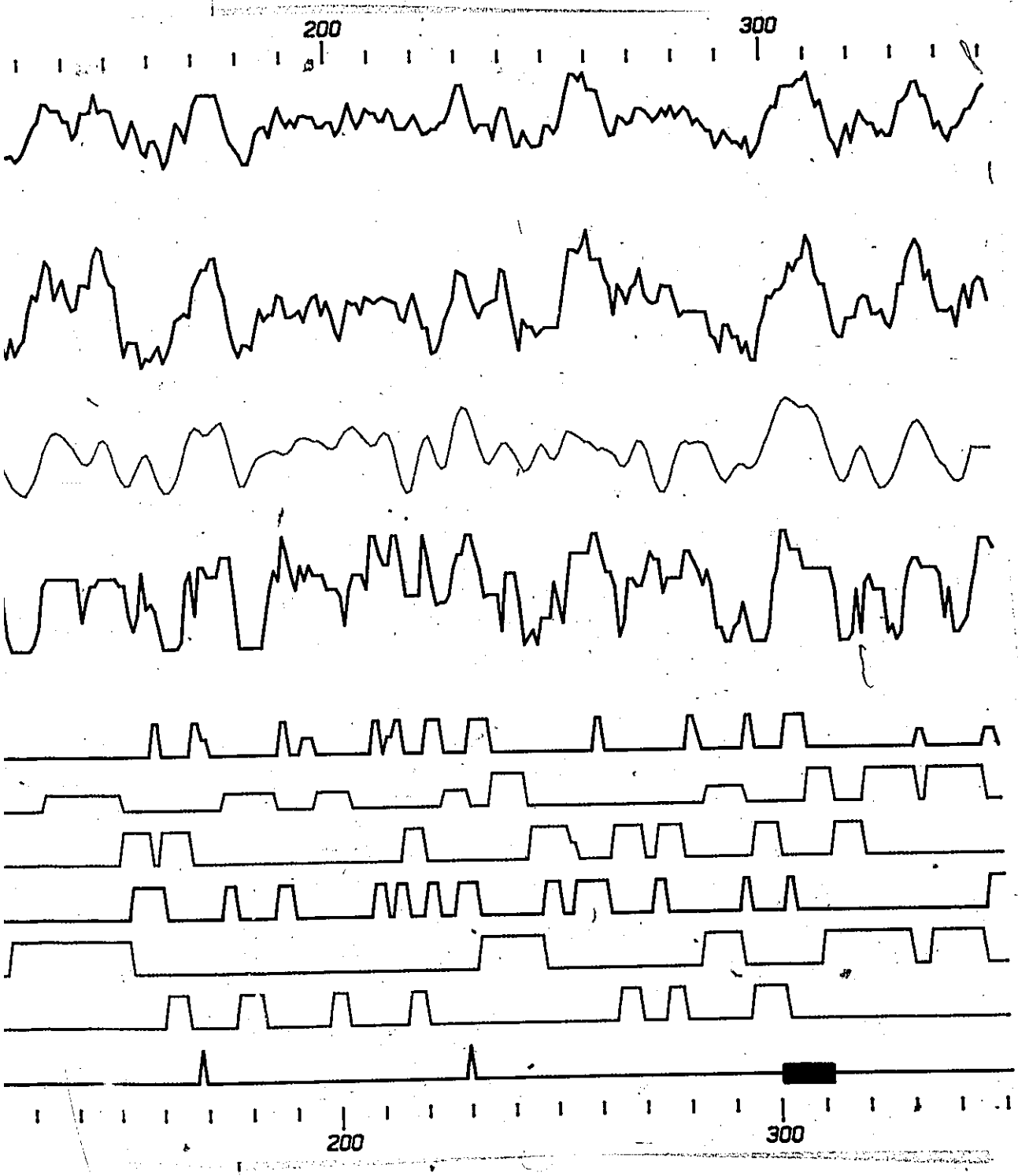
Table B.2. Codon useage in the P47 reading frame

TTT Phe	7 (2.0)	TCT Ser	2 (0.6)	TAT Tyr	5 (1.4)	TGT Cys	4 (1.1)
TTC Phe	13 (3.7)	TCC Ser	6 (1.7)	TAC Tyr	5 (1.4)	TGC Cys	3 (0.9)
TTA Leu	4 (1.1)	TCA Ser	3 (0.9)	TAA End	1 (0.3)	TGA End	0 (0.0)
TTG Leu	5 (1.4)	TCG Ser	1 (0.3)	TAG End	0 (0.0)	TGG Trp	6 (1.7)
CTT Leu	2 (0.6)	CCT Pro	6 (1.7)	CAT His	1 (0.3)	CGT Arg	0 (0.0)
CTC Leu	2 (0.6)	CCC Pro	4 (1.1)	CAC His	5 (1.4)	CGC Arg	2 (0.6)
CTA Leu	1 (0.3)	CCA Pro	3 (0.9)	CAA Gln	2 (0.6)	CGA Arg	2 (0.6)
CTG Leu	13 (3.7)	CCG Pro	1 (0.3)	CAG Gln	10 (2.8)	CGG Arg	2 (0.6)
ATT Ile	11 (3.1)	ACT Thr	7 (2.0)	AAT Asn	8 (2.3)	AGT Ser	5 (1.4)
ATC Ile	10 (2.8)	ACC Thr	4 (1.1)	AAC Asn	8 (2.3)	AGC Ser	5 (1.4)
ATA Ile	1 (0.3)	ACA Thr	3 (0.9)	AAA Lys	16 (4.6)	AGA Arg	6 (1.7)
ATG Met	8 (2.3)	ACG Thr	2 (0.6)	AAG Lys	17 (4.8)	AGG Arg	8 (2.3)
GTT Val	3 (0.9)	GCT Ala	4 (1.1)	GAT Asp	11 (3.1)	GGT Gly	2 (0.6)
GTC Val	2 (0.6)	GCC Ala	10 (2.8)	GAC Asp	11 (3.1)	GGC Gly	6 (1.7)
GTA Val	2 (0.6)	GCA Ala	6 (1.7)	GAA Glu	16 (4.6)	GGA Gly	8 (2.3)
GTG Val	10 (2.8)	GCG Ala	0 (0.0)	GAG Glu	12 (3.4)	GGG Gly	8 (2.3)

