THE MOLECULAR NATURE OF KERATINOCYTE-DERIVED INTERLEUKIN-1

STUDIES ON THE MOLECULAR NATURE OF KERATINOCYTE-DERIVED INTERLEUKIN-1

by

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A Thesis

Submitted to the School of Graduate Studies

in Partial Fulfilment of the Requirements

for the Degree

Master of Science

McMaster University,

MASTER OF SCIENCE (1992)
(Biochemistry)

McMASTER UNIVERSITY
Hamilton, Ontario

TITLE: Studies on the Molecular Nature of Keratinocyte-Derived Interleukin-1

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NUMBER OF PAGES: xi, 121

ABSTRACT

Interleukin-1 (IL-1), originally defined as a product of activated macrophages, has since been found to be produced by many cell types including keratinocytes. The nature of this IL-1 activity in keratinocytes, originally known as epidermal cell-derived thymocyte activating factor (ETAF) has been the subject of many studies. In the course of this work it was found that the human keratinocyte cell line COLO 16 contains mRNA homologous to human monocyte-derived IL-1β. A 1.2 kbp cDNA was selected with a human IL-1β probe from a λgt11 library constructed from COLO 16 mRNA. Sequence analysis revealed that this cDNA was nearly identical to the 3' 1.2 kb of human monocyte IL-1β.

In addition, a partial cDNA (F8) was isolated from COLO 16 cells which has a distinct sequence from either IL-1α or β. There is evidence to suggest that the F8 message may be derived from differential splicing of a region of the human genome which also gives rise to the cGMP-gated ion channel in rod photoreceptor cells. The F8 cDNA hybridized on Northern blots of COLO 16 mRNA to a 1.6 kb message of low abundance. Antisera generated against a synthetic peptide based on inferred protein sequence from the cDNA reacted with a 20 and 30 kDa species in both COLO 16 cells and PMA-stimulated normal human keratinocytes. Expression of the partial cDNA in COS-1 cells resulted in activity in the thymocyte co-stimulation and D10.G4.1 T-cell stimulation assays, suggesting that ETAF activity may be due to a combination of IL-1 and F8.

ACKNOWLEDGEMENTS

I wish to thank my supervisory committee for helpful criticism during the course of this work. I would also thank my supervisor, Cal Harley, for his support and patience. I thank Dan Sauder for his enthusiasm and Roddie McKenzie for his helpful discussions and collaborative assistance.

I would like to acknowledge the superb technical assistance of Dawn Stetsko for providing the tissue culture samples for the various RNA studies and for performing the hybrid selection experiment. I would also like to thank Karen Neilson for performing the oocyte microinjections. I wish to thank Roddie McKenzie for the mammalian expression studies that he performed with the F8 cDNA clone and Sofija Matic for performing the thymocyte assays.

In addition, I wish to acknowledge the financial support of the Medical Research Council of Canada and McMaster University.

A special thanks goes to my parents for their support and encouragement. I would also like to express my deepest gratitude to my husband Todd who has provided me with the courage and conviction to overcome the difficulties I encountered along the way.

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LIST OF ABBREVIATIONS

ACH	allergic contact hypersensitivity
ACTH	adrenocorticotropin hormone
AMV	avian myeloblastosis virus
ATCC	American Type Culture Collection
bp	base pair
C	carboxy
cDNA	complementary DNA
Ci	Curie
cm	centimetre
cpm	counts per minute
DEAE	diethylaminoethyl
DMEM	Dulbecco's modified minimal Eagle's medium
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
dNTP	deoxynucleoside triphosphate
EDTA	ethylene diamine tetraacetic acid
EP	endogenous pyrogen
ETAF	epidermal cell-derived thymocyte activating factor
FBS	fetal bovine serum
g	unit gravitational force
h	hour
Hfl	high frequency lysogeny
HPLC	high pressure liquid chromatography
Ig	immunoglobulin
IL	interleukin
IPTG	isopropylthiogalactoside
kb	kilobase
kbp	kilobase pair
kDa	kilodalton
LAF	lymphocyte activating factor
LP	leukocyte pyrogen
LPS	lipopolysaccharide
µl	microlitre
µM	micromolar
mg	milligram
ml	millilitre
mM	millimolar
mmol	millimole
M_r	relative molecular mass
mRNA	messenger RNA
mw	molecular weight
N	amino
ng	nanogram
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
pI	isoelectric point

PMN	polymorphonuclear
pmol	picomole
RNA	ribonucleic acid
s	second(s)
SDS	sodium dodecyl sulphate
SSC	saline sodium citrate
TBE	Tris borate EDTA
TE	Tris-EDTA
TNF	tumour necrosis factor
Tris	tris(hydroxymethyl)aminomethane
U	unit
XGAL	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

INTRODUCTION

1.1 THE IMMUNE RESPONSE AS A COMPLEX SYSTEM OF PROTECTION

1.1.1 Overview of the Immune System

A system of protection known as the immune system exists within all vertebrates which allows them to combat foreign invaders such as bacteria, parasites and viruses and to recognize abnormal cells as in the case of cancer. It is made up of a number of different cell types which, upon being challenged, interact to produce an immune response. The immune system is generally portrayed as consisting of two branches known as humoral and cellular immunity. Humoral immunity involves a population of lymphoid cells, called B lymphocytes, which mature largely in the bone marrow. Each cell expresses a specific immunoglobulin protein on its surface. In response to interaction with its specific antigen, the cell differentiates into a plasma cell which then proliferates and secretes antibody. The humoral immune response is especially effective in recognizing the surface antigens of bacteria, viruses and abnormal cells. The other arm of the immune system, the cellular immune response, is mediated by another population of lymphoid cells called T lymphocytes which mature largely in the thymus gland. Cellular immunity is usually involved in the defense against parasites, host cells infected with bacteria or virus, foreign and abnormal cells, and fungal infections. T lymphocytes can be subdivided into several groups including helper and suppressor T-cells. Helper T-cells are

involved in the positive regulation of the immune response, augmenting the maturation of plasma cells and the expansion of T-cell populations. Suppressor T-cells, on the other hand, have a role in the down-regulation of the immune response.

1.1.2 The Role of Cytokines in an Immune Response

In order for the various cell types of the immune system to interact with one another and effect their actions, a complex communication network has evolved. There are three different ways that cells can communicate with each other: (i) through surface contact involving membrane-bound signals; (ii) direct contact between cell cytoplasms via gap junctions; and (iii) secretion of soluble signals (Alberts, 1983). A large majority of the signals involved in the execution and regulation of the immune response are carried out by the third mechanism involving a group of soluble mediators known as cytokines (Hamblin, 1988) or secretory regulins (Paul, 1988). These factors are either proteins or glycoproteins which mediate their effects by interaction with target cells via specific receptors. Cytokines can be subdivided into several categories: interleukins, interferons, colony stimulating factors, growth factors and suppressor factors; for this discussion, only the interleukins will be highlighted.

1.1.3 Interleukins: A Family of Immunologically Important Molecules

As the number of cytokines being discovered continued to grow, it was found that the method for naming these factors, essentially based on their biological activities, was becoming increasingly cumbersome.

The problem was exacerbated by the fact that many of these factors had multiple activities, some of which could be attributed to more than one cytokine. As a result, the name interleukin (between leukocytes) was adopted and assigned to new cytokines along with a number in numerical order.

At the present time, there are no fewer than eight interleukins described in the literature. These factors and their major activities are presented in Table 1.1.1. Of these factors, interleukin-1 (IL-1) is of most importance to this discussion.

1.2 INTERLEUKIN-1

1.2.1 The Discovery of IL-1

IL-1 is a polypeptide hormone with a multitude of inflammatory and immunoregulatory activities. It was this vast number of activities which led to the initial confusion on the path to its characterization. Many independent groups were unknowingly studying different activities of the same molecule and assigning it a name based on this activity. As a result, prior to appointing the name of IL-1 to all of these activities, the literature is filled with reports on: endogenous pyrogen (Murphy et al, 1974), lymphocyte activating factor (Gery and Waksman, 1972), and leukocyte endogenous mediator (Kampschmidt, 1981). All of these factors turned out to be the same molecule once further characterization was performed. In addition, some of these initial studies were done on impure preparations which were later found to contain several factors. This merely added to the confusion surrounding

Table 1.1.1 The Interleukin Family

INTERLEUKIN	ALTERNATE NAMES	MAJOR ACTIVITIES
IL-1	Lymphocyte activating factor, Endogenous pyrogen	-stimulates thymocyte proliferation -induces IL-2 production by T-cells -induces fever
IL-2	T-cell growth factor	-stimulates growth of activated T-cells -induces cytotoxic T-cell reactivity
IL-3	Multipotent colony stimulating factor	-stimulates growth of mast cells
IL-4	B-cell stimulatory factor I	-stimulates growth of B-cells and resting T-cells
IL-5	B-cell growth factor II	-augments proliferation of activated B-cells
IL-6	B-cell stimulatory factor II, Interferon-β_2	-induces hepatic production of acute phase proteins -stimulates growth of B-cells
IL-7	Lymphopoietin 1	-stimulates proliferation of lymphoid progenitor cells
IL-8	Neutrophil activating factor	-stimulates histamine release by basophils

the biological activities attributed to IL-1. There are a number of other factors which initially were thought to be distinct species that have since been shown to be IL-1, these include: B-cell activating factor, osteoclast-activating factor (Dewhirst et al, 1985), mononuclear cell factor (Krane et al, 1985) and hemopoietin-1 (Mochizuki, 1987).

The discovery of IL-1 can trace its origins to studies which were done in the early 1940s on febrile responses in animals. The first evidence of a fever-inducing factor stemmed from experiments in which peritoneal fluid from rabbits injected with an inflammator was shown to cause fever in a second set of animals (Menkin, 1944). This fluid, which was rich in leukocytes, was given the name pyrexin. Confirmation of the presence of a fever-inducing factor had to wait until the the possibility of contaminating bacterial endotoxin (also a fever inducer), was eliminated. This was accomplished when a preparation of endotoxin-free polymorphonuclear (PMN) leukocytes was shown to cause fever upon intravenous injection into rabbits (Bennett and Beeson, 1953a,b). At this time it was thought that leukocytes alone produced the substance responsible for fever induction and as a result, it was given the name leukocyte pyrogen (LP) or endogenous pyrogen (EP). In 1972, lymphocyte activating factor (LAF), named for its ability to augment T-lymphocyte responses to mitogens or antigens, was described (Gery and Waksman, 1972). It was found that this factor was identical to the originally described EP. Further characterization of this factor led to the discovery that there were two different sizes of molecules possessing pyrogenic activity (Dinarello et al, 1974), with apparent molecular masses of approximately 15,000 and 38,000 respectively. In addition to

the two different sizes, there also appeared to be differences in isoelectric points (pI). The 15,000 Mr species was made up of a pI 5.1 and pI 6.8 form while the 38,000 Mr species had a pI of 5.0-5.3. At the Second International Lymphokine Workshop in 1979, this factor was given the name interleukin-1 (IL-1)

1.2.2 IL-1 Activities

IL-1 was first characterized in macrophages stimulated with bacterial lipopolysaccharide (LPS) based on its ability to increase the proliferation of murine thymocytes in the presence of a sub-optimal amount of LPS (Gery et al, 1972). The thymocyte co-stimulatory activity of IL-1 has been adopted as a classical assay for IL-1 activity. This in itself, however, is not a specific test for IL-1 since there are a number of other factors which also exhibit thymocyte co-stimulation in this particular assay. These include: interleukins 2, 4 and 6, TNF and LPS (Gearing and Thorpe, 1989). This fact stresses the point that a number of additional activity assays are needed in order to define a source of thymocyte co-stimulatory activity as IL-1. Additional IL-1 activities include: induction of fever, augmentation of IL-2 production by T cells, augmentation of fibroblast, epithelial, synovial and B cell proliferation, induction of muscle proteolysis, stimulation of prostaglandin E_2 release by: fibroblasts, synovial cells, endothelial cells, chondrocytes, brain and muscle cells, augmentation of natural killer activity, stimulation of collagenase synthesis by fibroblasts and synovial cells, induction of hepatic acute phase protein synthesis, induction of slow wave sleep, stimulation of ACTH production by

pituitary cells, stimulation of corticotropin-releasing hormone and somatostatin by the hypothalamus, and the ability to be chemotactic for neutrophils, T cells and macrophages.

1.2.3 The IL-1 Gene Family: Dissimilar Sequence Yields Similar Function

The cloning of the complementary DNAs (cDNAs) for IL-1 led to the discovery that this factor represented a gene family with at least two members termed IL-1α and IL-1β (Lomedico et al, 1984; Auron et al, 1984). These two genes encode for proteins which correspond to the previously identified pI5 and pI7 species respectively. IL-1α and β cDNAs for a variety of species have been isolated (for review see Gubler et al, 1989). The sequence homology between IL-1α and β of the same species is approximately 35-45% at the nucleotide level and 25% at the amino acid level (March et al, 1985). Among species, the level of homology for the same gene is approximately 60-70% for IL-1α and 75-78% for IL-1β (Dinarello, 1989). The limited homology between the two proteins is concentrated into four regions denoted: A, B, C-D and E. Regions A and B are contained within the precursor portion of the proteins while regions C-D and E are found in the mature proteins (Auron et al, 1985).

Although the IL-1α and IL-1β genes share very little sequence homology and encode proteins which are also quite dissimilar in sequence, both code for the production of a precursor (approximate Mr of 30,000) which is post-translationally processed to give a mature form of approximately 17,000 Mr. This explains the initial findings of

Dinarello (1974) which showed two different sizes of protein species possessing pyrogenic activity. In addition to the precursor and processed forms, there are several protein species which are intermediate or smaller in size which react with IL-1 specific antibodies. These peptides have been observed both in natural preparations of IL-1 and in recombinant protein preparations (Cannon and Dinarello, 1985; Kimball et al, 1984; Dinarello et al, 1984). It has been suggested that these smaller peptides as well as the mature form of IL-1 are generated as the result of protease cleavage. Evidence for this includes: the absence of the peptides from preparations to which serine protease inhibitors have been added (Auron et al, 1987), and the presence within the IL-1β sequence of several putative recognition sites for serine proteases, some of which give rise to fragment sizes corresponding to the observed peptides (Palaszynski, 1987; Matsushima et al, 1986). One of the smallest peptide species (Mr 4,000) has been shown to possess all of the activities associated with the full length IL-1 molecule (Dinarello et al, 1984).

Surprisingly, both IL-1α and IL-1β share the same spectrum of biological activities despite their low percentage of sequence homology at the DNA and protein level. There have, however, been a few reports which suggest that there are some differences between the activity profiles of these two molecules. These include reports of IL-1β but not IL-1α being responsible for: a cytotoxic effect on islets of Langerhans in the pancreas (Bendtzen et al, 1986), the stimulation of ACTH release (Uehara et al, 1987), and the induction of an inflammatory response in rat testis (Bergh and Söder, 1990).

The fact that two proteins quite dissimilar in sequence share a vast array of biological activities has led to intense investigation into the specific sequences required for these activities. Initial studies revealed that the expression of the C-terminal amino acids (113-271 for IL-1α; 117-269 for IL-1β) was sufficient for biologic activity (March et al, 1985). The sequence homology between IL-1α and β which is clustered in the C-D region has led to speculation that this area may be responsible for biological activity. Several peptides have been produced corresponding to this region to determine if they alone can manifest the activities of the entire molecule. One of these peptides, corresponding to amino acids 163-171, was able to stimulate thymocyte proliferation and induce IL-2 production in spleen cells (Antoni et al, 1986). Although this nonapeptide possessed immunostimulatory activities it appeared to be devoid of inflammatory activity due to its inability to induce human fibroblasts to synthesize prostaglandin E_2 and induce fever in mice. In addition, it was unable to compete with the 17,500 Mr IL-1 for receptor binding. Another synthetic peptide coding for the carboxy-terminal 33 amino acids was found to block the binding of native IL-1 (Palaszynski, 1987). More recently, antibodies specific for either N-terminal or C-terminal sequences were used to determine regions important for biological activity. One such study found that antibodies to either terminus were capable of partial inhibition of IL-1 activity (Massone et al, 1988). In another study, the deletion of histidine 30 from IL-1β results in a decreased ability to compete for IL-1 receptors on T cells as compared to the wild-type protein (MacDonald et al, 1986). These studies suggest that although IL-1 activities can, at

least partially, be mapped to certain small peptides within the C-D region, the histidine residue at position 30 in IL-1β and either the N-terminus or C-terminus or both may be important for biological activity and perhaps play a role in receptor recognition and binding. Although there are conflicting reports, there have been studies in which there is evidence that the human IL-1β precursor exhibits biological activity (Jobling et al, 1988).

X-ray crystallographic studies on the human IL-1β mature form protein have revealed its tertiary structure at a resolution of 3.0 Å (Priestle et al, 1988). The three-dimensional structure of IL-1β is comprised of a high percentage of β-structure with 12 β-strands. The tertiary folding of these β-strands is similar to that seen for soybean trypsin inhibitor. A rather interesting finding of the crystallography work has been the fact that both the N and C-terminus are exposed on the outer surface of the folded molecule and are in the proximity of one another. In light of the antibody studies which have shown that sequences at both the N and C-terminus appear to be important for biological activity, the three-dimensional structure data seem to support the hypothesis that the N and C-termini may participate together to facilitate receptor binding.

The IL-1 receptor was first characterized by affinity crosslinking studies using radiolabelled IL-1β (Dower et al, 1985). In these initial studies, the IL-1β protein was found to consistently associate with a protein species with an Mr of 80,000. Cloning of the cDNA and elucidation of its sequence revealed that the IL-1 receptor is a member of the immunoglobulin (Ig) gene superfamily (Sims et al, 1988).

Members of this family include, in addition to Ig constant and variable domains, several other receptors and membrane glycoproteins such as: cell-surface adhesion molecules, T-cell antigen receptors, platelet-derived growth factor receptor and colony-stimulating factor receptor (Williams and Barclay, 1988). Although members of this family share little homology at the amino acid level, they possess domains which are similar in their three-dimensional structure. The IL-1 receptor's extracellular portion is made up of three of these domains, each consisting of two β sheets joined by a disulfide bond. The relative positions of several residues are highly conserved including the cysteine residues responsible for this disulfide bond. The method of signal transduction for the IL-1 receptor is not yet known although there is evidence which suggests that it is not mediated through the classical post-receptor binding events such as those of the protein kinase C pathway (reviewed in Dower et al, 1989). Surprisingly, despite the lack of homology between IL-1α and IL-1β, it has been shown that both proteins effect their actions via the same receptor (Dower et al, 1986; Kilian et al, 1986).

In addition to IL-1α and β, a monocyte-derived protein with interleukin-1 inhibitory activity has been purified and shown to bind to the IL-1 receptor (Hannum et al, 1990). This inhibitor, called interleukin-1 receptor antagonist (IL-1ra) has been shown to bind to the IL-1 receptor with approximately the same affinity as Il-1 itself. It is interesting to note that although IL-1ra binds to the IL-1 receptor, it does not appear to possess IL-1 bioactivity as determined by its inability to stimulate PGE_2 synthesis in fibroblasts. The cDNA for this

molecule has been isolated and sequence analysis reveals approximately 26% homology to IL-1β and 19% homology to IL-1α (Eisenberg et al, 1990).

The amount of IL-1β mRNA in stimulated monocytes is approximately 10-50 times greater than the amount of IL-1α message (Auron et al, 1984; Demczuk et al, 1987), however, the amount of IL-1β protein associated with these cells is less than that of IL-1α. This suggests that the translation efficiency may be less for IL-1β.

One of the great mysteries associated with IL-1 is the fact that neither the IL-1α nor IL-1β protein contains a recognizable signal sequence and yet the proteins are able to get out of the cell. The lack of a typical signal sequence coupled with the fact that neither IL-1 species has been found associated with the endoplasmic reticulum or the Golgi apparatus, has led to the conclusion that these proteins do not exit the cell via the conventional secretory pathway (Bakouche et al, 1987; Singer et al, 1988). Until very recently, the only theory put forth to explain the presence of IL-1 outside the cell suggested that the molecules simply leaked out of damaged cells. This would not explain, however, the fact that human monocyte supernatants appear to contain IL-1β protein almost exclusively, while cell lysates are found to contain both IL-1α and IL-1β (Rubartelli et al, 1990). A novel secretory pathway for IL-1β has now been proposed in which the molecules are contained and transported to the surface within intracellular vesicles. This secretory mechanism is based on the finding that, in monocytes, IL-1β appears to be associated with a subcellular fraction which imparts protection from trypsin digestion while IL-1α is sensitive to proteolysis. This parallels the observation of the presence of IL-1β

but not IL-1α in the cell supernatant and suggests a possible link between association with this vesicular fraction and secretion. This vesicular secretion appears to be unrelated to the classical secretory pathway involving the endoplasmic reticulum and Golgi since the appearance of IL-1β outside the cell is not inhibited by either brefeldin A or monensin (Rubartelli et al, 1990).

A large amount of IL-1, however, never seems to get out of the cell and is found either intracellularly or associated with the outer membrane (Auron et al, 1987; Matsushima et al, 1986). The majority of cell-associated IL-1 appears to be the 31,000 Mr precursor and a 22,000 Mr form. It has been proposed that the 22,000 Mr form is the membrane-associated species and that it is biologically active (Kurt-Jones et al, 1985; Beuscher et al, 1987). There do indeed appear to be degrees of ability to secrete IL-1 depending on the cell type. Monocytes seem to be the best secretors of IL-1. Other IL-1 producing cells such as keratinocytes, smooth muscle cells and endothelial cells, although capable of synthesizing the protein, seem to accumulate a large amount of it intracellularly. (Dinarello, 1989). There is also evidence that there may be differences in the efficiency of secretion of the two members of the IL-1 family. Results of one study showed that the majority of membrane-bound IL-1 is IL-1α while the majority of IL-1 in the extracellular fluid is IL-1β (Conlon et al, 1987). However, IL-1β has also been found associated with the cell in the case of activated human monocytes and in tumor cells (Bayne et al, 1986; Ree et al, 1987). A recent study involving pulse-chase experiments on murine macrophages (Beuscher et al, 1990) suggests that the majority of IL-1β is secreted

in its inactive precursor form and then proteolytically processed once outside the cell by an LPS-inducible protease.

Perhaps differential rates of secretion or differences in the mechanism by which the two interleukins are secreted and processed represents a way in which a level of biological regulation can be achieved for the two different IL-1 molecules which appear to be almost identical in their biological activity profiles. Indeed, in light of these studies, it has been proposed that an active membrane-bound IL-1α may play a more significant role in activities localized to the cell as in the case of local inflammation and infection. IL-1β, on the other hand, may travel far from its site of production after being secreted and processed into its active form to play a major role in the systemic effects of IL-1.

The genomic sequences for human IL-1α and β have been isolated (Clark et al, 1986; Furutani et al, 1986). It is interesting to note that both genes are located on chromosome 2 (Webb et al, 1985) and are organized similarly, each being composed of seven exons. The fact that there is substantial structural similarity between the two genes has led to the postulate that one of the genes arose from the other as a result of a gene duplication event. This would explain the seemingly unneccessary occurrence of two genomic sequences encoding for what appears to be identical functions. Analysis of the genomic sequences identified certain characterisitics in the IL-1β gene which are reminiscent of a form of gene duplication via reverse transcription called retrotransposition (Clark et al,1986). The result of retrotransposition, a "retroposon", is characterized by the presence of

direct repeats flanking each end of the gene and the presence of a 3' poly(A) tail (Weiner et al, 1986). The presence of these features in the genomic sequence of human IL-1β suggest that IL-1β may be a retroposon derived from IL-1α.

1.2.4 Cell Sources of IL-1 Activities

In recent years, it has been found that cells other than those of macrophage lineage are sources of IL-1 activities. These include: B lymphocytes, endothelial cells, melanoma cell lines, fibroblasts, astrocytes, polymorphonuclear neutrophils, mesangial cells, epithelial cells, glioma cell lines, dendritic cells, Langerhans cells, microglial cells and keratinocytes. It is interesting to note that cells of the largest organ in the body, the skin, have been found to be a source of activities attributed to IL-1 as well as several other immunoregulatory and inflammatory factors (reviewed in Kupper, 1988).

1.3 IMMUNOLOGICAL PROPERTIES OF THE SKIN

1.3.1 Overview of Skin Structure

Skin is made up of the epidermis and dermis which are separated by the basement membrane (Curtis, 1979). The epidermis is made up of a number of different cell types: keratinocytes, dendritic cells, melanocytes and T lymphocytes. Below the epidermis is the dermis which is largely made up of connective tissue and contains numerous blood vessels, nerves and hair follicles as well as sweat and sebaceous glands (Figure 1.3.1). In this discussion, the epidermis will be emphasized

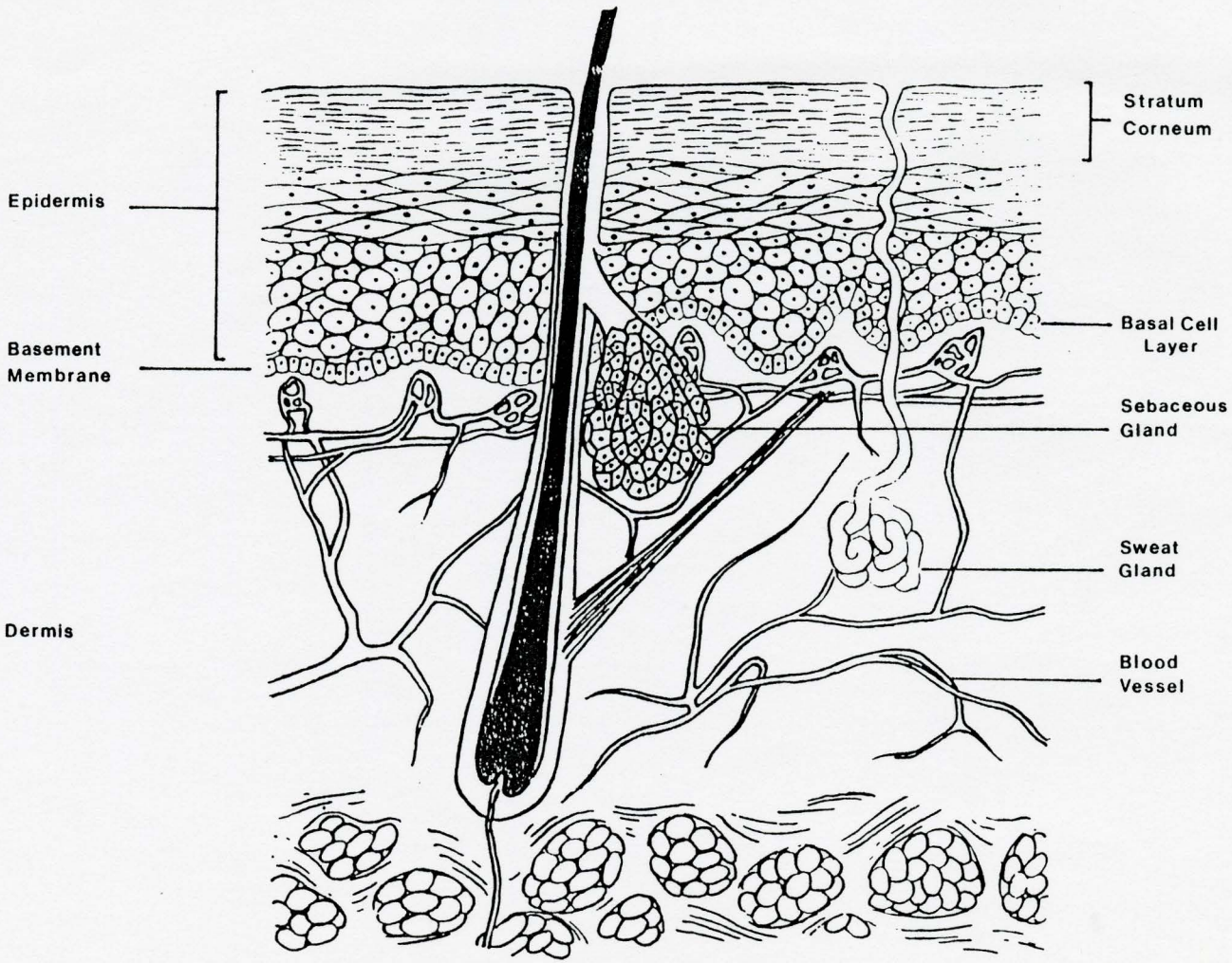

Fig. 1.3.1. Cross-sectional view of skin. A simplified representation of the cellular organization is shown. Modified from Johnson et al (1971).

for the sake of simplicity. The most abundant cell found in the epidermis is the keratinocyte, so named for the presence of large amounts of the protein keratin which is produced by fully differentiated cells. It is the proliferation of the keratinocyte which is responsible for the continual turnover of epidermal tissue. Bone marrow-derived dendritic cells within the epidermis can be subdivided into macrophage-like (Langerhans cells) and T cell-like (Thy1 dendritic epidermal cells). Melanocytes are of neural crest origin and produce the protein melanin which is responsible for the pigmentation of skin (Karp and Berrill, 1981). This cell type is found in the basal layer next to the basement membrane which separates the dermis and epidermis. The epidermis can, in fact, be thought of as a differentiation gradient of keratinocytes starting at the innermost basal layer where cells continously divide, followed by an intermediate region of differentiating cells which eventually denucleate to form the outermost dead layer of cells called the stratum corneum. There is a continual migration as cells of the basal layer divide and move to the surface to replenish cells which are lost. As the cells move away from the basal layer they lose the ability to divide and begin to differentiate, resulting in the production of keratin and changes in morphology, going from columnar to cuboidal and finally to a flattened shape known as a squame. Eventually, the differentiated cells form associations with each other and die, resulting in the sheets of dead cells which form the stratum corneum. The continual differentiation of the epidermis has been shown to be dependent on its interaction with the dermis although direct contact is not required.

1.3.2 Immunologically Important Skin Cells

Historically, the skin has not been thought to play an active role in immunity, but rather, acts only as a "passive barrier to penetration by pathogens" (Hood et al, 1984). It is now known, however, that this is not the case. In recent years, it has been found that certain types of skin cells possess activities similar to those attributed to cells of the immune system and there is growing evidence that skin cells produce many of the factors which play an important role in the regulation of the immune response. Indeed, an immunologic function for the skin was suggested by virtue of the fact that it displayed structural similarities to an immunologically important organ, the thymus gland. Both the thymus and the epidermis contain a mixture of epithelial and dendritic cells. Immunofluorescence studies revealed that the thymic epithelial cells were capable of producing the epidermal keratinocyte protein keratin, and that the keratinocyte cytoplasm contains a thymic hormone important for T-cell maturation called thymopoietin (Chu et al, 1982). The notion that epidermal cells could contribute to immunity by being more than just a barrier was pursued in studies on cutaneous T-cell lymphoma (Edelson, 1976) and allergic contact dermatitis (Baer, 1964)

The first of these, a lymphocytic malignancy called cutaneous T-cell lymphoma (CTCL), is characterized by the presence of malignant helper T-cells in the epidermis. It was this observation which led Edelson to postulate that the epidermis may, under normal conditions, contain this subset of T lymphocytes and that CTCL was the result of the

proliferation of malignant epidermal T lymphocytes (Edelson, 1976). The possibility of the presence of T lymphocytes under normal circumstances within the epidermis raised the question as to the possible role of skin cells in the immune response and their interactions with cells of known immunologic function such as T lymphocytes

Around the time that Edelson and colleagues were studying CTCL, work was being done on this very question involving a possible relationship between Langerhans cells and T lymphocytes. These studies involved the skin disease allergic contact dermatitis. This disease, also called allergic contact hypersensitivity (ACH), is an immunologic response triggered by the application of a sensitizing antigen to the skin. Morphological studies revealed that when a contact allergic reaction was induced experimentally, Langerhans cells were found in close association with lymphocytes at the site of application of the irritant (Silberberg et al, 1976), leading to the postulate that this cell type was somehow involved in the activation of the lymphocytes. This idea was further substantiated by the fact that Langerhans cells were shown to express two types of receptors found on monocytes/macrophages and Ia antigens (Stingl et al; Rowden et al; Klareskog et al, 1977), suggesting that these cells were capable of performing the macrophage function of antigen presentation. This surprising finding led to an explosion of studies on the cells of the epidermis and their possible participation in immune responses within the skin. Further studies have shown the Langerhans cell to be of bone marrow origin (Fithian et al, 1981) and have identified another bone marrow-derived cell type in murine epidermis which is similar to T-

lymphocytes (Tschachler et al,1983; Bergstresser et al, 1983; Bergstresser et al, 1984). This cell type is dendritic in nature and expresses a murine T-cell differentiation antigen called Thy-1. Little is known about the function of the Thy-1$^+$ dendritic epidermal cell (Thy-1 DEC) and, to date, a human equivalent has not been identified, although preliminary characterization of a potential equivalent has been reported (Kupper, 1988).

1.3.3 ETAF: A Keratinocyte Product Possessing IL-1 Activities

The finding that Langerhans cells are capable of presenting antigen to sensitized T cells led to studies aimed at determining whether these cells, like macrophages, could produce IL-1 (Sauder et al, 1982; Luger et al, 1981). Initially, supernatants from heterogeneous populations of skin cells were tested for IL-1 activity using the thymocyte co-stimulator assay. It was found that the supernatants from both murine and human skin cell populations contained thymocyte stimulatory activity. Due to the heterogeneous nature of the cell samples, it was not clear whether the Langerhans cells were the sole source of the IL-1 activity; it was possible that the keratinocytes or melanocytes could also be responsible for the activity. In order to determine whether the Langerhans cells were alone responsible for the thymocyte stimulation, populations of skin cells were treated with anti-Ia antiserum and complement which would specifically destroy only those cells possessing Ia antigens, in this case, the Langerhans cells. When the Langerhans cell-depleted population was tested for IL-1 activity, it was found it also contained a factor capable of stimulating thymocytes

(Sauder et al, 1982). In another study, the supernatant of the murine keratinocyte cell line Pam 212 was shown to contain a factor capable of thymocyte co-stimulation and the stimulation of IL-2 production by mitogen-stimulated spleen cells (Luger et al, 1981). Since the name IL-1 pertained to a factor derived from cells of the monocyte/macrophage lineage, this potentially new factor with IL-1-like activities was given the name epidermal cell thymocyte-activating factor (ETAF). Further studies on the physical characteristics of this factor were carried out using a human squamous cell carcinoma cell line, COLO 16. Squamous cell carcinoma is characterized by malignant keratinocytes resembling normal squames which are found at locations outside the epidermis (see Figure 1.3.2). Studies on ETAF using this human keratinocyte cell line revealed a number of functional similarities with IL-1 (Sauder, 1984). Both were capable of mediating fever, stimulating muscle proteolysis, and stimulating hepatic synthesis of acute phase proteins. In addition to the similarities in biological activities between the two factors, there were also structural similarities. Like IL-1, ETAF was found to have an apparent molecular mass in the range of 15,000-25,000 as determined by gel filtration. In addition, the temperature and pH stabilities of the two factors was shown to be similar. Inactivation of ETAF activity by phenylglyoxal as assayed by the thymocyte assay, revealed that like IL-1, ETAF activity was inhibited. It was found, however, that a higher concentration of phenylglyoxal was needed to inactivate ETAF than was needed for IL-1. This last fact raised the possibility that ETAF may have some structural differences to monocyte-derived IL-1. Indeed, the finding that IL-1 was composed of at least

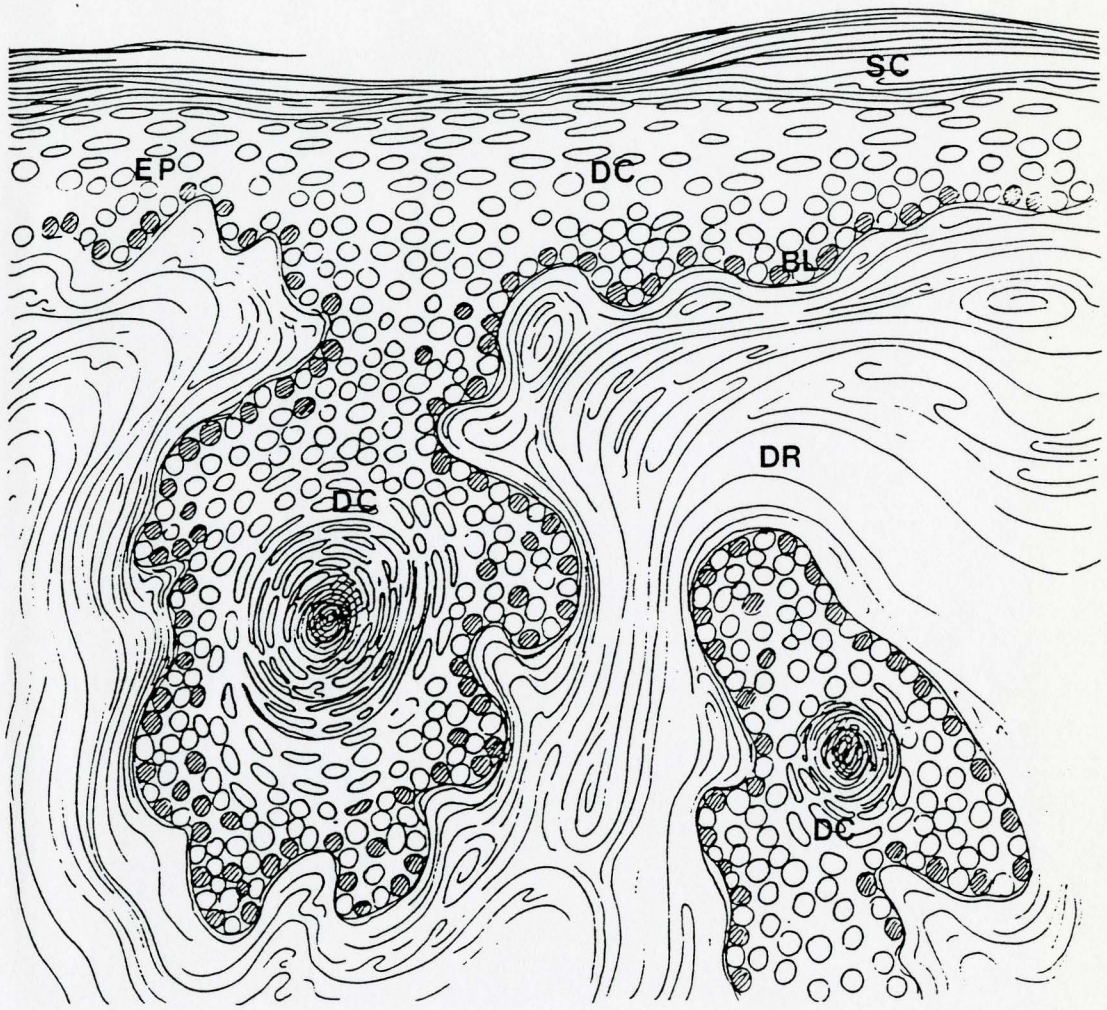

Fig. 1.3.2. Squamous cell carcinoma. This disease is characterized by a disruption in the normal organization of the skin's cellular structure, resulting in the presence of keratin squames at locations outside the epidermis. EP, epidermis; DR, dermis; SC, stratum corneum; BL, basal cell layer; DC, differentiating cells. Modified from Cairns (1975).

two distinct genes α and β, raised the question as to whether the keratinocyte-derived IL-1-like activity could represent a third member of this gene family.