Triplet frequencies were determined using Beckman Microgenie software. Values in parenthesis indicate the particular codon as a percent of total codons used in the reading frame. Values shown are for the Trp^{92} variant.

Fig. B.2. Comparison of various parameters used to predict surface regions and secondary structure of P47. Plots were based on the following methods: HW, Hopp and Woods (1981); surface probability, Emini et al (1985); flexibility, Karplus-Schultz (GCG manual); antigenic index, Jameson and Wolf (GCG manual); CF, Chou and Fasman (1978); GOR, Garnier et al (1978). Generated with the PLOTSTRUCTURE routine of the UWGCG software package. Solid bars on the glycosylation line indicate positions of the best predicted PKC site and the putative Ca^{2+}-binding EF-hand (from left to right, respectively).


0
100
5.0
HW Hydrophilicity
-5.0
200.0
Log Surface Prob.
0.1
1.2
Flexibility
0.8
1.7
Antigenic Index
-1.7
CF Turns
CF Alpha Helices
CF Beta Sheets
GOR Turns
GOR Alpha Helices
GOR Beta Sheets
Glycosyl. Sites
0
100


200
300
200
300

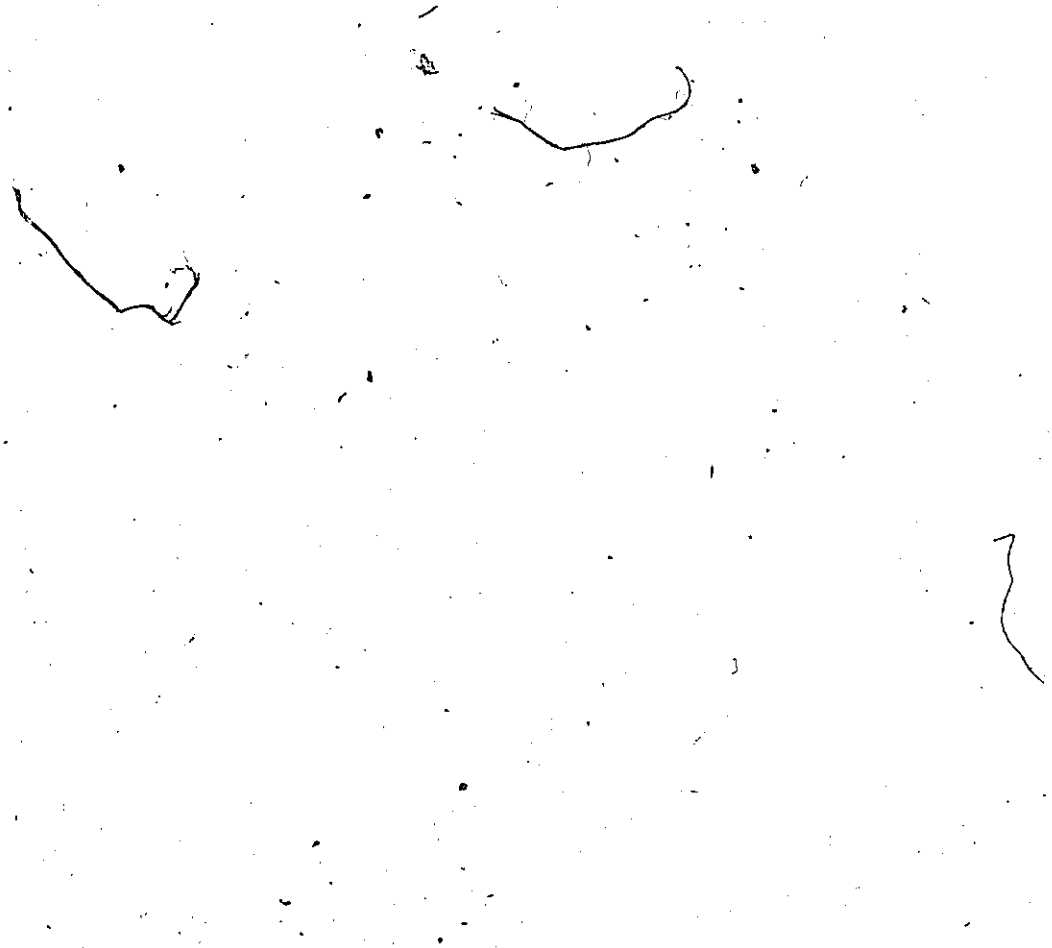
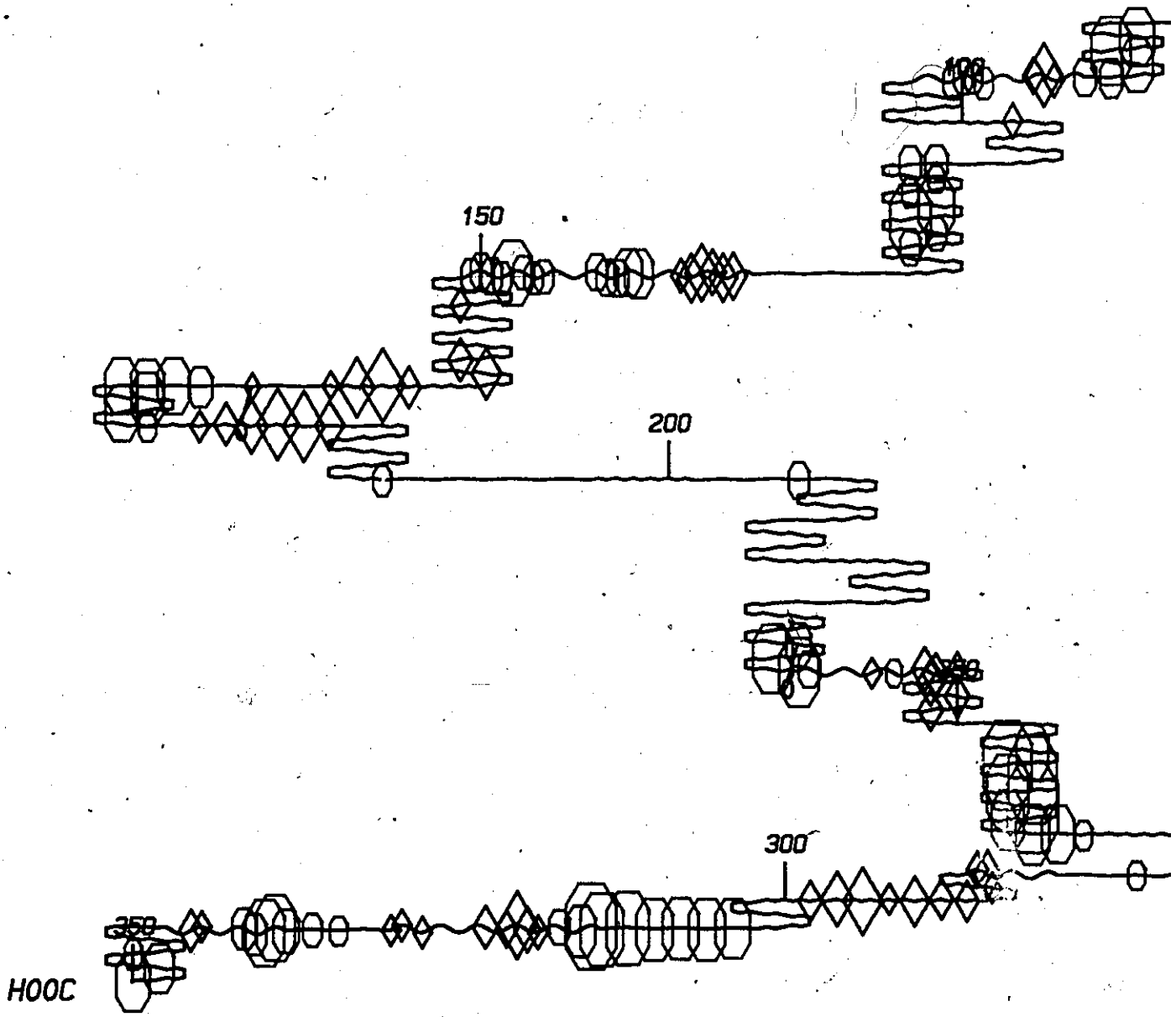