A new opinion of the epidermis is emerging as a result of these studies. No longer considered just an inert barrier, the skin functions as the body's first line of defense against the external environment. Indeed, the skin is perfectly situated for this purpose since it is the outermost layer and at the same time is closely linked to the rest of the body via the vascular and nervous systems of the dermis. The importance of the skin having its own immune function is that it does not have to rely entirely on the components of the classical immune system thereby facilitating a more expedient response to a local threat. At the same time, however, through the use of its own soluble mediators, it can recruit an overall immune response by the body. Due to its newly-defined role, the skin is now considered to be an important, active interface between the external environment and the rest of the body, providing an immunological dialogue between the two.

1.4 SCOPE OF THIS WORK

The focus of this work has been on the molecular aspects of biological activities in the human keratinocyte which correspond to those attributed to the cytokine called interleukin-1 (IL-1). Previous studies had shown that keratinocytes produce a factor, named epidermal cell thymocyte activating factor (ETAF), with both functional and structural similarities to monocyte-derived IL-1. In the absence of any

purified ETAF protein, information on the gene responsible for ETAF activity was deemed necessary. The focus of this project was on the characterization and subsequent isolation of the gene(s) responsible for this IL-1-like activity in the keratinocyte cell line COLO 16. In undertaking this study, it was hoped that information could be gleaned on the extent of similarity between IL-1 and ETAF at the molecular level.

A major part of this thesis involved the cloning and sequencing of a partial cDNA sequence from the human keratinocyte cell line COLO 16 which is virtually identical to human monocyte-derived IL-1β cDNA sequence described in the literature. Although a complete cDNA clone was not obtained, it is a significant finding which directly demonstrates that this keratinocyte cell line produces mRNA which is virtually identical to IL-1β. Despite the fact that the 5' end of the gene is missing, when expressed, it is capable of manifesting at least some of the IL-1 bioactivities. This finding may be an important contribution to the growing literature on studies designed to dissect the IL-1β sequence in order to determine areas important for biological function.

The second major portion of this thesis describes the cloning and characterization of a second cDNA species isolated from COLO 16 cells which has, except for a small region of sequence identity, very little homology to human monocyte-derived IL-1β. The subsequent finding that this partial cDNA clone appears to possess at least some of the activities associated with IL-1 demonstrates that IL-1 may not be the only molecule that is responsible for IL-1-like activities in the

keratinocyte cell line. Alternatively, these findings could suggest that the IL-1 activities associated with the clone are due to the small region of homology to IL-1 found at the 3' terminus. This result again may provide further information on those regions of the IL-1 molecule which are important for biological activity.

MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 Chemicals

Specialty chemicals or reagents and their sources are listed below:

IPTG	Gibco/BRL
XGal	Gibco/BRL
Oligo d(T) cellulose	Collaborative Research
GeneClean	Bio/Can Scientific

2.1.2 Radiochemicals

[α-^{32}P]-dCTP and [α-^{32}P]-dATP (3000 Ci/mmol) were purchased from either Amersham or ICN Radiochemicals. [γ-^{32}P]-ATP (3000 Ci/mmol) was obtained from New England Nuclear via Dr. P. Branton, Department of Pathology, McMaster University. [^{35}S]-dCTP (500 Ci/mmol) was obtained from New England Nuclear.

2.1.3 Enzymes

Restriction endonucleases were obtained from several sources: Pharmacia, Gibco/BRL and New England Biolabs. Additional enzymes were purchased from various companies. Pharmacia: AMV reverse transcriptase, Mo-MLV reverse transcriptase, T4 polynucleotide kinase, T4 DNA ligase DNA polymerase I, DNA polymerase Klenow fragment and RNase H; Gibco/BRL:

T4 DNA ligase, S1 nuclease; Promega Biotech: EcoRI methylase. The Sequenase sequencing kit was purchased from United States Biochemical and the sequencing system later used was bought from Pharmacia.

2.1.4 Cloning Vectors and Host Strains

The λgt10 and λgt11 vectors (digested and dephosphorylated arms), Y1090, C600 and C600 Hfl host cells, and packaging extracts were obtained from Promega Biotech. The pUC118/119 vectors and MV1193 host cells (Viera and Messing, 1987) were obtained from Dr. A.B. Futcher, Department of Biochemistry, McMaster University. The bacterial expression vector pKK233-2 and host cells were obtained from Pharmacia. The mammalian expression vector pXM (Yang et al, 1986) was kindly provided by Dr. G. Wong, Genetics Institute, MA.

2.1.5 Oligonucleotides

The list of oligonucleotides used in this project are given in Appendix B. A number of the oligonucleotides were purchased from the Central Facility of the Institute for Molecular Biology and Biotechnology, McMaster University. These include: oligo-$d(T)_{18}$, and all of those with names beginning AB. The murine IL-1α and human IL-1β oligonucleotides were synthesized using the SAM ONE system (use of the equipment was kindly provided by the Department of Biology, McMaster University).

2.1.6 cDNA Probes

The partial human IL-1β clone (pA26) and the full length human

IL-1β cDNA contained in an Okayama-Berg vector (pcD1218) was kindly provided by P.E. Auron, Massachussetts Institute of Technology, MA. The actin probe consisting of a plasmid containing a 2000 bp chicken β-actin cDNA insert was obtained from D. Cleveland (Cleveland et al, 1980).

2.1.7 Antisera and Synthetic Peptides

A rabbit serum which precipitates human IL-1α was kindly provided by Dai Nippon Pharmaceuticals Ltd., Japan. An anti-human IL-1β rabbit antiserum was provided by Cistron Technology, Piebrook, N.J. Three synthetic peptides corresponding to short regions of the F8 open reading frame and antiserum generated against these peptides were kindly provided by Hoffmann-La Roche, Nutley, N.J. A goat anti-rabbit alkaline phosphatase conjugate was purchased from Bio-Rad. A protein A horseradish peroxidase conjugate was kindly provided by Dr. B. Underdown, Department of Immunology, McMaster University.

2.2 BACTERIAL CULTURE AND MANIPULATION

Bacterial cells were grown in standard culture media (YT or LB) at 37°C in a rolling drum incubator. When necessary, antibiotics were added to the media at concentrations specified in Maniatis (1982). Liquid cultures containing bacterial cells were stored at 4°C for up to one month. For prolonged storage, bacterial cultures were grown to stationary phase (14-18 h), adjusted to contain 15% glycerol (v/v) and stored in 1 ml aliquots at -70°C. Transformation of bacterial cells was performed using either commercially prepared competent cells (BRL) or

cells made competent following the procedure of Hanahan (1985).

2.3 BACTERIOPHAGE CULTURE AND MANIPULATION

2.3.1 λgt11

λgt11: predigested λgt11 arms, packaging extracts and host Y1090 cells were obtained from Promega Biotech. Viral stocks were stored and diluted in SM buffer (0.1 M NaCl, 0.01 M $MgSO_4$ $7H_2O$, 0.05 M Tris-HCl, pH 7.5, 0.01% gelatin). Packaging of the ligation mixture containing DNA inserts and λgt11 arms into viral particles was performed following the guidelines provided by Promega. Briefly, a portion of the ligation mix not exceeding 10 µl was added to a packaging extract (50 µl) and incubated at room temperature for 2 h. To this was then added 500 µl of SM buffer and 25 µl of chloroform. The resulting λgt11 library was titred as follows: various ten-fold dilutions of the library were made in SM buffer and 10 µl of each dilution was added to 200 µl of freshly prepared Y1090 plating cells which had been grown to an OD_{600} of approximately 0.7 in YT broth containing 50 µg/ml Ampicillin, 0.2% maltose, 0.01 M $MgSO_4$. This mixture was incubated at 37°C for 30 min after which it was added to 3 mls of top agar containing 0.05% XGal, 0.5 mM IPTG and plated onto YT agar plates containing 50 µg/ml Ampicillin. Plates were incubated at 37°C for 7-8 h and then stored at 4°C. In order to quantitate the percentage of recombinants, the ratio of recombinant plaques (clear) to nonrecombinant plaques (blue) was determined for each plate, (in most cases, the percentage of

recombinants was between 85-95%). The total number of recombinants represented in a library was determined from the number of recombinants on selected plates, correcting for the dilution involved. The titre of the library was determined similarly except that the total number of plaques on the plate (both recombinant and nonrecombinant) were used in the calculation.

In order to screen a particular λgt11 library and isolate an individual bacteriophage clone, the λgt11 library was plated at a density of 10,000-15,000 plaques/90 mm plate or 50,000 plaques/150 mm plate following the same procedure as outlined for titering the phage above except for the omission of the XGal and IPTG. Once plaques were visible, plates were stored overnight at 4°C prior to doing plaque transfers. It was found that this last step helped in preventing the top agar from adhering to the nitrocelluose filter and peeling off of the plate during the plaque transfer step. Plaques were transferred in duplicate to nitrocellulose filters following a previously described procedure (Maniatis, 1982, p 321). Filters were then baked <u>in vacuo</u> for 2 h at 80°C and hybridized with the appropriate probes according to outlined procedures (see Radioactive Labelling and Hybridization of DNA Probes). Potential positive clones were plaque purified as follows: The positive signal on the X-ray film was aligned over the original plate and a plug of agar approximately 1 cm in diameter was removed from the area corresponding to the positive signal. The agar plug was placed in 1 ml of SM buffer containing two drops of chloroform and left at room temperature for at least 2 h to allow the phage to elute. The supernatant containing the eluted phage was titered as described

previously and then plated at a plaque density of approximately 1000 plaques/90 mm plate for secondary screening. Following plaque transfer and hybridization with desired probe it was usually possible to pick an isolated positive clone. For this purpose, a sterile pasteur pipette was used in order to isolate the smallest plug possible and thus avoid contamination by adjacent plaques. The viral particles were eluted from the plug as described above. Occasionally, a tertiary screening was necessary in order to isolate an individual plaque.

Once a single clone had been isolated, a culture was grown in order to amplify the virus and isolate DNA for subcloning into bacterial vectors for further manipulation. Small amounts (approximately 5 µg) of relatively pure DNA was isolated following the procedure of Davis et al (1986). A fraction of the DNA was then digested with EcoRI and electrophoresed on 1% agarose/1xTBE gels to determine the size of the inserted fragment. The insert DNA was isolated from the gel using either electroelution (Maniatis et al, 1982) or the product GeneClean (Bio/Can Scientific) incorporating the method of Vogelstein and Gillespie (1979). The insert DNA was quantitated spectrophotometrically following standard procedures and ligated into the appropriate bacterial vector.

2.3.2 λgt10

λgt10: predigested λgt10 arms, packaging extracts and the two bacterial strains C600 and C600 Hf1 (high frequency lysogeny) were obtained from Promega Biotech. Packaging of the ligation mixture containing DNA fragments and λgt10 arms was performed following the

procedures provided by Promega and outlined in the λgt11 section. The titering of λgt10 libraries was performed similarly to that of λgt11 except that the Y1090 cells were replaced with C600 and C600 Hf1 (high frequency lysogeny) cells. The C600 Hf1 cells were used to plate the λgt10 library for screening purposes as only the recombinant phage are able to lyse the cells efficiently to form plaques. Infection of these cells with nonrecombinant wildtype phage results in efficient and virtually complete lysogeny therefore no plaques are formed. The recombinant phage are unable to lysogenize because the presence of the DNA insert disrupts the portion of the viral genome necessary for the formation of lysogens. The C600 cells were used for the determination of the ratio of recombinants/nonrecombinants for a given library as λgt10 does not possess the blue/white selection system of λgt11. Infection of these cells with a mixture of recombinant and nonrecombinant phage allows for the distinction between the two as the former produce clear plaques while the latter are able to lyse a small percentage of the cells resulting in turbid plaques.

2.4 MAMMALIAN CELL CULTURE

The human skin carcinoma cell line COLO-16 and the monkey COS-1 cell line were cultured in Dulbecco's modified minimal Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) as described (Baumann et al, 1984; Rosenwasser et al, 1986). FBS (Gibco/BRL) was heat inactivated at 56°C for 1 h. Normal human breast keratinocytes (NHEK) were obtained from Clonetics and grown in modified MCDB153 medium

purchased from Clonetics. For some experiments, cells were stimulated overnight (18 h) with 5 ng/ml phorbol myristate (PMA). Monocytes were prepared from human peripheral blood by Ficoll-Hypaque centrifugation and plastic adherence following a 1 h incubation in culture (Baumann et al, 1984). Stimulated monocytes were prepared by treating the adherent cells for 4 h with bacterial lipopolysaccharide (LPS) (10 µg/ml) (Baumann et al, 1984). HL60 cells were cultured in RPMI 1640 containing 20% FBS (Gallagher et al, 1979). Differentiation along the monocyte lineage was induced by incubation with 10 nM 12-O-tetradecanoylphorbol-13-acetate (TPA) for 48 h. Fibroblasts were prepared by placing a small section of skin biopsy (approximately 1 mm) under a coverslip and culturing in DMEM containing 10% FBS. After 2-3 weeks, explants were surrounded by a dense outgrowth of fibroblasts. Subcultures were established and used for RNA preparations. All lines were grown at 37°C in a humidified incubator which maintained a 5% CO_2 atmosphere. Cells were stored in liquid nitrogen in 1 ml vials (NUNC) at a density of $5x10^6$ cells/ml in their appropriate media containing 10% (v/v) FBS and 10% (v/v) DMSO.

2.5 ANALYSIS OF NUCLEIC ACIDS

2.5.1 Radioactive Labelling and Hybridization of DNA Probes

Oligonucleotide probes were 5' end labelled under the following conditions: 6 pmol of oligonucleotide in 0.05 M Tris-HCl (pH 8.0), 0.01 M $MgCl_2$, 5 mM dithiothreitol, 10 µCi [γ^{32}P]-ATP (3000 Ci/mmol), 6 U of T4 polynucleotide kinase in a total volume of 30 µl for 1 h at 37°C.

Radionucleotide incorporation was estimated by spotting 1 µl of the reaction mix onto DE81 paper, washing with 5 x 50 ml of 0.1 M Na_3PO_4, 10 mM $Na_4P_2O_7$ and determining Cerenkov counts. Typical Cerenkov counts were 10^6 cpm/pmol of oligonucleotide. cDNA probes were labelled following the random primer method (Feinberg and Vogelstein, 1983; 1984). Plasmid vectors containing the cDNA probes were digested with appropriate restriction enzymes to either linearize within the vector portion of the construct or release the cDNA insert from the vector. The latter method was often employed to eliminate false positive signals due to hybridization of the vector with target sequences as in the case of bacterial colony hybridization. Digested DNA was electrophoresed in 1% agarose/0.5xTBE gels and desired bands were excised and isolated either by electroelution in dialysis bags (Maniatis et al, 1982) or by the method of Vogelstein and Gillespie (1979) which employed the Gene-Clean kit (Bio/Can Scientific). DNA was then labelled as follows: 50 ng of DNA fragment in 20 µM dCTP, dGTP, and dTTP, 20 mM Tris-HCl (pH 7.5), 5 A_{260} units/ml of $(dN)_6$, 50 µCi [α^{32}P]-ATP (3000 Ci/mmol), 5 U of Klenow fragment in a total volume of 50 µl for 16 h at 20°C. Reactions were adjusted to a final volume of 100 µl by the addition of 50 µl of TE pH 7.5 and labelled probe was separated from unincorporated radionucleotide by centrifugation through a 1 ml Sephadex G-50 spin-column preequilibrated with TE pH 7.5 (Maniatis et al, 1982). Typical Cerenkov counts were 10^8-10^7 cpm/µg fragment.

Nitrocellulose filters were prehybridized in sealed bags under conditions appropriate for the particular probe being used. Labelled probes were denatured by boiling for 5 min and added to the

prehybridization solution to give a final probe concentration of $5x10^5$ cpm/ml. For oligonucleotides other than $(dT)_{18}$, prehybridization was for at least 1 h in 5 x SSC, 1 x P wash, 5 x Denhardt's at 48°C, followed by hybridization for 14-18 h. Filters were washed twice in 2 x SSC at room temperature for 5 min and twice in 1 x SSC at 48°C for 10 min. For $(dT)_{18}$ oligonucleotide, prehybridization was for at least 1 h in 5 x SSC, 1 x P wash, 5 x Denhardt's at room temperature, followed by hybridization for 4-6 h. Filters were washed in 1 x SSC at room temperature, 4 x 5 min. Conditions for cDNA probes were as follows: prehybridization was for at least 3 h in 5 x SSC, 50% formamide, 0.5 x P wash, 5 x Denhardt's, 250 µg/ml sheared and denatured salmon sperm DNA, 0.1% SDS at 48°C, followed by hybridization for 14-18 h. Filters were washed twice in 2 x SSC, 0.1% SDS for 10 min at room temperature, then twice in 0.1 x SSC, 0.1% SDS for 30 min at 48°C.

After washing, filters were air dried on 3 MM paper, wrapped in plastic film and exposed for the desired length of time using either Kodak XAR or XRP film at -70°C with a Dupont Lightning or Cronex intensifying screen. After the appropriate exposures had been achieved, the probes were removed from the filters by washing twice in 0.1 x SSC, 0.1% SDS at 95-100°C for 10 min.

2.5.2 Synthesis and Purification of the hIL-1β Oligonucleotide

A human IL-1β oligonucleotide based on published sequence was synthesized on the SAM ONE system (Biosearch) in the Biology Department, McMaster University. The blocking groups were removed and the oligonucleotide was cleaved from the solid support following the

procedure outlined in the SAM ONE manual. The silica support containing the oligonucleotide was first removed from the column and placed in a 1.5 ml microcentrifuge tube. The support was washed twice with dry acetonitrile and then treated with 1 ml of freshly prepared solution containing 0.3 M 1,1,3,3-tetramethylguanidine and 0.3 M 2-pyridine aldoxime in dry acetonitrile for 20 min at room temperature. The sample was centrifuged 2 min at 12,000xg and the supernatant containing the blocking groups was discarded. The oligonucleotide was then removed from the support by treating with 1 ml of concentrated NH_4OH for 5 h at 55°C. After centrifugation at 12,000xg for 2 min, the supernatant was divided into two microcentrifuge tubes and evaporated in a rotary evaporator. The dry product was resuspended in a total of 1 ml of H_2O.