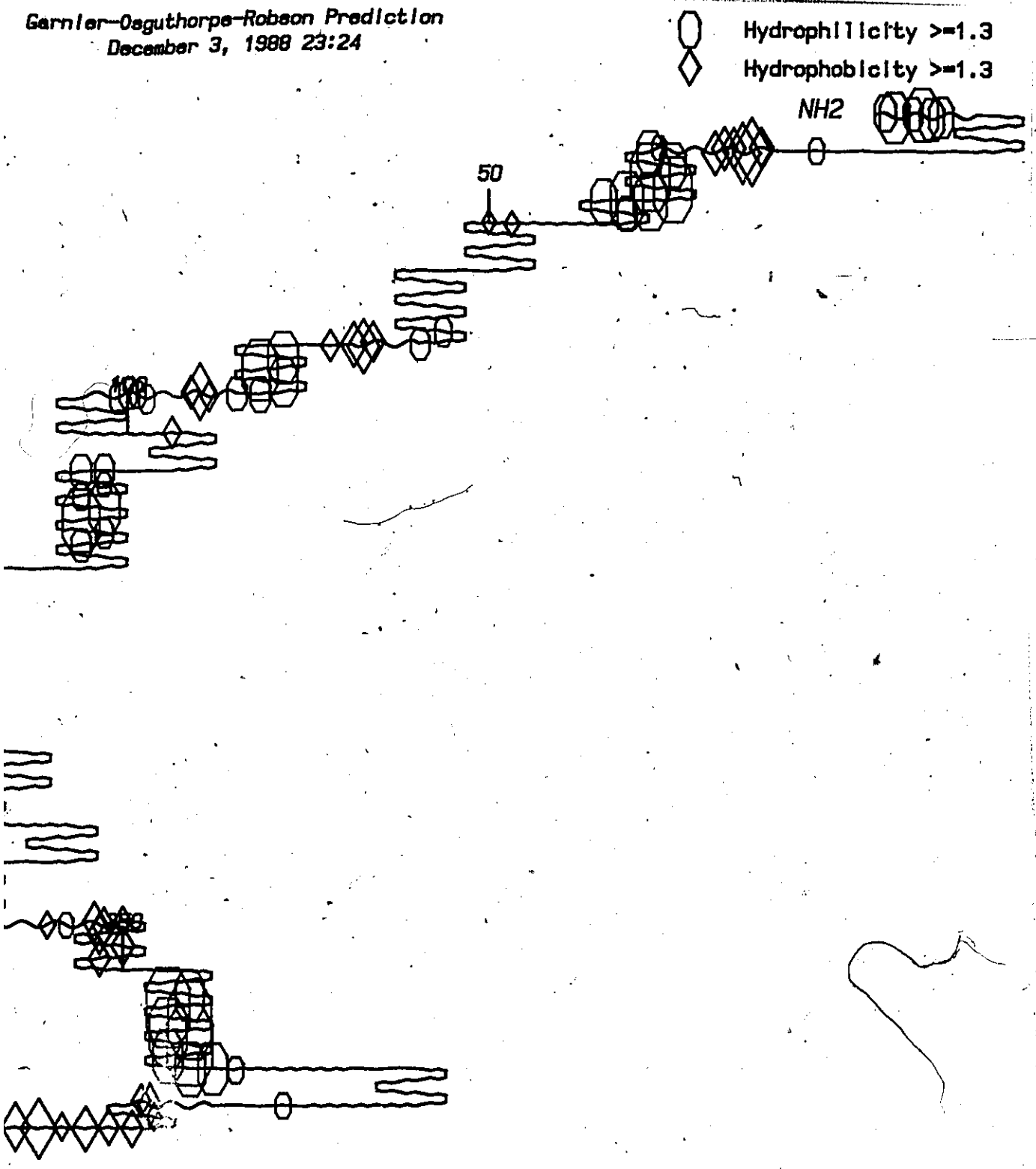
Fig. B.3. Stylized 2-dimensional plot of features predicted in the P47 protein sequence. This representation of part of the data shown in Fig. B.2 was also generated with the PLOTSTRUCTURE routine of the UWGCG software package.

PLOTSTRUCTURE of: p47pro.seq ck: 1500

P47 - HUMAN

Garnier-Osguthorpe-Robson
December 3, 1988 2

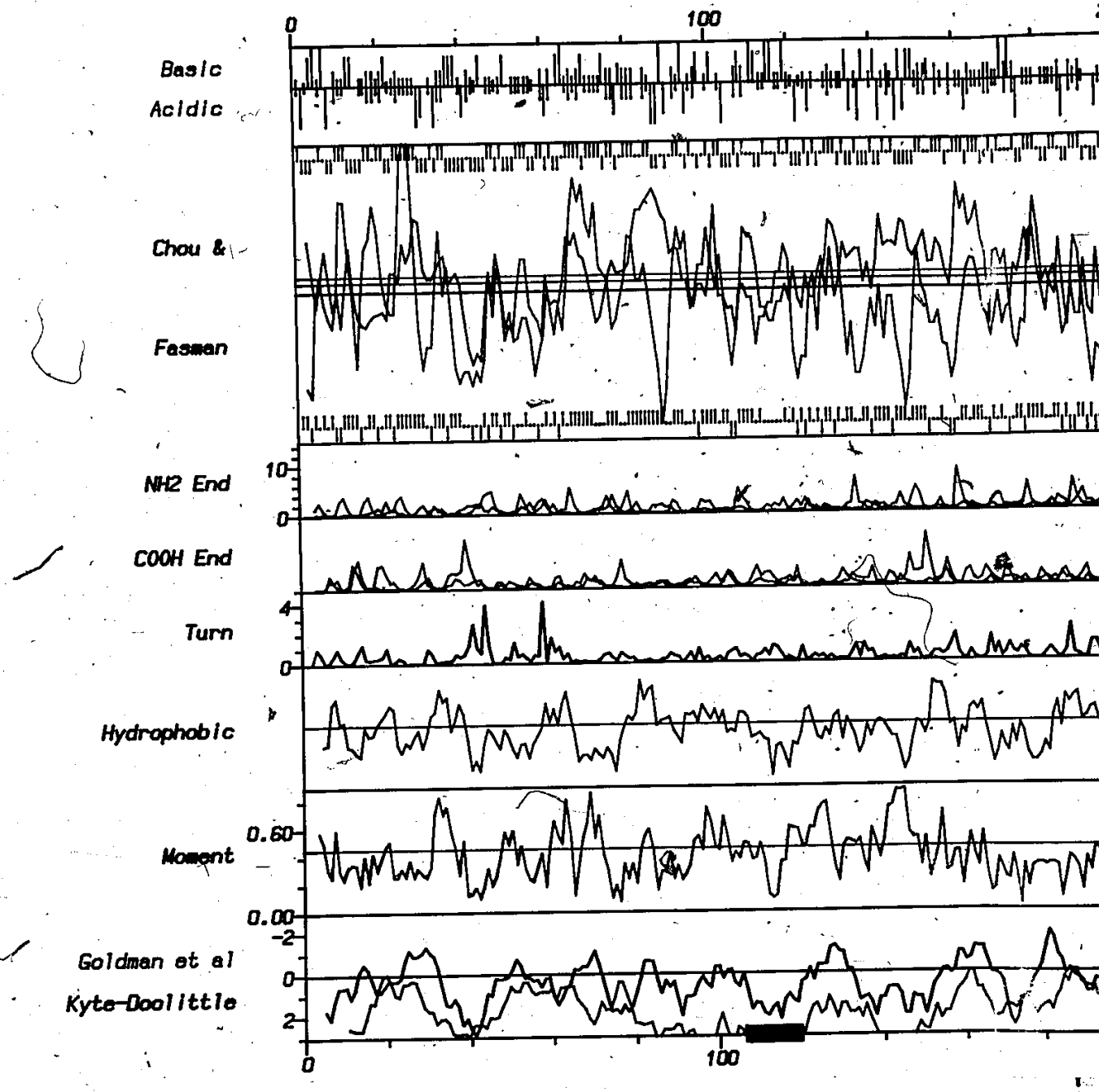


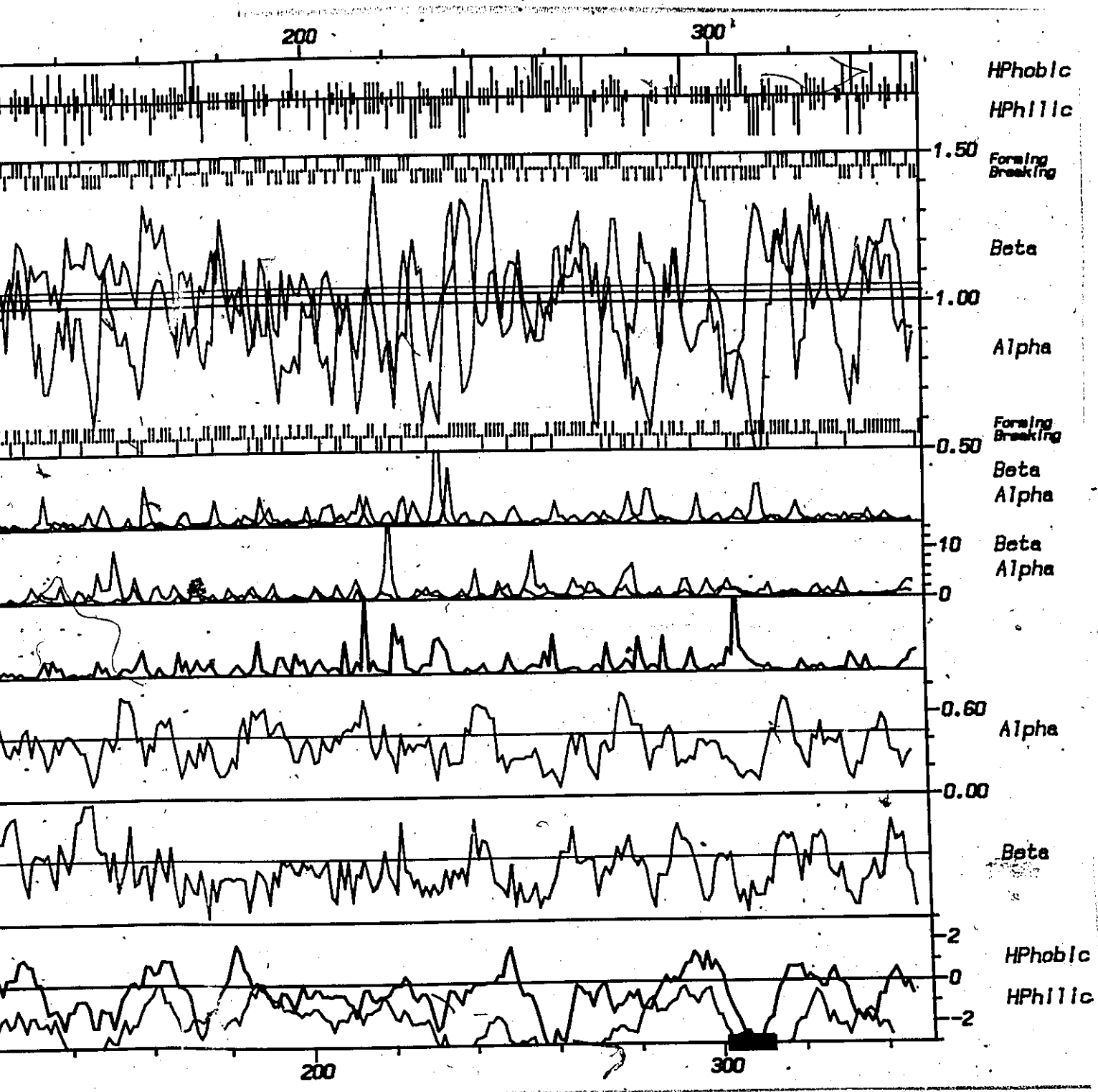

Garnier-Osguthorpe-Robson Prediction
December 3, 1988 23:24
Hydrophilicity >=1.3
Hydrophobicity >=1.3
NH2
50
100

dimensional plot of P47 based on the predictions in Fig. B.2 is shown in Fig. B.3.