In order to assess the purity of the 24-base nucleotide, a small portion of the material was radioactively labelled and subjected to electrophoresis. Briefly, 0.5 µl of oligonucleotide solution was kinased in 0.05 M Tris-HCl pH 8.0, 0.01 M $MgCl_2$, 5 mM dithiothreitol, using 10 µCi [γ^{32}P]-ATP (3000 Ci/mmole) and 6 U T4 polynucleotide kinase for 1 h at 37°C followed by electrophoresis on a 20% acrylamide/ 8 M urea gel which was autoradiographed for 1-2 h at -70°C. Purification of the crude product involved electrophoresis of the oligonucleotide mixture on a 20% acrylamide/ 8 M urea gel and visual detection of the separated species by the "shadow-cast" method. Briefly, the gel containing the electrophoresed material is placed over a fluorescent screen (DuPont "Cronex") and subjected to ultraviolet (short wave) irradiation from above. Absorption of the ultraviolet light by the DNA in the gel results in a lack of fluorescence on the screen directly

below the location of the DNA in the gel. The topmost shadow, corresponding to the 24-base oligonucleotide, was then outlined on the gel and this area of the gel was excised. The gel slice was placed in a 1.5 ml microcentrifuge tube and ground to a fine powder with a plastic rod. To this was then added 500 µl of 1 M NH_4HCO_3 and the sample was incubated at 50°C for 10 min followed by incubation for 16 h at room temperature. The purified oligonucleotide was then desalted by passing the supernatant over a Sephadex G-50 spin column equilibrated with 0.1 M NH_4HCO_3. The eluant was lyophillized and the dried product was resuspended in 100 µl of sterile distilled H_2O. Purified oligonucleotide was radioactively end-labelled, as described above, and its purity determined by electrophoresis (see Figure 2.5.1).

2.5.3 RNA Analysis

Total RNA was prepared from tissue or cell culture by the method of Chirgwin et al (1979), following the modifications of Rachubinski et al (1985). Pellets containing up to $8x10^8$ cells were resuspended by vigorous agitation in 2 ml of 4M guanidinium isothiocyanate, 0.25mM sodium citrate (pH 7.0), and 0.1M 2-mercaptoethanol. This suspension was then layered onto 2.5 ml of 5.7M cesium chloride and centrifuged in a Sorvall TST 41.4 rotor at 27,000 rpm or in a Beckman SW 50.1 rotor at 30,000 rpm for 16-19 h at 20°C. The supernatant was removed by aspiration and the tubes were allowed to drain in an inverted position. The remaining film of CsCl was removed from the walls using a sterile cotton swab. Pellets were dissolved in 100 µl of 0.5% SDS and transferred to 1.5 ml Eppendorf tubes. Samples were heated at 56°C for

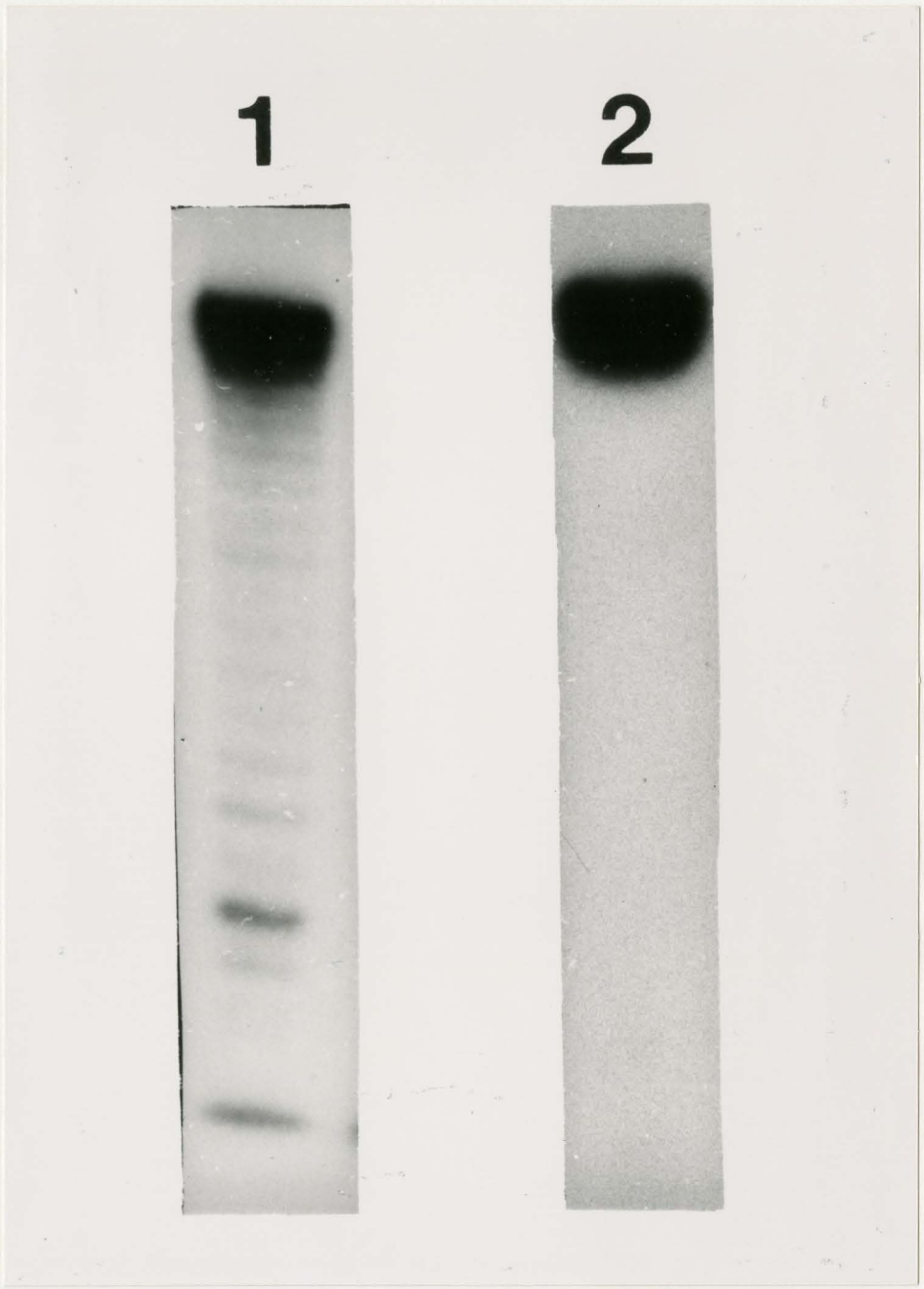

Fig. 2.5.1. Purification of the human IL-1β oligonucleotide. Autoradiograms of kinased oligonucleotide before (lane 1) and after (lane 2) purification. Electrophoresis was performed on a 20% acrylamide/7 M urea gel at 700 V. In both cases, 0.1% of the total sample was kinased and subsequently electrophoresed. Exposure conditions for the autoradiograms were: 1, 1h at -70°C with intensifying screen; 2, 1.5h at -70°C with intensifying screen.

5 min, chilled rapidly on ice to room temperature, and then centrifuged in an Eppendorf microfuge for 5 min at room temperature. RNA was precipitated by addition of 260 µl of distilled H_2O, 40 µl of 2M potassium acetate (pH 5.5) and 1 ml of cold (-20°C) absolute ethanol. After incubation at -20°C for at least 30 min, RNA was pelleted by centrifugation for 10 min at 12,000x g, 4°C. Pellets were washed with cold 70% ethanol, dried briefly under vacuum and then resuspended in 50-500 µl of sterile distilled H_2O. Average yield was 3-5 µg of RNA per 10^6 cells.

Poly(A)+ RNA was isolated by oligo-dT cellulose chromatography following a modified procedure of Maniatis et al (1982). Typically, 1-2 mg of total RNA was chromatographed on a 0.2 ml oligo-dT cellulose column in a sterile 1 ml syringe plugged with siliconized glass wool. Total RNA was diluted to a concentration of 2 µg/µl and sufficient 0.5M EDTA was added to achieve a final concentration of 1mM. Samples were heated at 70°C for 1 min and cooled rapidly on ice to room temperature. The RNA solution was then adjusted with concentrated stock solutions to equal the composition of the binding buffer: 0.5M NaCl, 10mM Tris-HCl (pH 7.5), 1mM EDTA, 0.5% SDS, and applied to the column which had been pre-equilibrated with binding buffer. The effluent was collected, heated at 70°C for 1 min and reapplied to the column. The column was then washed with 20 column-volumes of binding buffer and the poly(A)+ RNA was then eluted with elution buffer: 10mM Tris-HCl (pH 7.5), 0.2% SDS, 1mM EDTA. Fractions of approximately 250 µl were collected into Eppendorf tubes and the RNA was precipitated by adding 0.04 volumes of 5M potassium acetate and 2.5 volumes of cold absolute ethanol and

incubating at -20°C for 30 min. Poly(A)+ RNA was pelleted by centrifugation at 12,000x g for 10 min at 4°C, washed with cold 70% ethanol, dried and then resuspended in 10-20 µl of sterile distilled H_2O. Recovery was approximately 2% of initial quantity of total RNA chromatographed.

Pretreatment of the nitrocellulose membrane for all types of blots involved floating the membrane on H_2O until fully wetted and then soaking in 10x SSC for approximately 30 min. The conditions for dot and slot blot were identical and as follows: 3-5 µg of total or poly(A)+ RNA was made up to 50 µl in 10mM Tris-HCl (pH 7.5), 1mM EDTA and an equal volume of 12x SSC/ 15% formaldehyde was added. Samples were then heated at 60°C for 15 min prior to being applied to the nitrocellulose membrane (Schleir and Schuell or Gelman Sciences) under gentle suction. For Northern blots, 5-10 µg of total or poly(A)+ RNA was prepared in 6% formaldehyde, 0.02 M sodium borate, 0.2 mM EDTA in a total volume of 20-24 µl and heated at 70°C for 10 min prior to being electophoresed on 1% agarose/6.6% formaldehyde gels at 80 mA for 4-5 h. The RNA was then transferred to nitrocellulose in 10 x SSC for 16-24 h using the wick method (Maniatis et al, 1982). After RNA application or transfer, nitrocellulose was baked in vacuo at 80°C for 2 h.

2.5.4 DNA Analysis

Plasmid DNA was isolated from bacterial cells by two methods. For small scale applications, the alkaline lysis miniscreen method (Birnboim and Doly, 1979) as modified by Rommens et al, (1983) was used. For large scale preparations, DNA was isolated according to the method

of Pulleyblank et al, (1983).

For Southern analysis, 0.5-1.0 µg (for recombinant DNA) or 10 µg (for genomic DNA) of DNA digested with the appropriate restriction enzyme was electrophoresed on 1% agarose/1xTBE gels and transferred to nitrocellulose membranes following the wick method (Maniatis et al, 1982). Blots were then hybridized with the appropriate probe as described in section 2.5.1.

2.5.5 Primer Extension Studies

First-strand cDNA was synthesized from 1.0 µg poly(A)+ RNA with 40 U AMV reverse transcriptase (Pharmacia) using 8 pmol of labelled human IL-1β oligonucleotide as a primer in 2 mM methyl mercury, 20 mM mercaptoethanol, 1 mM vanadyl ribonucleoside complex, 100 mM Tris-HCl pH 8.3 at 48°C, 140 mM KCl, 10 mM $MgCl_2$, and 0.5 mM each deoxynucleoside triphosphate (dNTP), at 42°C for 2 h. Transcripts were denatured at 90°C for 5 min in 50% formamide and analysed on 5% polyacrylamide, 8 M urea gels (Maniatis et al, 1982).

2.6 CLONING AND SEQUENCING

2.6.1 Construction of cDNA Libraries

Two different methods of cDNA synthesis were employed over the course of this work to construct cDNA libraries. The initial library in pBR322 was constructed using cDNA synthesized following the procedure of Rachubinski et al, (1985), which is a modification of Wickens et al, (1978). First strand cDNA was synthesized from 10 µg denatured poly(A)+

RNA with 50 U AMV reverse transcriptase using 200 pmol human IL-1β oligonucleotide as primer in 50 mM Tris-HCl pH 8.3 at 42°C, 100 mM KCl, 6 mM $MgCl_2$, 10 mM dithiothreitol, 1 mM of each dNTP at 42°C for 1 h. The mixture was then boiled for 2 min, quick-cooled on ice, and the second strand synthesized using 30 U DNA polymerase I in 0.2 M HEPES, 0.07 M KCl, 12 mM $MgCl_2$, 20 mM dithiothreitol and 34 mM of each dNTP at 15°C for 4 h. Double-stranded cDNA was digested with 2000 U S1 nuclease in 50 mM sodium acetate pH 4.5, 0.2 M NaCl, 1 mM $ZnCl_2$ for 30 min at 37°C, and purified by phenol extraction and ethanol precipitation. The resulting double-stranded cDNA was resuspended in 300 µl of sterile distilled H_2O and a fraction (1/12) of this cDNA was dC-tailed with 27 U terminal deoxynucleotidyl transferase in 0.2 M potassium cacodylate, 0.5 M dCTP, 1 mM $CoCl_2$ for 15 min at 15°C. The reaction was stopped by the addition of EDTA to a final concentration of 5 mM and purified by phenol extraction and ethanol precipitation. The dC-tailed cDNA was resuspended in 25 µl of sterile distilled H_2O and half of this amount was added to 0.5 µg dG-tailed PstI-cut pBR322. The mixture was heated to 70°C and then allowed to cool slowly to 4°C. Various dilutions of the annealed mixture were used to transform competent LE 294 cells and plated onto YT agar plates containing 15 µg/ml tetracycline. Resulting colonies were replica plated onto another plate containing tetracycline as well as a plate containing 50 µg/ml ampicillin in order to distinguish those colonies containing cDNA inserts from those containing parent vector alone. In conjunction with this, colony hybridization with the human IL-1β oligonucleotide was performed on the tetracycline plates in order to isolate clones containing sequences homologous to IL-

1β. The method followed was a modification of Hanahan and Meselson (1980) and Woller (1983). Colonies were transferred onto nitrocellulose discs in duplicate and treated as follows: 2x 2 ml 0.5 M NaOH, 3 min; 2x 2 ml 1 M Tris-HCl pH 7.6, 3 min; 2x 2 ml 1.5 M NaCl, 0.5 M Tris-HCl pH 7.4, 3 min; air dried 6 min; 1x 2 ml 0.3 M NaCl, 5 min

The solution for each step was placed onto a piece of plastic film and the nitrocellulose filter was floated on top of the solution for the required time. Filters were then dried in vacuo and treated for hybridization with the oligonucleotide probe as described in section 2.5.1.

All subsequent libraries were constructed with cDNA synthesized following the procedure of Sartoris et al, (1986). Using this method, 5 µg of poly(A)+ RNA was mixed with 0.5 µg of oligo $(dT)_{18}$ in H_2O to a final volume of 20 µl. The mixture was boiled for 2 min and then quickly frozen at -70°C. Once thawed, first strand synthesis was performed under the following conditions: 50 mM Tris-HCl pH 7.5, 75 mM KCl, 3 mM $MgCl_2$, 10 mM dithiothreitol, 2 mM each of dATP, dCTP, dGTP, dTTP, 0.1 µg/µl bovine serum albumin, 400 U M-MuLV reverse transcriptase, 37°C for 30 min. Another 400 U of M-MuLV reverse transcriptase was added and reaction was incubated a further 30 min at 37°C. Second strand synthesis was then performed on this mixture after adjusting to the following conditions: 25 mM Tris-HCl pH 7.5, 100 mM KCl, 6.5 mM $MgCl_2$, 5 mM dithiothreitol, 10 mM $(NH_4)_2SO_4$, 1 mM each of dATP, dCTP, dGTP, dTTP, 0.05 µg/µl bovine serum albumin, 1 U RNase H, 20 U DNA polymerase I. The reaction was incubated at 14-16°C for 5 h and

double-stranded cDNA was purified by phenol/ chloroform extraction and ethanol precipitation. To monitor the first and second strand synthesis, radioactive pilot reactions were performed in which 1 µCi [α^{32}P]-ATP (3000 Ci/mmole) was added to the first strand reaction mixture. Prior to the adjustment for the second strand conditions, 2 µl of the first strand reaction was removed and diluted to 10 µl with H_2O. After second strand reaction was complete, 2 µl of this mixture was also removed and diluted to 10 µl with H_2O. These pilot reactions were analysed by electrophoresing 5 µl of each of the diluted reaction mixtures on 5% polyacrylamide/ 8M urea gels at 500V. Gels were autoradiographed for 16 h at room temperature.

2.6.2 Construction of Genomic Libraries

Peripheral lymphocyte DNA was isolated as described in the section for Southern blotting. The DNA (100 µg) was digested with EcoRI in 20 mM Tris-acetate, 20 mM magnesium acetate, 100 mM potassium acetate, 200 U of EcoRI in a total volume of 400 µl for 21 h at 37°C. A portion of the digest (10 µl) was electrophoresed on a 1% agarose/0.5xTBE gel in order to check for completion. The entire digest was then heated to 68°C for 5 min to eliminate reannealed ends, layered onto a 5%-40% sucrose gradient and centrifuged at 26,000 rpm at 20°C for 24 h. The gradient was fractionated into approximately 150 µl fractions and the fragment size range for every fraction was determined by electrophoresis on 1% agarose/0.5xTBE gels. Fractions containing the greatest number of fragments in the 1.7 kbp range were pooled and diluted with distilled H_2O to obtain a solution with a final

concentration of less than 10% sucrose. DNA was then precipitated by addition of one tenth volume of 3 M sodium acetate and 2.5 volumes of cold (-20°C) absolute ethanol. DNA was recovered by centrifuging at 9,000 rpm for 30 min at 4°C. The pellet was washed with cold (-20°C) 70% ethanol and then resuspended in 80 µl of 10 mM Tris-HCl pH 7.5, 1 mM EDTA.

A small portion of the genomic DNA (approximately 50 ng) was combined with 1 µg of EcoRI digested λgt11 arms and ligated under standard conditions: 10 mM Tris-HCl, pH 7.9, 10 mM $MgCl_2$, 1 mM dithiothreitol, 1 mM ATP, 0.1 mg/ml BSA, 1 U T4 DNA ligase, 16-18 h at 4°C. The entire ligation mixture was then packaged into λgt11 and titred as described (see section 2.3)

2.6.3 DNA Sequencing

Double-stranded sequencing: Double-stranded plasmid DNA for sequencing was isolated from bacterial culture following the alkaline lysis miniscreen method (Birnboim and Doly, 1979). It was found that template isolated by this method was far superior to that obtained by large scale methods. Many different conditions for double-stranded DNA sequencing were tried over the course of this work. Originally, the method of Zagursky et al (1985) was employed with a variable success rate. This was replaced by the method of Zhang et al (1988) which used a modified T7 DNA polymerase (Tabor and Richardson, 1987) supplied in the Sequenase kit (United States Biochemical), using supercoiled DNA as template.

Single-stranded sequencing: Single-stranded DNA was obtained for

sequencing using the phagemid system of Viera and Messing (1987). DNA to be analyzed was inserted into either pUC118 or pUC119 and used to transform MV1193 host cells. Cells containing the plasmid were then infected with M13K07 phage and single-stranded template was isolated as follows: Cultures were inoculated and grown in 2xYT broth containing 150 µg/ml ampicillin until reaching an optical density of 0.05-0.10 at 600 nm. Cells were then infected with M13K07 at an m.o.i. of approximately 10 and allowed to continue growing for 1.5 h. Cultures were diluted 1:4 into media supplemented with 70 µg/ml kanamycin and grown for an additional 14-18 h to select for infected cells. Phage were then harvested and single-stranded DNA was isolated following the procedure of Davis et al (1986). The quality and quantity of single-stranded template was assessed by electrophoresis on 1xTBE/1%agarose gels.

For both methods, either ^{35}S or ^{32}P were used and reactions were electrophoresed on 6% or 8% acrylamide/7 M urea gels at 1200-1600 V for desired lengths of time.