Other secondary structure data is presented in Fig. B.4. The Chou-Fasman algorithm can be augmented by statistical data from known protein structures (Chou and Fasman, 1978; Richardson and Richardson, 1988); the propensity of residues to disrupt α or β secondary structures and the prefered residues to begin and end regions of secondary structure are shown above and below the Chou-Fasman prediction. Some of these coincide with the appropriate points in predicted α-helical regions. In particular, the 2 potential helices which bound the putative Ca^{2+}-binding loop begin with correct amino acids. The ratio of charged to apolar residues in P47 (approximately 1.0) is suggestive of an elongated structure (Cohen and Parry, 1986); this would concur with the anomalously large Stokes radius of P47 (Imaoka et al, 1983). Since P47 has a relatively high predicted α-helical content, it is tempting to speculate that the overall structure of P47 is based on a 4 α-helical bundle motif (Cohen and Parry, 1986). A plot of hydrophobic moment assuming either α or β secondary structure (Eisenberg et al, 1984) did not suggest any extended amphipathic regions indicative of secondary structure packing (Fig. B.4). However, this method would not detect amphipathic tendencies over long stretches, particularly if local discontinuities were present (Eisenberg et al, 1984). Finally, the hydropathy profile of P47 reflected the hydrophilic amino acid content of the protein as no potential membrane spanning regions were evident (Kyte and Doolittle, 1982). Two of the prominent hydrophilic regions in P47 corresponded to the best candidate PKC site and the Ca^{2+}-binding

Fig. B.4. Comprehensive analysis of P47 protein sequence. Charge profile, predicted secondary structure (Chou and Fasman, 1974), N- and C-terminal propensity of secondary structures (Richardson and Richardson, 1988), hydrophobic moment of secondary structures (Eisenberg et al, 1984) and hydropathy profile (Engelman et al, 1986; Kyte and Doolittle, 1982) were plotted using the PEPPLOT routine of the UWGCG software package. Solid bars on the bottom axis indicate positions of the best predicted PKC site and the putative Ca^{2+}-binding EF-hand (from left to right, respectively).


0
100
Basic
Acidic
Chou &
Fasman
NH2 End
10
0
COOH End
Turn
4
0
Hydrophobic
Moment
0.60
0.00
Goldman et al
Kyte-Doolittle
-2
0
2
0
100


200
300
HPhoblc
HPhllic
1.50
Forming
Breaking
Beta
1.00
Alpha
Forming
Breaking
0.50
Beta
Alpha
10
Beta
Alpha
0
0.60
Alpha
0.00
Beta
2
HPhoblc
0
HPhllic
-2
200
300

loop of the putative EF-hand.

Although experimentally unfounded, it is possible to speculate on the structure of P47 based on the above theoretical computer analyses. Post-translational modification of P47, such as phosphorylation by PKC, may be necessary to uncover Ca^{2+}-binding activity. Since Ca^{2+}-binding proteins usually contain 2, 3 or 4 EF-hand domains that bind Ca^{2+} in a cooperative fashion, deviations in the sole EF-hand of P47 may reflect looser structural requirements for binding a single Ca^{2+}. The lysine in the P47 Ca^{2+}-binding loop occupies a location normally required for hydrophobic packing of different EF-hand domains (Kretsinger, 1980). As a matter of extreme speculation, this function could be replaced in P47 by an electrostatic interaction between the lysine and a phosphate residue added by PKC. Recently, it has been demonstrated that negative surface charges contribute significantly to the free energy of Ca^{2+}-binding in bovine calbindin D_{K9} (Linse et al, 1988). Phosphorylation near the P47 EF-hand may increase its affinity for Ca^{2+}. It is also interesting that the anomalous lysine forms part of a weak potential PKC site within the EF-hand itself.

A single attempt to demonstrate Ca^{2+}-binding by recombinant P47 in a Ca^{2+}-overlay blot was unsuccessful, even though binding to muscle proteins and purified calmodulin was detected (Fig. B.5). In contrast, preliminary results indicate that phosphorylated platelet P47 can bind Ca^{2+} at 10 fold lower molar ratios than the unphosphorylated form (Dr. R.J. Haslam, personal communication). Whether this reflects true Ca^{2+}-binding or merely arises from non-specific interaction of Ca^{2+} with phosphorylated residues remains to be seen.