2.7 PROTEIN ANALYSIS

Cell lysates were prepared from approximately $3x10^7$ cells by resuspending pelleted cells in an equal volume of 2x Laemmli loading buffer (Laemmli, 1970) and boiling for 10 min. After centrifugation at 12,000xg for 5 min to remove cellular debris, approximately 100 µg of soluble material was loaded onto 0.4% SDS/15% polyacrylamide gels. Electrophoresis was performed using the Bio-Rad "mini-protean" system

for 45 min at 200 V. Gels were then equilibrated for 15 min in transfer buffer (25 mM Tris, 192 mM glycine, 20%(v/v)methanol, pH 8.3) prior to electrophoretic transfer to nitrocellulose membrane, using a Bio-Rad miniblot apparatus (18 h at 30 V, 4°C). After transfer, the lane containing the molecular weight markers (Pharmacia) was cut off and stained 5-10 min in 0.2% amido black in 7% acetic acid , followed by a brief destaining in 30% methanol/10% acetic acid. The remainder of the nitrocellulose blot was blocked with blocking buffer consisting of tris-buffered saline (TBS, 10 mM Tris-HCl, pH 7.5, 170 mM NaCl) containing either 1%(w/v)hemoglobin or 0.5%(w/v)non-fat dry milk, for 2-3 h at room temperature. Blots were then cut into strips and placed in test tubes for probing with primary antisera. Appropriate dilutions of the various antisera were made in blocking buffer and 1-2 ml was added to each tube. Tubes were rocked at room temperature for 3-5 h and then washed with TBS for 1 h. Immunoreactive proteins were visualized by incubating in blocking buffer containing either a protein A horseradish peroxidase conjugate (1:200 dilution) or a goat anti-rabbit alkaline phosphatase conjugate (1:3000 dilution) for 3-16 h at room temperature. Blots were washed in TBS for 1 h prior to colour development following the procedure specific for each conjugate as outlined in Harlow and Lane (1988).

2.8 EXPRESSION OF F8 SEQUENCE

2.8.1 Indirect Expression in Xenopus Oocytes

The F8 clone was initially assayed for biological activity using

hybrid select/oocyte injection. Briefly, plasmid DNA containing the clone of interest was denatured, fixed to nitrocellulose and then hybridized with poly(A)+ RNA from COLO-16 cells. The hybrid-selected mRNA was then microinjected into Xenopus laevis oocytes. Fifty nanolitres of mRNA (dissolved in sterile distilled H_2O at a concentration of 0.5-1 mg/ml) was injected into each oocyte which had been previously treated with trypsin to remove the follicle. After injection, the oocytes were placed at 4°C for 45 min, then transferred to a microtitre plate and incubated in fresh oocyte medium for 18 h at 23°C. After incubation, oocytes were harvested, the supernatant concentrated 10x and chromatographed on a TSK-2000 size exclusion HPLC column to remove an IL-1 inhibitor produced by the oocytes. The material was then tested for IL-1-like activity using the thymocyte co-stimulator assay described in the Appendix.

2.8.2 Direct Expression in Prokaryotic Systems

Initially, attempts were made to insert the F8 open reading frame into the bacterial expression vector pKK233-2 (Figure 2.8.1). The F8 open reading frame was excised from the pBR322 clone (pBF8) in a two step process. Firstly, the clone was digested with the restriction endonuclease Ssp I of which there were two sites, one within the F8 sequence itself and one within the pBR322 vector. The SspI site within the F8 sequence is located 21 bases from the proposed initiation codon for the open reading frame. Upon digestion with SspI, the majority of the F8 sequence needed for expression studies would be located on a

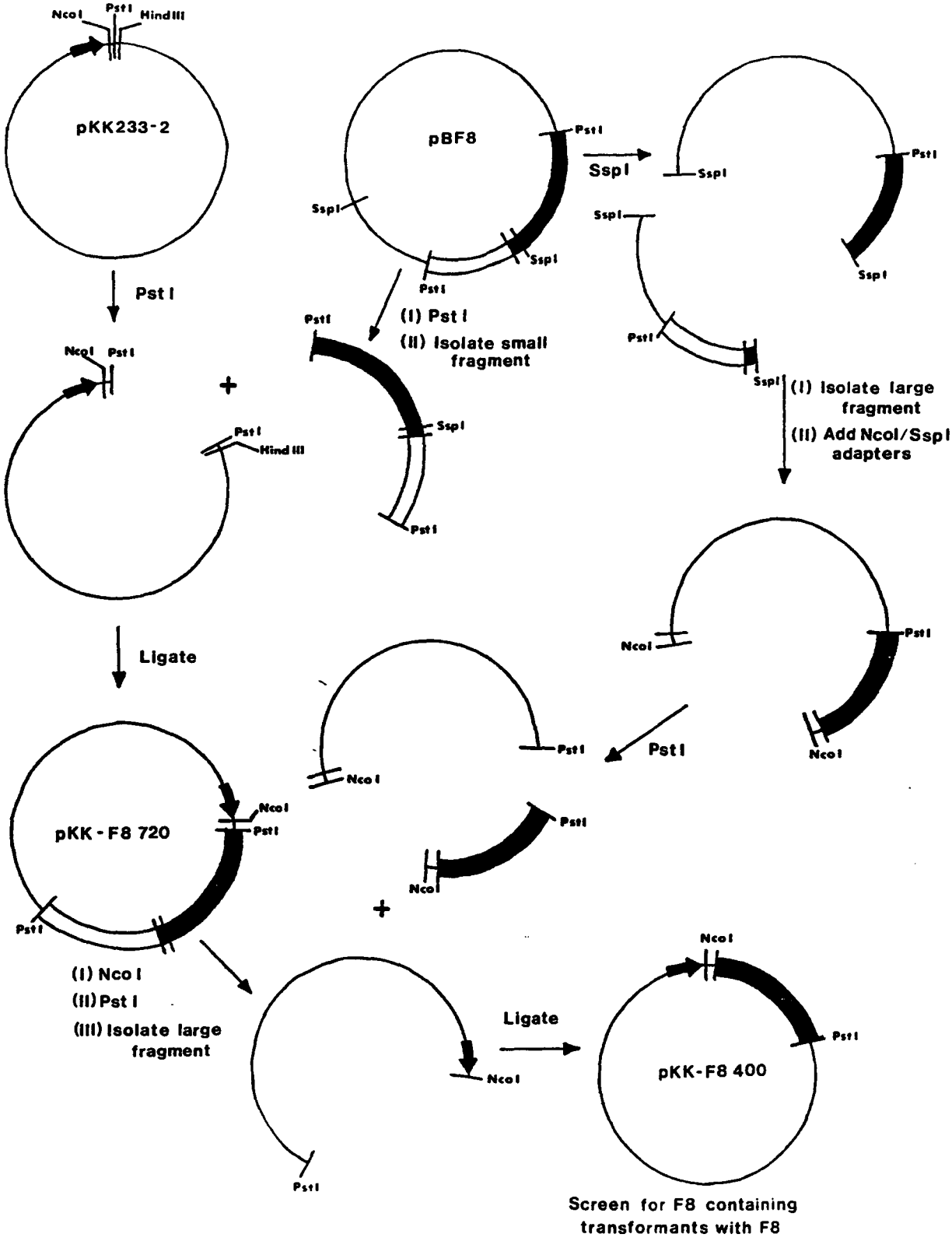

Fig. 2.8.1. Initial cloning strategy for the expression of the F8 open reading frame in bacterial cells.

4.1kbp fragment. After isolation of this fragment using GeneClean, an SspI/NcoI adaptor would be ligated onto the fragment which would regenerate the 5' end of the F8 open reading frame. This would then be digested with PstI to release the F8 open reading frame from the remainder of the vector sequence. This could then be ligated into pKK233-2.

The revised strategy for inserting the F8 open reading frame into pKK233-2 is outlined in Figure 2.8.2. This strategy was developed in an attempt to reduce the number of purification steps involved thereby increasing the yield of the final product and increasing the possibility of obtaining a transformant.

The cloning scheme devised for the insertion of the F8 open reading frame into the alternate bacterial expression vector pRIT2T is identical to that outlined in Figure 2.8.1. The vector-insert junction region is shown in Figure 2.8.3.

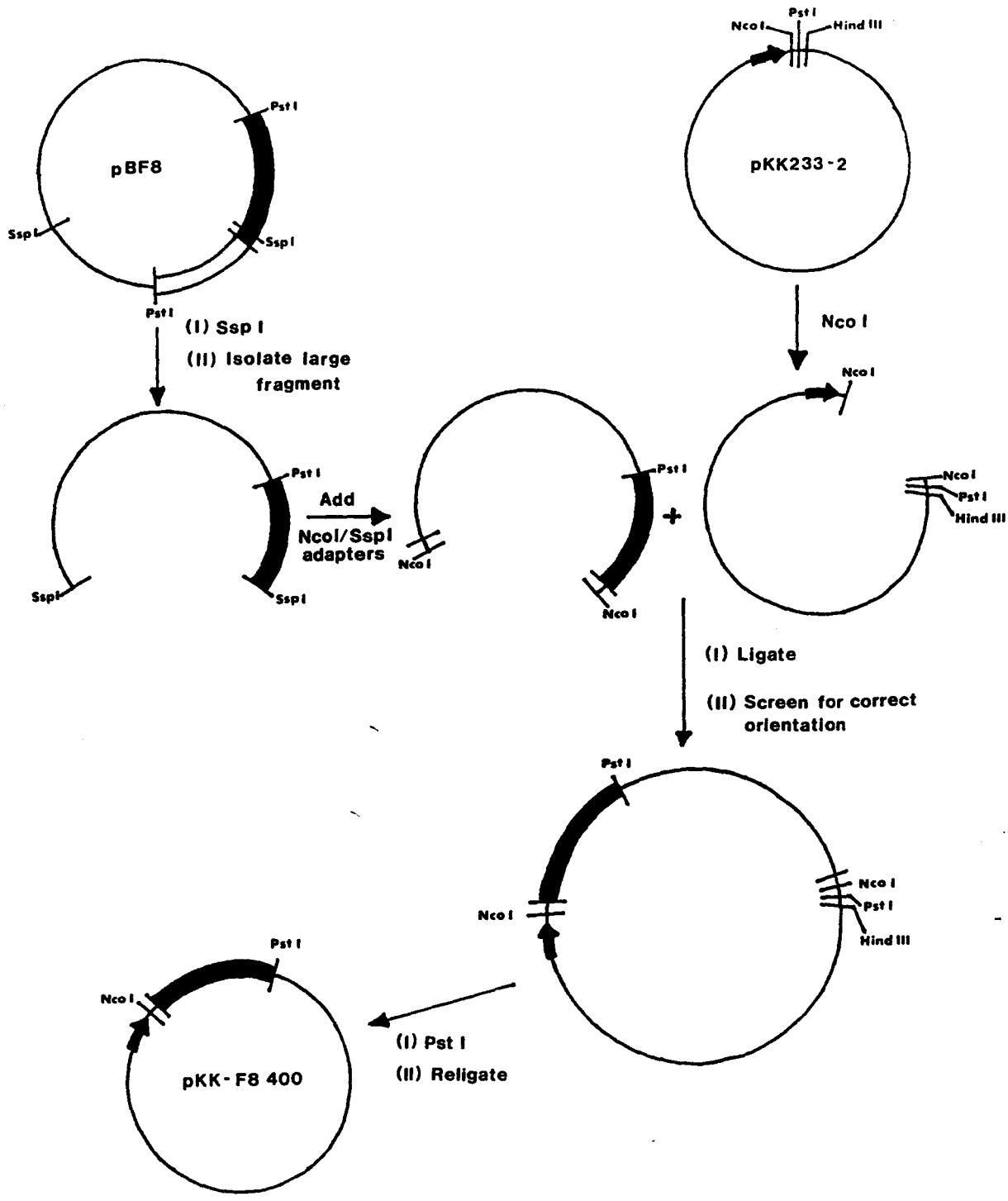

Fig. 2.8.2. Revised cloning strategy for inserting F8 open reading frame into pKK233-2.

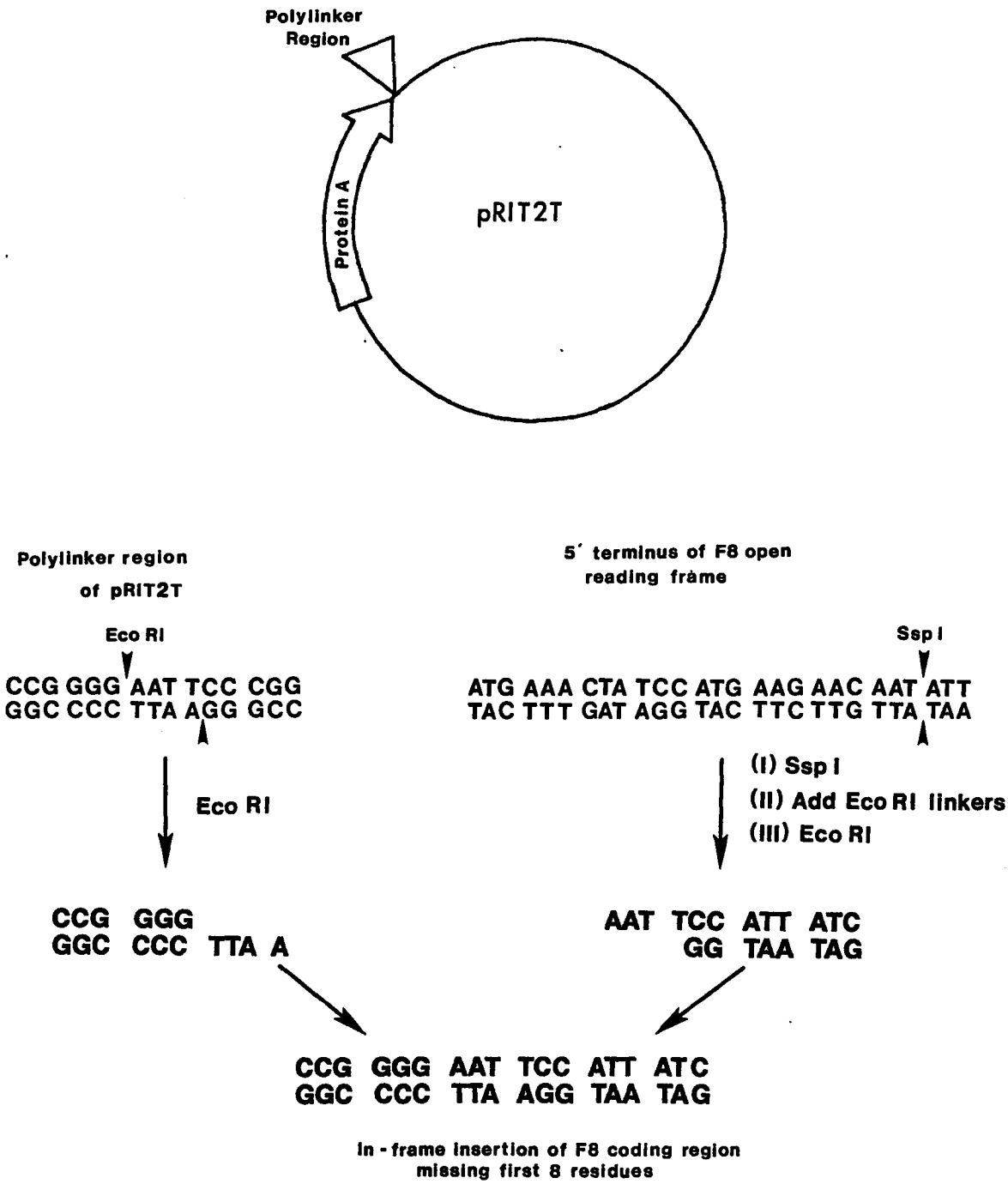

Fig. 2.8.3. Cloning strategy for inserting F8 into pRIT2T.

RESULTS AND DISCUSSION

3. THE DETECTION AND ISOLATION OF SEQUENCES HOMOLOGOUS TO IL-1β FROM COLO 16 CELLS

3.1 Studies on the Presence of IL-1-like Sequences in COLO 16 Cells

It had been previously shown that a human keratinocyte cell line (COLO 16) produced a factor with IL-1-like activities, including both immunostimulatory and inflammatory functions (Sauder et al, 1982). The factor, termed epidermal cell-derived thymocyte activating factor (ETAF), had been partially characterized, however, no information was available on the gene itself regarding its similarity to the monocyte-derived family of IL-1 genes (IL-1α and IL-1β).

Initially, it was thought that because ETAF shared at least some of the biological activities of IL-1, perhaps there would be some sequence homology in the areas important for biological function. On this premise, probes based on the known IL-1 sequences were synthesized in order to investigate the possibility that the keratinocyte cell line produced IL-1-like sequences. Synthetic oligonucleotides based on the published human IL-1β and murine IL-1α sequences were produced. The IL-1β probe consisted of a 24-base sequence from the 3' end of the coding region of human monocyte IL-1β (Auron et al, 1984), and the IL-1α probe consisted of an 18-base sequence from the published sequence of murine IL-1α (Lomedico et al, 1984). As the system under study was the human keratinocyte, in retrospect it would have been desirable to have probes

based on human sequences for both IL-1α and IL-1β. At that time, however, the sequence for a human equivalent to the murine IL-1α had not been found. In selecting the sequences for the two probes a number of criteria were followed: low frequency of codon redundancy, sequences selected were within the coding region and had little homology between members i.e. α and β.

The IL-1β oligonucleotide probe hybridized with monocyte RNA and with RNA derived from the human skin squamous cell carcinoma cell line, COLO 16, on dot blots hybridized in 6xSSC at 55°C (Figure 3.1.1, B). At this stringency, the probe did not hybridize with RNA from human fibroblasts or mouse liver, whereas, at lower stringency (6xSSC at 30°C) there was hybridization with RNA from all cell types (Figure 3.1.1, A). These results suggested that the oligonucleotide probe was specifically hybridizing to sequences present in monocytes and the keratinocyte cell line which were either absent from the other cell types or at least in very low abundance compared to monocytes and COLO cells. The murine IL-1α oligonucleotide probe was used to probe several dot blots but no signal was ever detected with any sample. As a result, further studies focussed on the human IL-1β oligonucleotide.

Northern transfers were then used to determine the size of the RNA hybridizing to the IL-1β probe. Specific hybridization of this probe to RNA from the keratinocyte cell line COLO 16 as well as to RNA from stimulated monocytes (a positive control) and the promyelocytic leukemia cell line HL-60 was observed (Figure 3.1.2, A). No significant hybridization was seen to RNA from normal human fibroblasts or unstimulated monocytes. Two bands of approximately 1.6 and 2.7 kb were

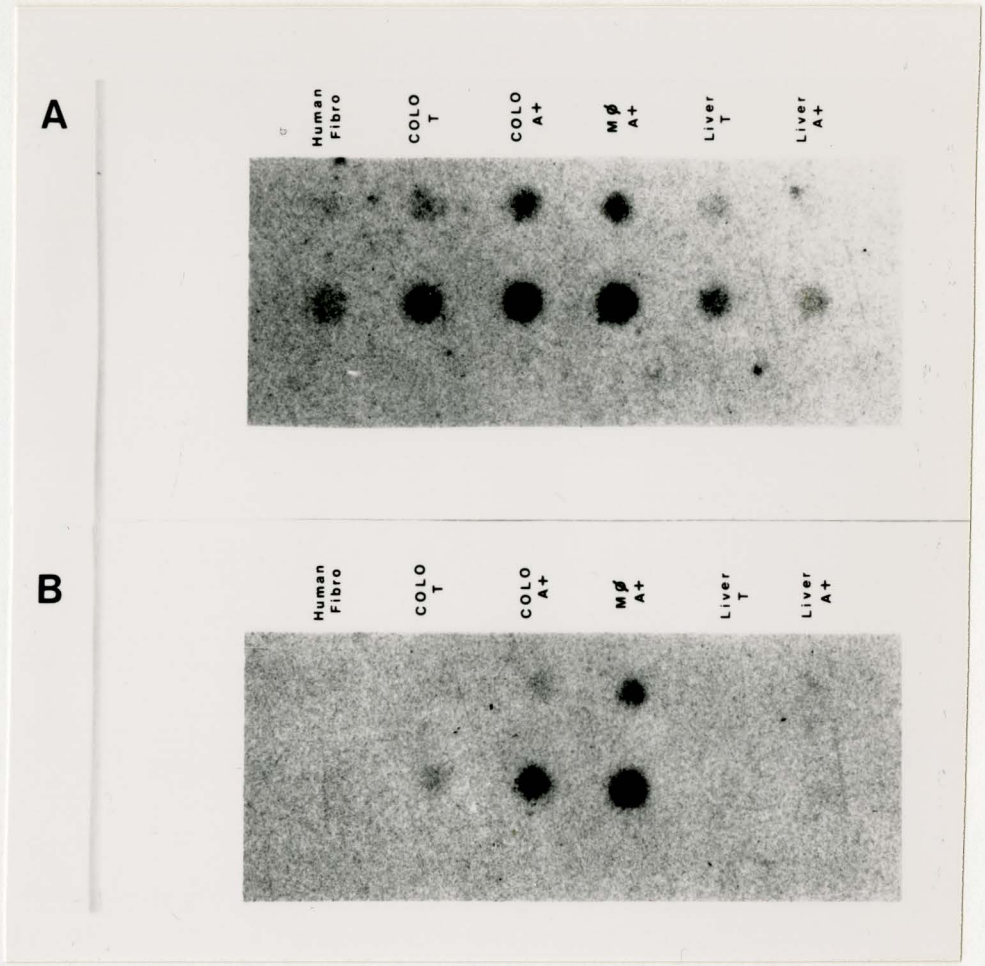

Fig. 3.1.1. Detection of IL-1β homologous RNA in macrophage and COLO 16 cells by dot blot analysis. RNA from various cell types (1 µg, top row; 4 µg bottom row) was hybridized with the IL-1β oligonucleotide in 6xSSC at 30°C (A) or 55°C (B). RNA samples from left to right are: total human fibroblast, total COLO 16, poly(A)+ COLO 16, poly(A)+ macrophage, total liver, poly(A)+ liver.

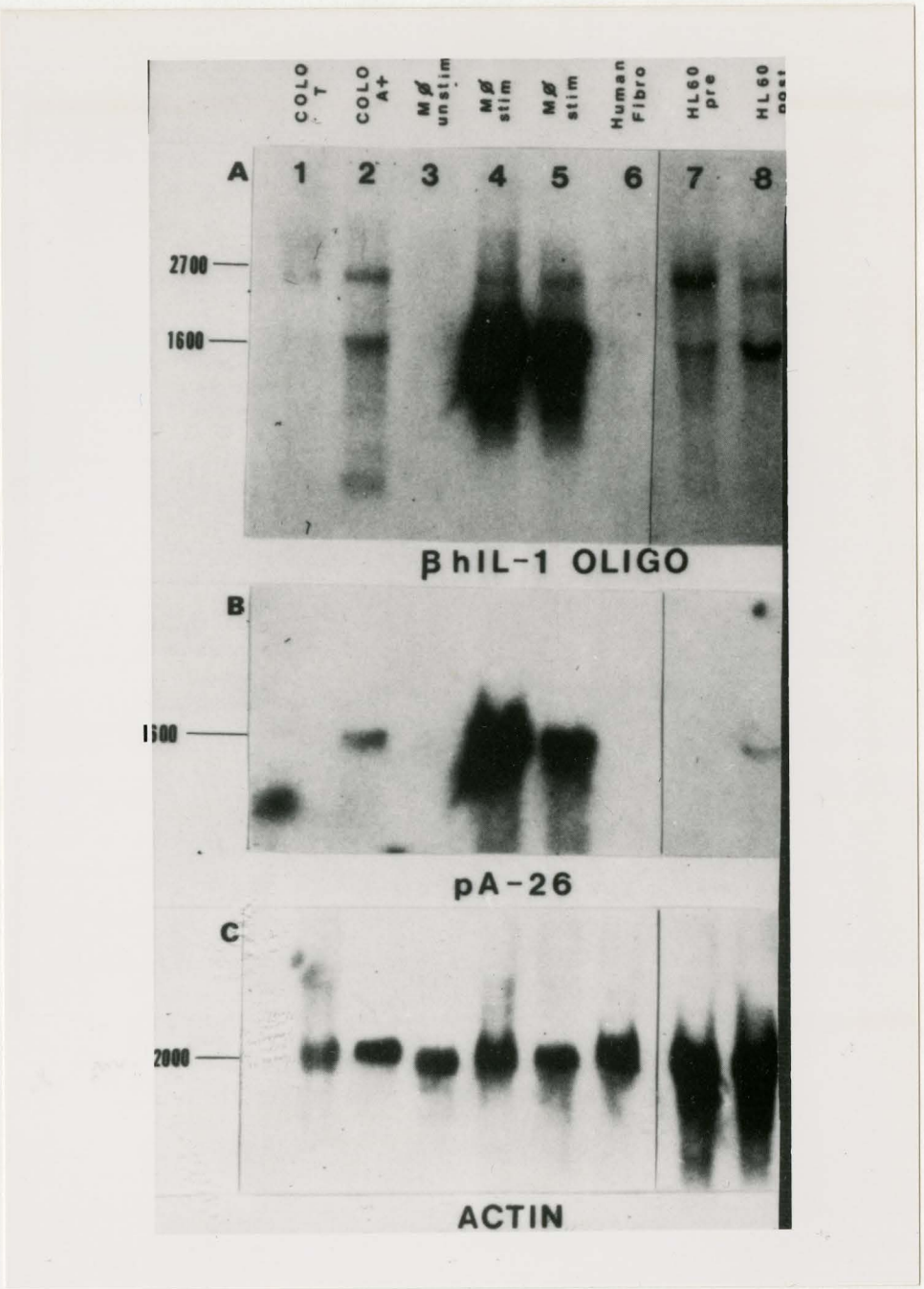

Fig. 3.1.2. Northern analysis of IL-1β homologous RNA in various cell types. The same blot was hybridized sequentially with (A) human IL-1β oligonucleotide, (B) human IL-1β cDNA (pA-26), (C) actin cDNA as outlined in sections 2.5.1 and 2.5.3. RNA samples were from the following cell types: COLO 16 (total and poly(A)+ RNA respectively) (lanes 1 and 2), unstimulated monocytes (lane 3), LPS-stimulated monocytes (lanes 4 and 5), cultured human fibroblasts (lane 6), and HL60 cells cultured without (lane 7) or with (lane 8) TPA. Conditions for LPS treatment of monocytes and TPA treatment of HL60 cells is described in section 2.4. The relative sizes, in bases, of the two RNA species observed are indicated to the left.

detected. The marked difference in amounts of the 1.6 kb message seen in stimulated monocyte RNA as compared to unstimulated showed that the human IL-1β oligonucleotide probe was specifically hybridizing to the IL-1β message in monocytes. The abundance of the two RNA species appeared to be different among the cell types. In COLO poly(A)+ and monocyte stimulated total RNA, the 1.6 kb species appeared to be more abundant than the 2.7 kb species. In the case of the HL-60 cells, undifferentiated cells appeared to contain more of the 2.7 kb species while those cells which had undergone differentiation by treatment with 10 nM TPA for 48 h contained more of the 1.6 kb species. At this time, the relationship between these two RNA species and possible control by differentiation has not been pursued further. The presence of a 1.6 kb species in TPA treated HL-60 cells was not too surprising based on the fact that these cells differentiate along the monocyte/macrophage lineage when treated with this phorbol ester (Collins, 1987). The fact that the IL-1β oligonucleotide probe was hybridizing to a 1.6 kb RNA species in COLO cells was interesting because this corresponded to the published size of monocyte-derived IL-1β mRNA. When the same Northern blot was probed with a 900-base human IL-1β cDNA (pA-26) it was found that this probe also hybridized specifically to the 1.6 kb RNA from COLO cells, stimulated monocytes and differentiated HL-60 cells, but not to the 2.7 kb RNA (Figure 3.1.2, B). This suggested that, although the 2.7 kb species possessed some homology to human IL-1β by virtue of the fact that it hybridized with the oligonucleotide, this homology was confined to a short sequence or sequences thereby preventing it from hybridizing with the 900 bp human IL-1β cDNA. It did appear, however, that there

was a message with considerable homology to IL-1β that was approximately 1.6 kb in size. To control for variability in the amount of RNA loaded and transferred during Northern blotting, an actin cDNA probe (Cleveland et al, 1980) was used to screen the same blot. A hybridization signal of similar intensity was seen in all RNA samples, (Figure 3.1.2, C) thereby eliminating the possibility that the differences in the intensities of the IL-1β signal seen in the different cells was due to actual discrepancies in the amount of total RNA present in each lane. These data provided the first clear evidence that the keratinocyte cell line COLO 16 contained RNA with significant homology to IL-1β (Bell et al, 1987).

Since strong hybridization was observed between human keratinocyte RNA and the IL-1β oligonucleotide, this oligonucleotide was used as a primer in primer extension studies on COLO 16 poly(A)+ RNA. This resulted in one major reverse transcript of approximately 750 bases and a minor transcript of approximately 500 bases (Figure 3.1.3). These transcripts fell into the size range of transcripts observed when the IL-β oligonucleotide probe was used to prime RNA from stimulated macrophages, although the transcripts were observed in much greater abundance in the case of the macrophages. The implication of this study was that the human keratinocyte cell line COLO 16 appeared to contain an RNA species with considerable homology and similar organization to monocyte-derived IL-1β.

Fig. 3.1.3. A primer extension experiment on COLO 16 RNA using the hIL-1β oligonucleotide. Reverse transcripts from COLO 16 (lane 1) and LPS-stimulated monocyte (lane 2) RNA primed with labelled IL-1β oligonucleotide. Reverse transcripts were synthesized and resolved on a 5% polyacrylamide/8 M urea gel. Marker sizes in bp are indicated to the left. The xylene cyanol dye front is denoted by XC.

3.2 Isolation of a unique cDNA sequence with homology to IL-1β

Once it had been determined that COLO 16 cells contained mRNA with homologies to monocyte-derived IL-1β, the next step was to construct a cDNA library from COLO mRNA in order to isolate and characterize the IL-1 homologous sequences. For the original library, it was decided that the human IL-1β oligonucleotide employed for the hybridization analysis and primer extension studies would be used to prime first strand cDNA synthesis from COLO mRNA. It was thought that in using this sequence rather than the conventional oligo d(T) primer, the library could be enriched for IL-1-like sequences, thereby facilitating screening of the library. This method cannot produce full-length cDNA clones, but this disadvantage was felt to be outweighed by the gained enrichment in the library for IL-1 homologous sequences. In addition, once a partial cDNA clone was isolated, it could then be used to screen a second library using oligo d(T) as primer for first strand synthesis in order to isolate a full length clone.

The IL-1β oligonucleotide-primed cDNA library was constructed in the bacterial vector pBR322 as described in section 2.6.1. This initial library consisted of approximately 1200 tetracycline-resistant, ampicillin-sensitive transformants. Of 300 transformants screened by colony hybridization with the human IL-1β oligonucleotide probe, 101 were positive. The DNA from all of these clones was isolated and subjected to electrophoresis after being treated with Eco RI to liberate the inserts. From this analysis, it was found that the cDNA inserts of the selected clones varied widely in their size (eg. Figure 3.2.1). Two inserts of particular interest were those of clones D6 and F8 which

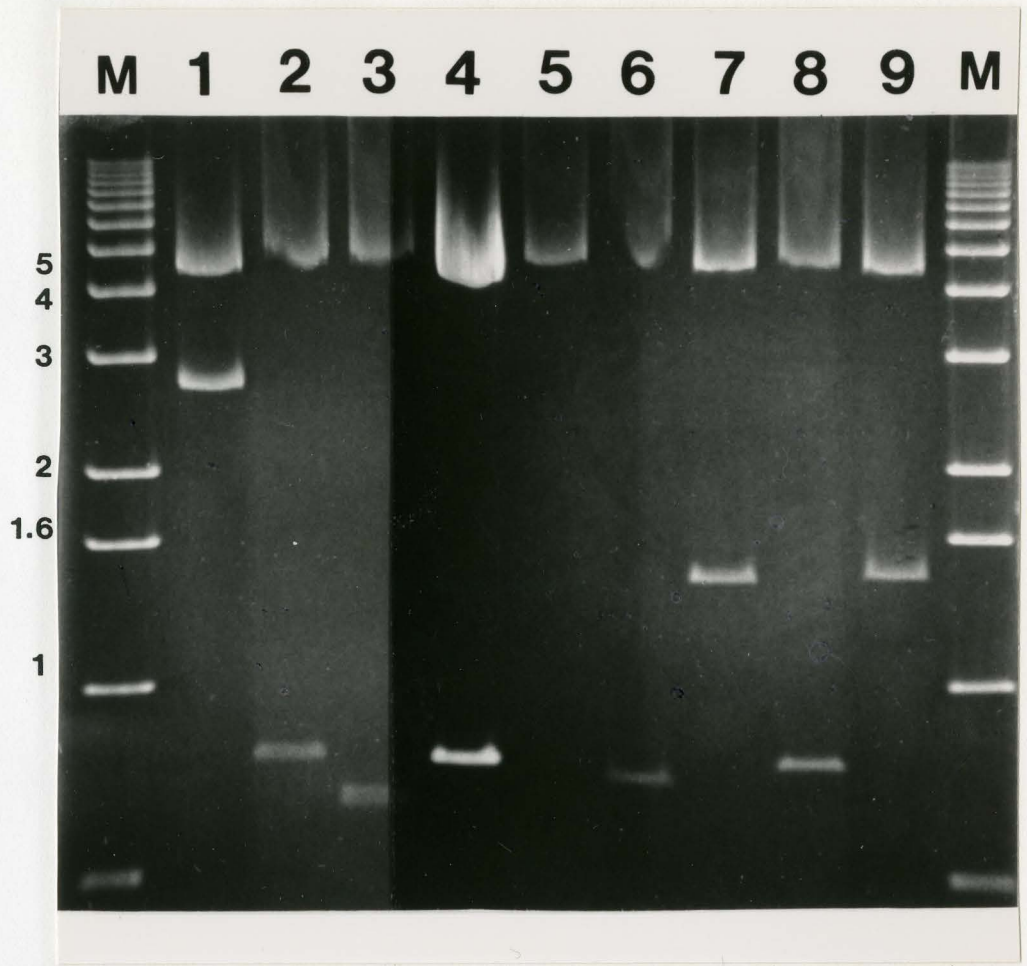

Fig. 3.2.1. Restriction analysis of various cDNA clones which hybridized with the hIL-1β oligonucleotide. Clones were digested with the restriction endonuclease PstI in order to release the cDNA fragments from the parent vector pBR322. Clones are as follows: (1) C10, (2) D6, (3) D9, (4) F8, (5) F9, (6) G10, (7) H3, (8) I8, (9) J8. Size markers (M) were the BRL 1 kb ladder; sizes in kbp are indicated to the left.

appeared to both be approximately 700 bp in length. These two clones were subjected to further study because of the similarity in the size of their inserts to that of the predicted size of a cDNA insert that would be generated from the monocyte-derived IL-1 sequence if the IL-1β oligonucleotide was used as primer. Initial studies on these two clones involved restriction analysis to determine whether their cDNA inserts were similar in sequence. Restriction analysis revealed identical fragment patterns suggesting that the D6 and F8 clones were indeed similar. The D6 cDNA clone was used as a probe on Northern blots of COLO 16 RNA to determine the size of the message and whether it corresponded to either of those species seen on Northerns probed with IL-1β oligonucleotide. Figure 3.2.2 B shows that the size of the message which hybridizes with the D6 clone is approximately 1.6 kb, corresponding to the smaller of the two messages which hybridizes to the IL-1β oligonucleotide (Figure 3.2.2 A). Northern analysis of COLO 16 poly(A)+ RNA with the F8 cDNA clone resulted in the detection of the 1.6 kb species as well (Arsenault et al, 1992). The size of the cDNA insert being significantly smaller than the RNA species to which it hybridized on the Northern suggested that the D6 and F8 cDNA clones were only partial length. This was not too surprising considering the fact that a sequence other than oligo-d(T) was used to construct the cDNA library and that it would be highly unlikely that the complementary sequence of that chosen for the primer would be found at the extreme 3' end of the message.

The fact that the D6 and F8 cDNA clones were similar in sequence as inferred through restriction analysis, contained a sequence

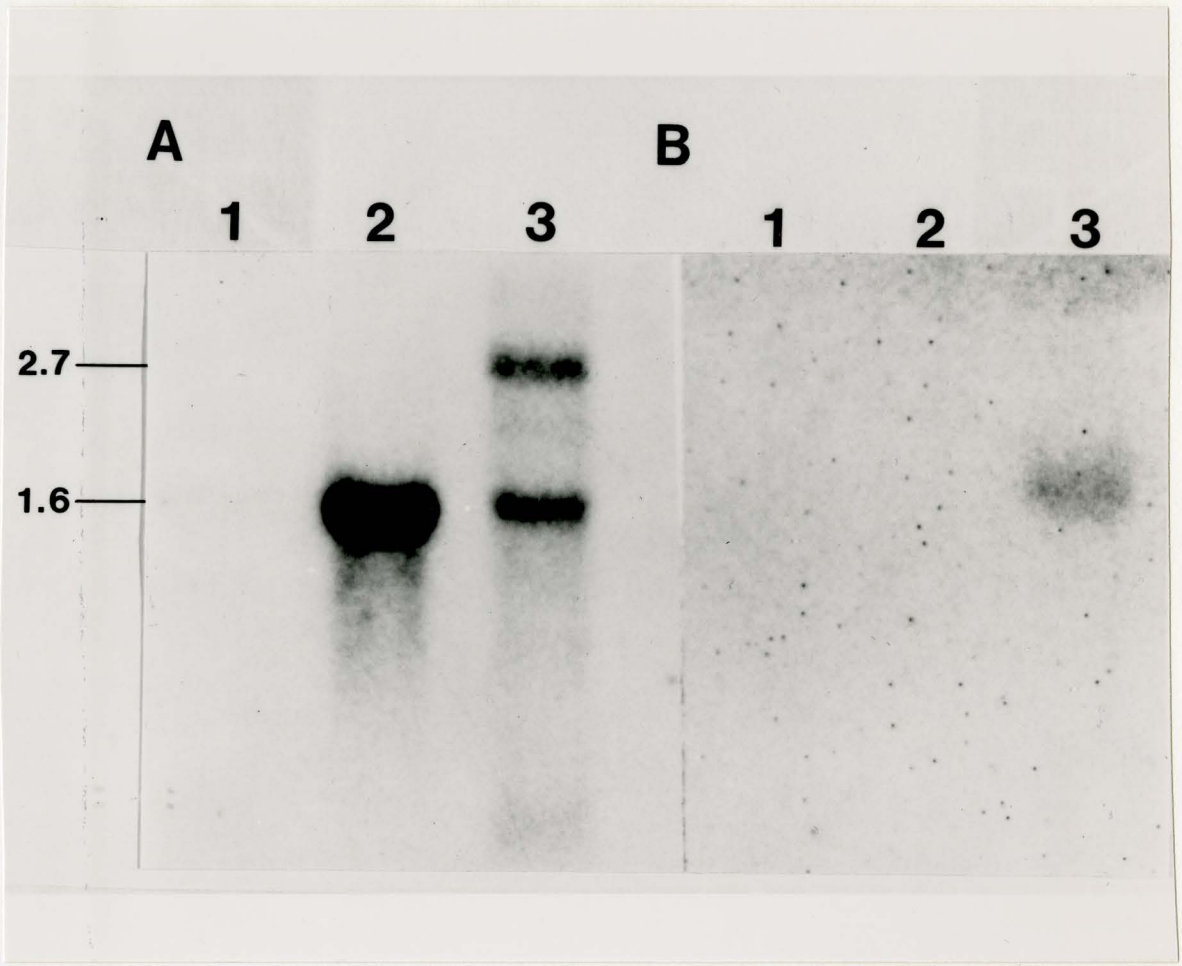

Fig. 3.2.2. Northern analysis of COLO 16 cells using the D6 cDNA clone. RNA from unstimulated monocytes (lane 1); LPS-stimulated monocytes (lane 2); and COLO 16 cells (lane 3) were hybridized with the human IL-1β oligonucleotide (A). The same filter was washed and rehybridized with the D6 cDNA clone (B). The relative sizes in kb of the two sizes of RNA species observed are indicated to the left.

homologous to a human IL-1β oligonucleotide, and the D6 clone hybridized to a 1.6 kb message suggested very strongly that we had isolated an IL-1-like cDNA species from COLO 16 cells. These similarities did not prove, however, that the two cDNA clones isolated from COLO 16 cells represent the species responsible for the IL-1-like activity which had been reported for this cell type. In order to investigate these clones further, studies were undertaken to determine their sequence and isolate a full length cDNA clone. Concurrent with this, it was decided that the partial clones should be assayed for biological activity.