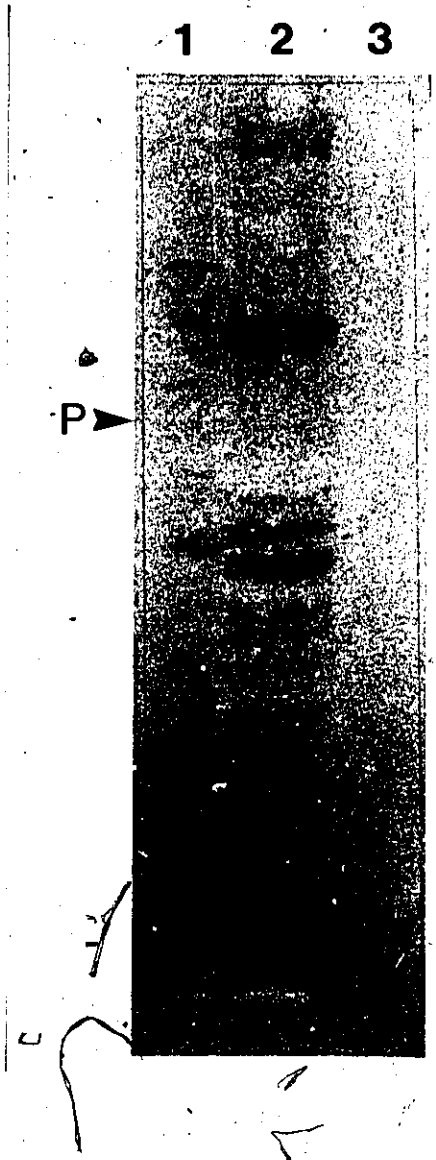


Fig. B.5. Ca^{2+}-overlay blot of recombinant P47. Samples were electrophoresed, transferred to nitrocellulose and probed with $^{45}Ca^{2+}$: bacterial lysate (100 µg) containing high levels of P47 (lane 1); lysate (approximately 80 µg) of mouse muscle tissue (lane 2); 5 µg purified calmodulin (lane 3). Arrow indicates position of recombinant P47 detected with 1% amido black stain. Ca^{2+}-overlay blots were carried out exactly as described by Maruyama et al (1985). Mouse skeletal muscle was minced and boiled in sample buffer for use as a positive control for Ca^{2+}-binding proteins.

Ultimately the role of the candidate Ca^{2+}-binding and phosphorylation sites of P47 must be tested by site-specific mutatgenesis in conjunction with functional assays. Two oligonucleotides were designed for this purpose. One degenerate oligonucleotide was synthesized to optimize the Ca^{2+}-binding loop by replacement of the lysine residue with either valine or isoleucine. A second degenerate oligonucleotide complementary to the putative PKC site was designed to substitute each serine and threonine residue in any combination. Each oligonucleotide and the corresponding permutations of amino acids generated are shown in Fig. B.6. All mutants would have to be defined by sequencing but in conjunction with high efficiency methods of mutagenesis (see Zoller and Smith, 1987) this should still be faster and cheaper than creating individual amino acid replacements.

As indicated in Section 4.6, some recently published sequences have marginal similarity to P47. These are aligned against P47 in Fig. B.7 and Fig. B.8.

(a)

wild type P47:	305-	Asn	Gly	Arg	Lys	Ser	Glu -310
mRNA:	5'-	AAU	GGC	AGG	AAG	AGU	GAG -3'
oligo:	3-	TA	CCG	TCC	C/T AG	TCA	CTC -5'
mutant P47:	305-	Asn	Gly	Arg	Val/Ile	Ser	Glu -310

(b)

wild type P47:	111-	Arg	Lys	Ser	Thr	Arg	Arg	Ser	Ile	Arg -119
mRNA:	5'-	AGG	AAA	UCU	ACC	AGG	AGG	UCC	AUU	CGA -3'
oligo:	3'-	TCC	TTT	C/A GA	C/T GG	GCG	TCC	C/A GG	TAA	G -5'
mutant P47:	111-	Arg	Lys	Ala/Ser	Ala/Thr	Arg	Arg	Ala/Ser	Ile	Arg -119
				----	-xx	xxx	xx	xxx		
				Alu I		Bst NI		Ava II		

Fig. B.6. Oligonucleotides synthesized to mutate P47. a, replacement of the anomalous lysine in the putative EF-hand with either valine or isoleucine. b, replacement of each potentially phosphorylated residue (either alone or in combination) in the best potential PKC site with alanine residues. Degenerate oligonucleotides were designed to create multiple amino acid changes in a single extension reaction. Mismatches between wild type P47 sequence and oligonucleotide sequence are underlined, as are the corresponding amino acid substitutions. Restriction enzyme sites either created (----) or destroyed (xxxxx) by the mutagenic oligonucleotide and the minimum 2 mismatches per incorporation will facilitate detection of mutant clones.