Sequence analysis was performed on the two COLO 16 cDNA clones in order to determine the extent of the homology with human IL-1β. Both of the clones were subcloned into pUC118 in order to do the sequencing. The D6 clone was chosen first in order to establish the conditions for double-stranded Sanger dideoxy sequencing. In the first few sequencing attempts, troubles were encountered in obtaining readable sequence from the D6 clone. Upon subsequent purification of the DNA it was found that the pUC118 clone had undergone a spontaneous deletion of a large amount of the cDNA insert. The fact that the cDNA clone suffered a spontaneous deletion suggested that the DNA construct may not be stable. Since the 700 bp insert was well within the limits of insert size for a bacterial vector, it was thought that the clone may contain some sort of destabilizing element in its sequence, such as a "hot spot" for recombination. As a result, the F8 clone was chosen to continue the sequencing analysis as it did not appear to have suffered the same fate as the D6 clone. Why the D6 clone suffered a deletion while the F8 clone appeared to be stable was not resolved.

The results of the sequence analysis on the F8 clone revealed no significant homology to IL-1β with the exception of the IL-1β oligonucleotide sequence present at the 3' terminus (Figure 3.2.3). Initially, database searches did not reveal significant homology to any other known sequence. Recently, however, the F8 cDNA has been found to have sequence homology to the cDNA for the bovine retinal rod photoreceptor cyclic GMP-gated channel (Kaupp et al, 1989). The lack of a termination codon suggested that the F8 cDNA clone represented the 5' end of a larger message. No cleavable signal peptide was predicted for the sequence using the PC/GENE protein analysis program (IntelliGenetics Inc., Geneva, Switzerland).

Numerous attempts to isolate a full length F8 cDNA clone were unsuccessful, suggesting that there may be a block to complete reverse transcription of this mRNA or that the full length cDNA is unstable for some reason. The fact that the D6 cDNA clone suffered a spontaneous deletion during routine manipulation may have been a result of such a problem. Difficulties in obtaining a full length cDNA could also be due to the fact that the F8 message appears to be low in abundance. Different batches of COLO 16 cells have been found to differ widely in the amount of message observed, in some cases being undetectable. Attempts were also made by an independent laboratory (U. Gubler, Hoffmann La-Roche) to clone a full length cDNA. Conventional methods as well as PCR were used without success.

3.3 Isolation of a cDNA sequence with considerable homology to IL-1β

Construction of a COLO cDNA library in λgt10 using oligo-d(T)$_{18}$

```
                                                                    60
CTGGCAGGAGTAGCAGCTGCCCCTTGGCGCGACTGCTGGAGCCGCGAACTAGAGAAACAC

                                                                   120
AGACACGCCTCATAGAGCAACGGCGTCTCTCGGAGCGTGGAGCCCGCCAAGCTCGAGCTG

                                                                   180
AGCTTTCGCTTGCCGTCCACCACTGCCCACACTGTCGTTTGCTGCCATCGCAGACCTGCT

                                                                   240
GCTGACTTCCATCCCTCTGGATCCGGCAAGGGCCTGCGATTTTGACAATGTCAAGATTTA

                                                                   300
CCGTATATCCCTGTTTGTTTGGATACACCAGTGACGTCCACTTCTAGAAGACAAAGTTAT

                                                                   360
ATTACTTAAACAACCAAAGATATGAAACTATCCATGAAGAACAATATTATCAATACACAG
                          MetLysLeuSerMetLysAsnAsnIleIleAsnThrGln

                                                                   420
CAGTCTTTTGTAACCATGCCCAATGTGATTGTACCAGATATTGAAAAGGAAATACGAAGG
GlnSerPheValThrMetProAsnValIleValProAspIleGluLysGluIleArgArg

                                                                   480
ATGGAAAATGGAGCATGCAGCTCCTTTTCTGAGGATGATGACAGTGCCTCTACATCTGAA
MetGluAsnGlyAlaCysSerSerPheSerGluAspAspSerAlaSerThrSerGlu

                                                                   540
GAATCAGAGAATGAAAACCCTCATGCAAGGGGTTCCTTTAGTTATAAGTCACTCAGAAAG
GluSerGluAsnGluAsnProHisAlaArgGlySerPheSerTyrLysSerLeuArgLys

                                                                   600
GGAGGACCATCACAGAGGGAGCAGTACCTGCCTGGTGCCATTGCCATTTTTAATGTGAAC
GlyGlyProSerGlnArgGluGlnTyrLeuProGlyAlaIleAlaIlePheAsnValAsn
                                                    K3
                                                                   660
AACAGCGACAATAAGGACCAGGAACCAGAAGAAAAAAAGAAAAAGAAAAAAGAAAAGAAG
AsnSerAspAsnLysAspGlnGluProGluGluLysLysLysLysLysGluLysLys

                                                                   720
AGCAAGTCAGATGATAAAAACGAAAATAAAAACGACCCAAAGAAGAAGATGGAAAAGCGA
SerLysSerAspAspLysAsnGluAsnLysAsnAspProLysLysLysMetGluLysArg
```

Fig. 3.2.3. Sequence of partial cDNA clone F8 from COLO 16 cells. Homology to the human IL-1β oligonucleotide sequence is denoted by the overlined region. The amino acid sequence of the synthetic peptide (K3) used to generate antisera is represented by the boxed area.

as primer was undertaken in an attempt to isolate a full length F8 clone in order to further analyse its role as a keratinocyte-derived factor with IL-1-like activities. For this second cDNA library it was decided that a bacteriophage vector system would be used instead of the bacterial system used for the first library. There were several advantages to this approach: the size limit of cDNA inserts is much larger for a bacteriophage vector as compared to a bacterial vector; the number of recombinants generated by a bacteriophage library is much greater with the ability to screen more recombinants at once. At the same time that the library was screened for F8 positive clones, it was also screened with a partial human IL-1β cDNA clone (pA-26) in attempts to isolate the species which hybridized with this probe on Northerns.

Screening of the COLO 16 cDNA library in λgt10 with the partial human IL-1β cDNA (pA-26) resulted in one positive clone (designated B7) out of approximately $8x10^4$ screened. Upon purification of the positive plaque and isolation of the viral DNA it was found that the cDNA insert within this clone was approximately 1.6 kb in size (see Figure 3.3.1). Southern analysis was performed on a restriction digest of this clone to confirm that the insert did indeed hybridize with the IL-1β cDNA sequence; afterwards, the insert was isolated using the GeneClean procedure (Vogelstein and Gillespie, 1979) and subcloned into the bacterial vector pUC118 for further analysis. Screening of this library with the F8 cDNA did not yield any positive clones from a total of $4x10^5$ plaques screened.

The B7 clone was sequenced using the Sequenase system (Pharmacia). A large portion of the 3' end of the B7 clone was found to

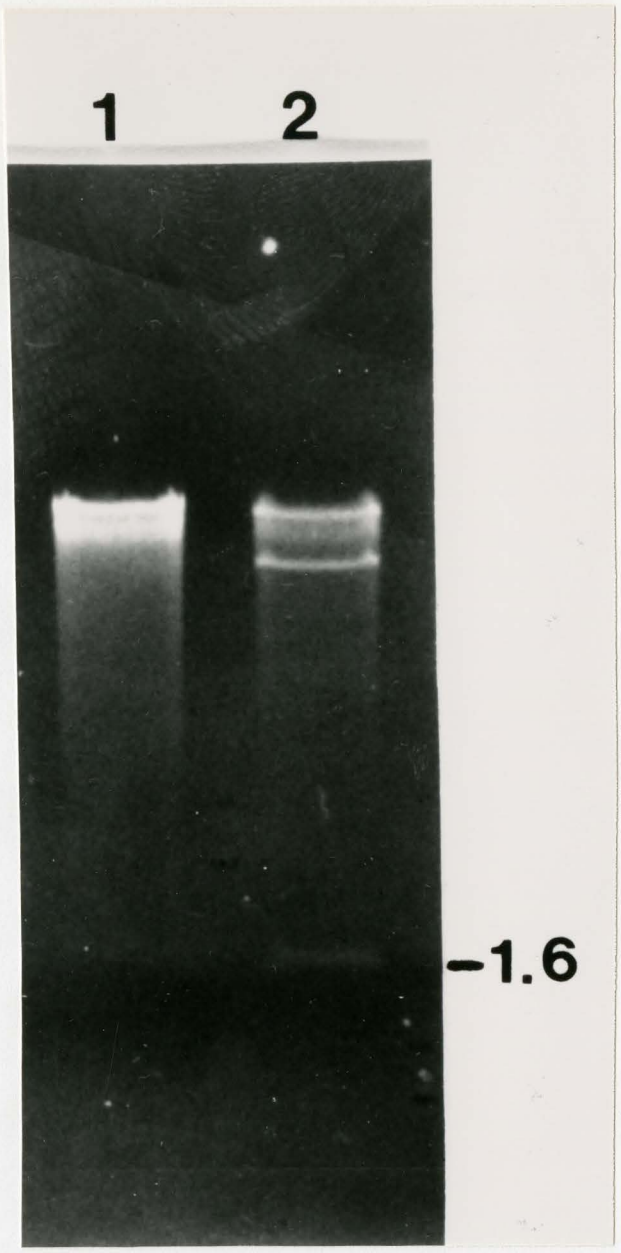

Fig. 3.3.1. Analysis of λgt10 cDNA clone B7 revealing a 1.7kb EcoRI restriction fragment. B7 DNA, either undigested (lane 1) or digested with EcoRI (lane 2), was electrophoresed on 1% agarose/1xTBE gels.

be virtually identical to the 3' end of the the coding region as well as the 3' noncoding region (including the poly(A) tail) of monocyte-derived IL-1β. This homology abruptly ended, however, with the remainder of the 5' end of the clone bearing no resemblance to IL-1. Instead, it was found that this part of the clone was homologous to human elongation factor-1α (Brands et al, 1986). Further sequence analysis revealed the presence of a second poly(A) tail at the 5' end of the clone. From these results, it was concluded that the B7 clone was a hybrid of two unrelated cDNA sequences which had become ligated to one another during the blunt end ligation step of the cDNA library construction. The presence of two poly(A) tails, one at either end of the clone, seems to corroborate this hypothesis.

Once it had been determined that the B7 clone exhibited almost complete homology to monocyte-derived IL-1β and that it was a partial clone, attempts were made to isolate a full length clone in order to obtain the 5' coding region and 5' noncoding terminus. Another cDNA library was constructed in λgt11 and was again screened with the I1-1β cDNA probe pA-26. Out of $4x10^5$ recombinants screened, two positive clones were identified, isolated and subcloned into pUC118 for sequence analysis. One of the clones, C22, was slightly larger than B7 while the other, K2 was slightly smaller in size. Both clones were sequenced and together with the B7 clone, a sequence was deduced for keratinocyte-derived IL-1 (see Figure 3.3.2). The longest cDNA clone was C22 at 1,236 bp which lacked 262 nucleotides of the 5' end of IL-1β, including 174 nucleotides of the coding region. Although the sequence was identical with monocyte-derived IL-1β in the protein coding region of