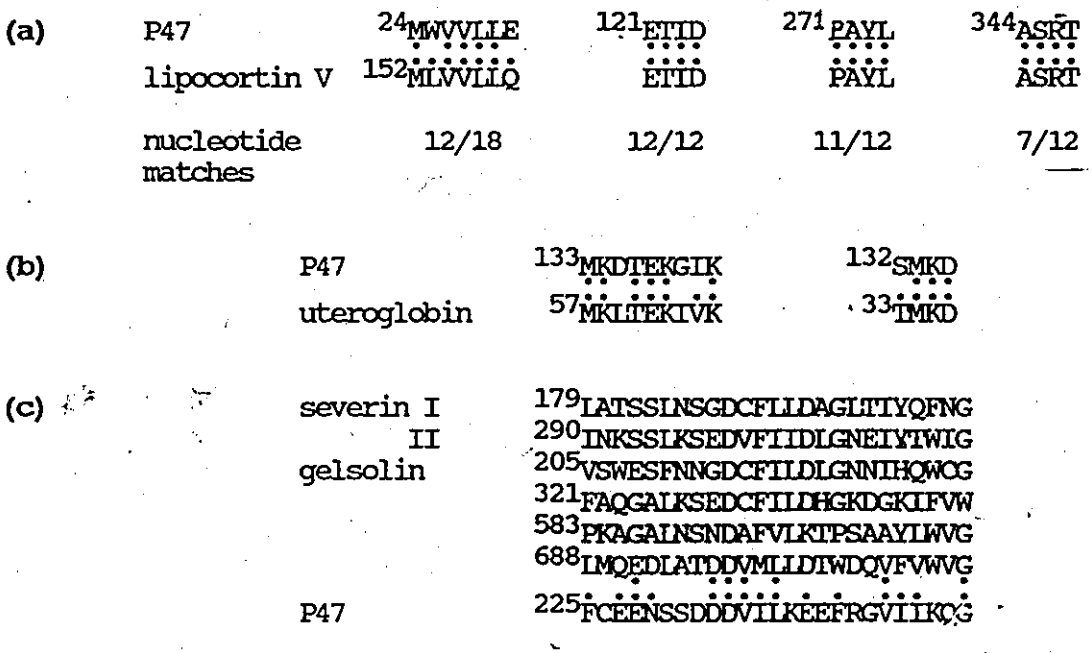
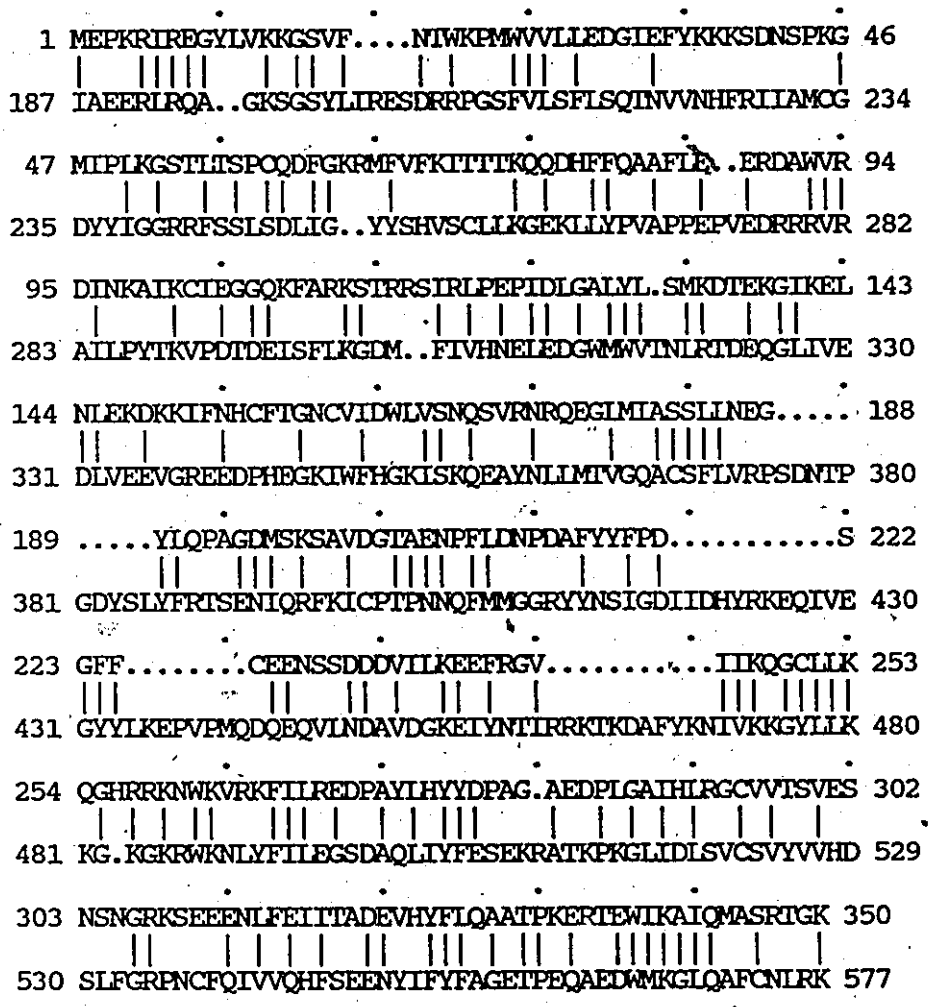


Fig. B.7. Alignment of P47 amino acid sequences with 3 recently published protein sequences. P47 residues were matched visually to those from: a, human lipocortin V (Pepinsky et al, 1988); b, rabbit uteroglobin (Miele et al, 1988); c, severin (from D. discoidium) and gelsolin (Andre et al, 1988).



Symbol comparison table: GenDataBase:SWGapPep.Cmp

Gap Weight:	3.000	Average Match:	0.540
Length Weight:	0.300	Average Mismatch:	-0.396
Quality:	123.8	Length:	398
Ratio:	0.354	Gaps:	12

Percent Similarity: 45.481

Fig. B.8. Best alignment of P47 and GAP. Note that the standard gap penalty for this program is 5.0; under this condition the similarity between P47 and GAP is reduced to 37 %.

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