A

```
GGCTTCAGGCAGGCCGCGTCAGTTGTTCTGGCCATGGACAAGCTGAGGAAGATGCTGGTTCCCTGCCCACAGACCTTC
 G  F  R  Q  A  A  S  V  V  L  A  M  D  K  L  R  K  M  L  V  P  C  P  Q  T  F

CAGGAGAATGACCTGAGCACCTTCTTTCCCTTCATCTTTGAAGAAGAACCTATCTTCTTCGACACATGGGATAACGAG
 Q  E  N  D  L  S  T  F  F  P  F  I  F  E  E  E  P  I  F  F  D  T  W  D  N  E

GCTTATGTGCACGATGCACCTGTACGATCACTGAACTGCACGCTCCGGGACTCACAGCAAAAAAGCTTGGTGATGTCT
 A  Y  V  H  D  A  P  V  R  S  L  N  C  T  L  R  D  S  Q  Q  K  S  L  V  M  S

GGTCCATATGAACTGAAAGCTCTCCACCTCCAGGGACAGGATATGGAGCAACAAGTGGTGTTCTCCATGTCCTTTGTA
 G  P  Y  E  L  K  A  L  H  L  Q  G  Q  D  M  E  Q  Q  V  V  F  S  M  S  F  V

CAAGGAGAAGAAAGTAATGACAAAATACCTGTGGCCTTGGGCCTCAAGGAAAAGAATCTGTACCTGTCCTGCGTGTTG
 Q  G  E  E  S  N  D  K  I  P  V  A  L  G  L  K  E  K  N  L  Y  L  S  C  V  L

AAAGATGATAAGCCCACTCTACAGCTGGAGAGTGTAGATCCCAAAAATTACCCAAAGAAGAAGATGGAAAAGCGATTT
 K  D  D  K  P  T  L  Q  L  E  S  V  D  P  K  N  Y  P  K  K  K  M  E  K  R  F

GTCTTCAACAAGATAGAAATCAATAACAAGCTGGAATTTGAGTCTGCCCAGTTCCCCAACTGGTACATCAGCACCTCT
 V  F  N  K  I  E  I  N  N  K  L  E  F  E  S  A  Q  F  P  N  W  Y  I  S  T  S

CAAGCAGAAAACATGCCCGTCTTCCTGGGAGGGACCAAAGGCGGCCAGGATATAACTGACTTCACCATGCAATTTGTG
 Q  A  E  N  M  P  V  F  L  G  G  T  K  G  G  Q  D  I  T  D  F  T  M  Q  F  V

TCTTCCTAAAGAGAGCTGTACCCAGAGAGTCCTGTGCTGAATGTGGACTCAATCCCTAGGGCTGGCAGAAAGGGAACA
 S  S

GAAAGGTTTTTGAGTACGGCTATAGCCTGGACTTTCCTGTTGTCTACACCAATGCCAACTGCCTGCCTTAGGGTAGTG

CTAAGAGGATCTCCTGTCCATCAGCCAGGACAGTCAGCTCTCTCCTTTCAGGGCCAATCCCCAGCCCTTTGTTGAGCC

AGGCCTCTCTCACCTCTCCTACTCACTTAAAGCCCGCCTGACAGAAACCACGGCCACATTTGGTTCTAAGAAACCCTC
                                                          T                  TCC

TGTCATTCGCTCCCACATTCTGATGAGCAACCGCTTCCCATTTATTTATTTATTTGTTTGTTTGTTTTATTCATTGG
                                                                          G

TCTAATTTATTCAAAGGGGGCAAGAAGTAGCAGTGTCTGTAAAAGAGCCTAGTTTTTAATAGCTATGGAATCAATTCA

ATTTGGACTGGTGTGCTCTCTTTAAATCAAGTCCTTTAATTAAGACTGAAAATATATAAGCTCAGATTATTTAAATGG
                                                                  T

GAATATTTATAAATGAGCAAATATCATACTGTTCAATGGTTCTGAAATAAACTTCACTGAAG
                                           C
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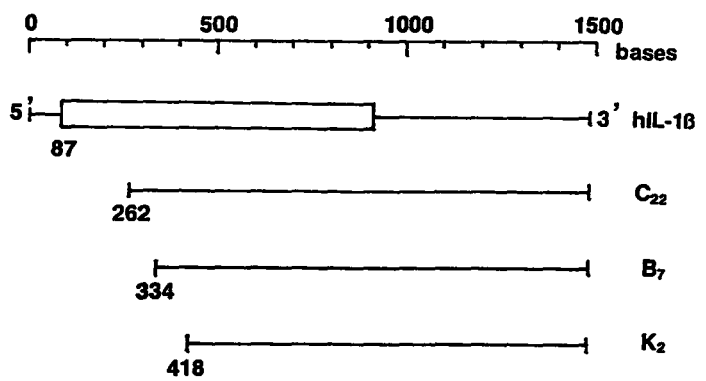
B



the molecule, a few differences were noted in the 3' noncoding region. Indeed, upon comparing published sequences for human monocyte IL-1β, it was found that there were differences between the sequences found in the literature (Auron et al, 1984; Nishida et al, 1987). Interestingly, our sequence possessed some of the differences of both published sequences, although it more closely resembled the sequence of Nishida et al (1987). These differences may represent errors in previous sequence data or true polymorphisms.

The longest clone, C22, was subsequently expressed in COS cells using a mammalian expression vector (this work was performed by Dr. R.C. McKenzie as described in McKenzie et al, 1990a). Despite the fact that this clone lacks 174 nucleotides of the 5' terminal IL-1β coding sequence, it exhibited IL-1 activity using assays for thymocyte, D10-T-cell and fibroblast proliferation. These results correlate with the findings of March et al (1985) which demonstrated that the C-terminal amino acids of IL-1β were sufficient for activity. Western analysis of C22-transfected COS cell lysates with human IL-1β antisera revealed an immunoreactive protein with an M_r of approximately 17,000 suggesting that the partial cDNA clone is expressed and processed to the mature form in this system.

3.4 Isolation of F8 Genomic Sequences

From Southern analysis, it was found that the F8 partial cDNA hybridized predominantly to a 1.7kb EcoRI fragment in DNA derived from both peripheral lymphocytes and COLO cells (see Figure 3.4.1, lanes 1

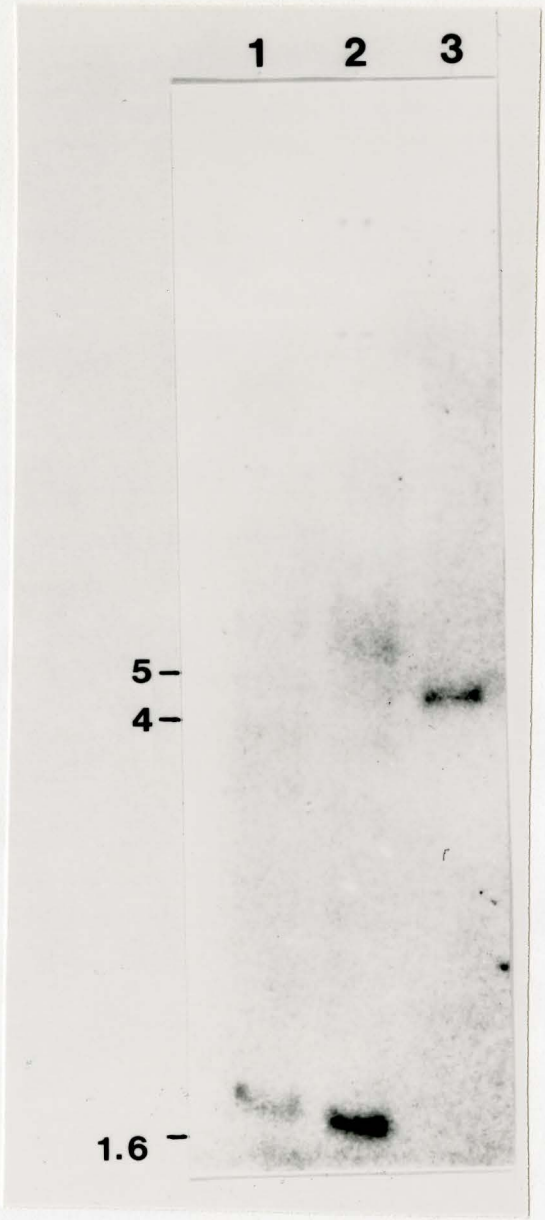


Fig. 3.4.1. Southern analysis of the F8 gene. Lane 1, EcoRI digested peripheral lymphocyte DNA, lanes 2 and 3, COLO 16 DNA digested with: EcoRI (lane 2), BamHI (lane 3). Ten µg of DNA was digested for each sample, electrophoresed on a 1% agarose gel, transferred to nitrocellulose and hybridized with [^{32}P]-labelled F8 cDNA. Exposure time was 3 days. Size markers indicated to the left in kbp are the BRL 1 kb ladder

and 2 respectively) as well as a 4.5kb BamHI fragment in COLO cells (Figure 3.4.1, lane 3). The fact that F8 sequences are present in normal human genomic DNA as well as the transformed cell line COLO 16 confirms that this sequence is not merely an artefact resulting from the cloning of cDNA sequences from the keratinocyte cell line mRNA. A genomic library of EcoRI fragmented lymphocyte DNA in λgt11 (previously constructed by Brett Stevens), was screened with the F8 cDNA resulting in the detection of 5 positive signals. These were purified to single plaques and DNA was isolated from each of them. The inserts, which ranged in size from approximately 2.8-7.0 kb, were subcloned into pUC118 for sequence analysis. Unfortunately, no 1.7kb inserts could be isolated from this library, since the DNA used to construct the library was sucrose gradient purified in the 3-7kb range.

All of these clones were sequenced using four different primers: universal and reverse universal (positioned at opposite ends of the insert), and two internal primers (AB64 and AB84) used previously for sequencing the F8 cDNA clone (from the 5' region and the 3' region of the cDNA respectively). None of the clones could be sequenced with the two internal F8 primers, suggesting that these sequences were not present in these clones. Using the other two primers it was found that two of the clones (P and 02) were identical and that they contained sequence homologous to F8 (100% homology over 112bp). The other three clones did not contain homology to F8 in the sequences obtained with the two universal primers. This, however, is inconclusive since the sequence obtained from these primers only corresponded to a small percentage of the entire clone. Analysis of P and 02 homologous

sequence revealed that it corresponded to the 5' terminus of the F8 cDNA sequence and ended at residue 156 in a putative splice site consensus sequence (see Figure 3.4.2) (Mount, 1982). The existence of an abrupt end in the homology between the genomic and cDNA sequence coupled with the observation of a putative splice junction at the same point in the sequence suggests that the F8 partial cDNA derived from an authentic mRNA species. This precludes the possibility that the F8 clone could have been a cloning artefact derived from genomic DNA contamination in the mRNA preparation.

In order to pinpoint the areas of F8 homology in the remaining genomic clones, the inserts were digested with various restriction enzyme combinations and the resulting patterns of bands were analyzed on Southern blots using the F8 cDNA as a probe. Subfragments of these clones which were positive with the F8 cDNA probe were then subcloned into pUC118 and sequence was obtained using the universal and reverse universal primers. One subfragment (R) yielded F8 homologous sequence, although the homology was only 85% over 90bp. The high frequency of mismatches and deletions between the F8 cDNA and this genomic sequence suggests that this genomic sequence may represent a pseudogene, perhaps arising from the duplication of part of the F8 sequence which has accumulated a number of mutations over the time of evolution. The presence of such pesudogenes could also explain the multiple bands seen on Southern blots of genomic DNA probed with the F8 cDNA sequence (eg. Figure 3.4.3).

In an attempt to isolate the 1.7kb EcoRI and 4.5kb BamHI F8 positive genomic fragments, lymphocyte DNA digested with these enzymes

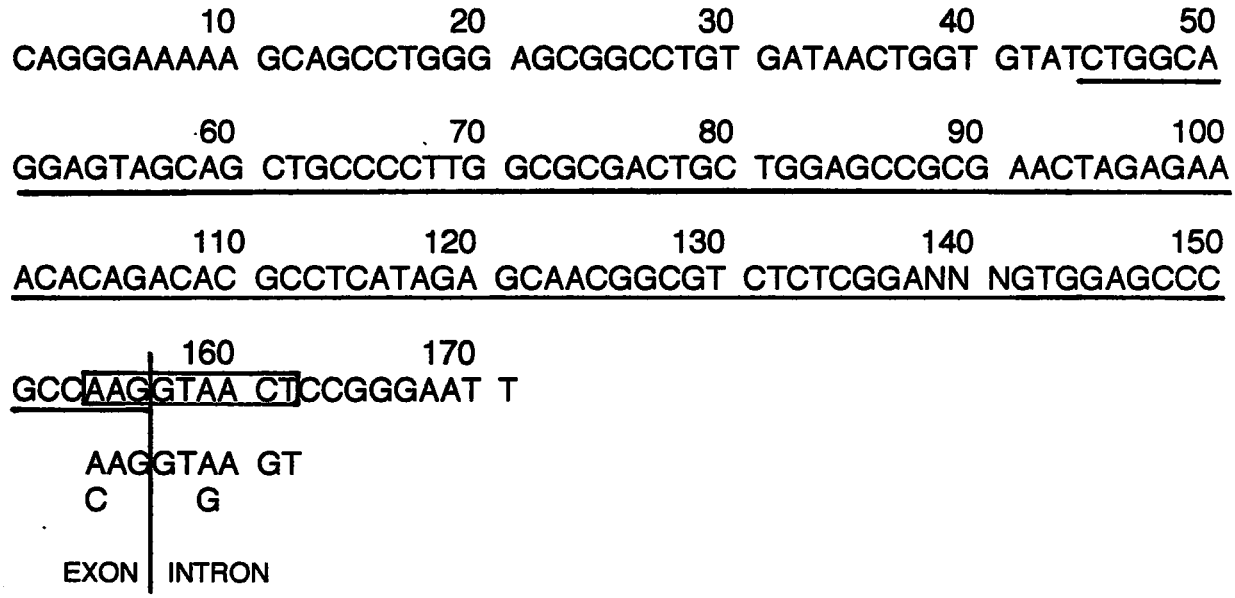


Fig. 3.4.2. Partial sequence of an F8 genomic clone showing a putative splice junction. Homology to the 5' terminus of the F8 cDNA sequence is denoted by the underline. The boxed region resembles the splice site consensus sequence shown directly below it. NNN denotes ambiguous sequence due to a compression on the sequencing gel.

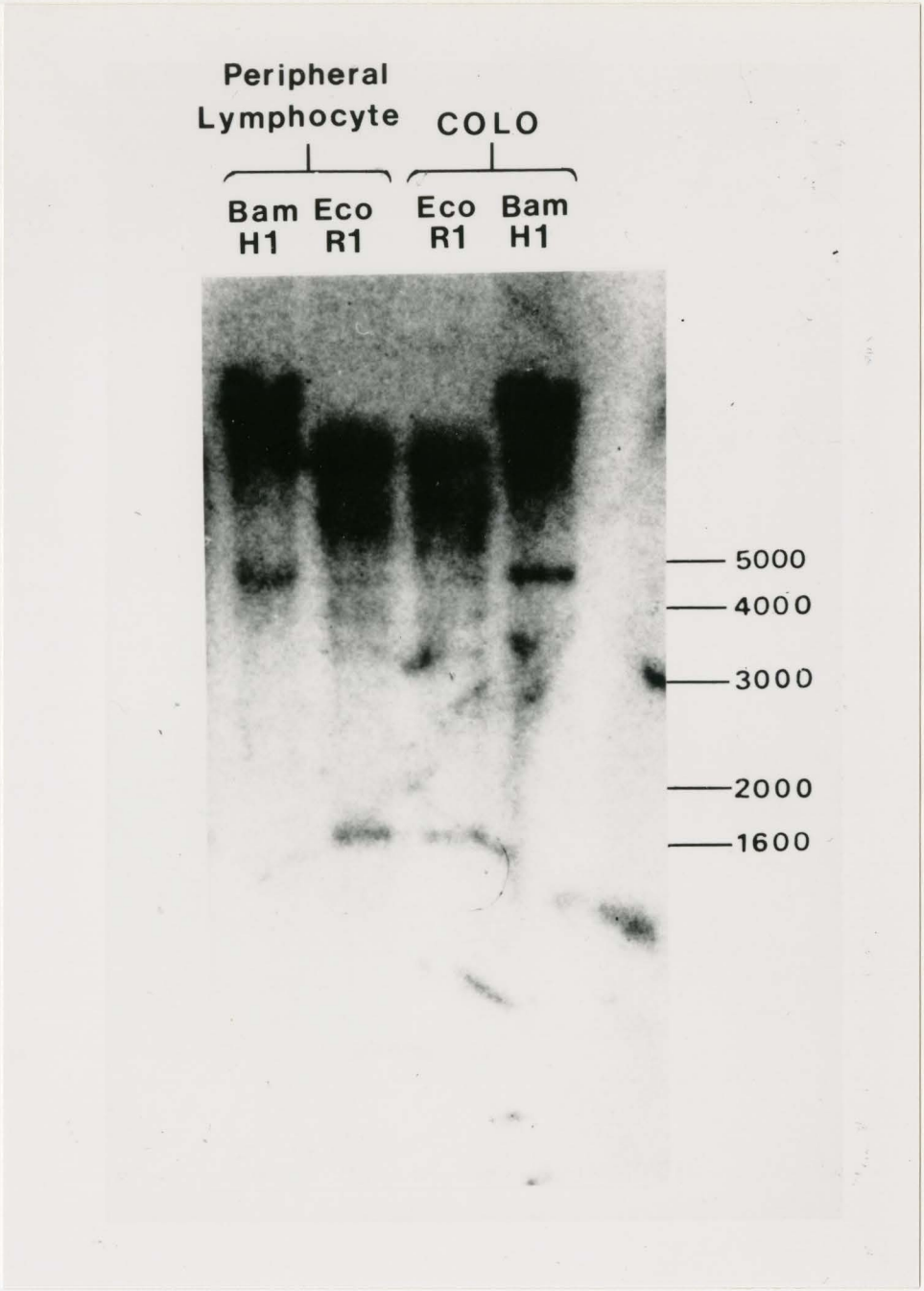


Fig. 3.4.3. Southern blot of F8 genomic sequences. Ten µg of DNA was digested with the indicated restriction endonucleases, electrophoresed on a 1% agarose gel, transferred to nitrocellulose and hybridized with [^{32}P]-labelled F8 cDNA. Size markers indicated to the right in bp are the BRL 1 kb ladder.

was electrophoresed and the appropriate size ranges were electroeluted. Attempts to clone this size selected DNA into either λgt11 (for the EcoRI fragments) or pUC118 (for the BamHI fragments) resulted in very few recombinants. To circumvent this problem (often seen in previous manipulations with gel purified fragments), DNA was size fractionated on sucrose density gradients. A library was then constructed in λgt11 using the size fractionated EcoRI fragments resulting in a library containing $2.1x10^6$ total recombinants. This library was screened with the F8 cDNA resulting in 12 positives from $3.6x10^5$ recombinants screened. The positive plaques were isolated and purified by secondary screening and the viral DNA was isolated from plate lysates following the procedure of Davis et al, 1986). Upon digestion with EcoRI, two of the clones appeared to contain inserts of approximately 1.7kb as determined by electrophoresis on agarose gels. A portion of the digest was added to a ligation mixture containing EcoRI digested pUC118 vector. The resulting ligation reaction was then used to transform competent DH5α cells. Transformants were picked and DNA was isolated employing the miniscreen method (Birnboim and Doly, 1979). The resulting DNA was digested with EcoRI and analyzed by gel electrophoresis. Sequencing of the resulting positive clone with all four sequencing primers used previously did not yield any F8 homologous sequence.

4. ANALYSIS OF F8 PROTEIN SPECIES

Three synthetic peptides based on the F8 coding region (designated K1, K2 and K3) were synthesized by P.L. Kilian and W. Danho at Hoffmann-La Roche and then injected into rabbits to generate F8 specific antisera. Initially, the antisera to all three synthetic peptides were used to probe a Western blot of COLO 16 cells. It was disturbing to find that the three antisera did not appear to have any immunoreactive proteins in common. Upon inspection of the sequences of the synthetic peptides provided, it was discovered that two of them (K1 and K2), were found to have mistakes in their amino acid sequence. This could explain why a common immunoreactive species was not observed on this initial blot. The discrepancies between the two synthetic peptide sequences and the putative F8 protein sequence could result in a significant fraction of the antisera that is incapable of recognizing the target sequences. As a result of these findings, the K1 and K2 specific antisera were not used extensively and subsequent studies concentrated on the immunoreactive species observed using the K3 antiserum. On the initial Western blot, the K3 antisera reacted with two proteins of apparent m.w.s of approximately 20,000 and 30,000 which did not appear to be present in the preimmune sera. The sizes of these two proteins were very similar to the precursor and mature forms of IL-1α and IL-1β protein species, suggesting possible cross-reactivity of the K3 antiserum with one or both of these proteins. Another Western

blot was performed on COLO 16 cells and probed with the K3 antiserum in order to determine whether the two immunoreactive species were distinct from those seen with either IL-1α or IL-1β specific antibodies (Figure 4.1.1). Probing the same blot of COLO 16 cells with both K3 and IL-1β antisera (Figure 4.1.1a) revealed that the two protein species reacting with the K3 antiserum (lane 2) were slightly different in size from those observed with the IL-1β antiserum (lane 4). This distinction was further strengthened by the fact that these K3-reactive species did not disappear when the K3 antisera was preincubated with recombinant IL-1β (lane 3). In a parallel experiment (Figure 4.1.1b), the K3 immunoreactive species could not be eliminated by competition with recombinant IL-1α (lane 3). There appeared to be a significant difference in the amount of the IL-1α immunoreactive species (lane 4) as compared to the amount of IL-1β species observed in COLO 16 cell lysates.

Although the possibility was eliminated that the K3-reactive bands on the Western blots were due to cross-reaction with IL-1α or β, the presence of a large and small K3 immunoreactive protein species was reminiscent of the precursor and mature forms of IL-1α and IL-1β. This suggests the possibility of a processed and unprocessed form of F8.

The presence of the 20,000 M_r K3 reactive protein was subsequently found to be variable, ranging from abundant in some preparations to not detectable at all in others. It could be possible that the expression of this protein is sensitive to the state of the cells from which it is isolated. It also could be that the 20,000 M_r species is a processed form of a higher Mr species (perhaps the 30,000

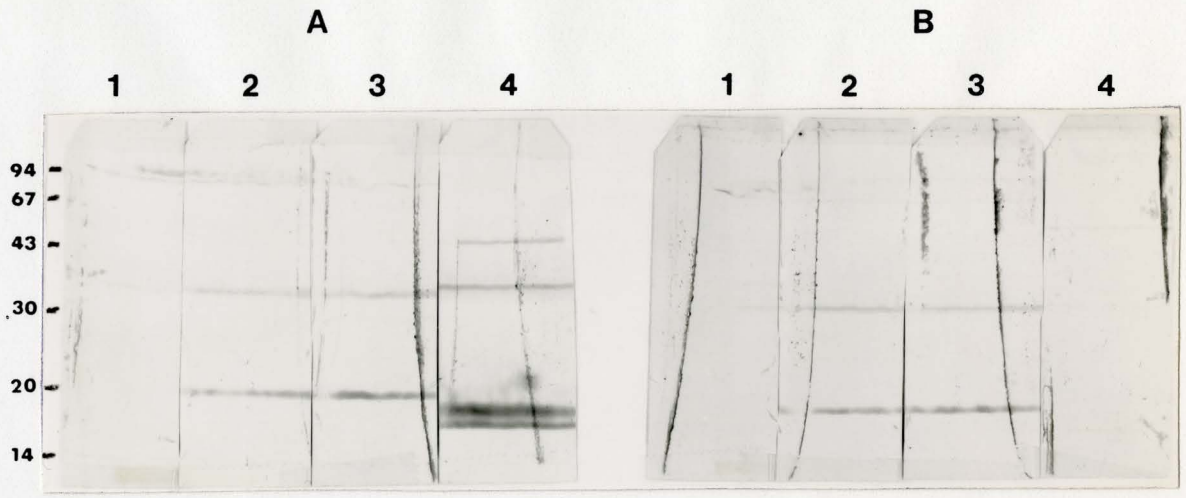


Fig. 4.1.1. Western blot of a COLO 16 cell lysate with antiserum generated against the K3 synthetic peptide. A crude cell lysate of COLO 16 cells was electrophoresed on duplicate 0.4% SDS/ 15% polyacrylamide gels, transferred to nitrocellulose and treated with: preimmune serum (A1, B1); K3 antiserum (A2, B2); K3 antiserum pretreated with recombinant IL-1β (A3); K3 antiserum pretreated with recombinant IL-1α (B3); IL-1β antiserum (A4); IL-1α antiserum (B4). M_r values of standard proteins electrophoresed in parallel are indicated on the left.

Mr protein observed) and that for some reason the protein is not being processed to the same extent in all preparations.

In an attempt to determine whether the K3-reactive proteins were F8 specific, competition experiments were performed in which the F8 synthetic peptides were used in attempts to block the binding of the various antisera to any of the observed protein species seen on the Westerns. In these experiments, 20 or 50 ng of F8 synthetic peptide was incubated with the F8 antisera prior to treatment of the Western blots. The addition of the K3 synthetic peptide to its respective antisera appeared to inhibit the appearance of the 20 and 30 kDa species on one blot (Figure 4.1.2). The competition experiment was repeated several times with the K3 synthetic peptide without successfully blocking the appearance of the 30 kDa species (on these later Westerns, the 20 kDA species could not be detected).

In order to ascertain whether the F8 protein species were also present in normal keratinocytes, cell lysates of normal keratinocytes were subjected to Western analysis. Figure 4.1.3B shows the presence of the 20 kDa protein species in normal human keratinocytes treated with PMA (lane 1) which is absent from the the preimmune control (lane 2). This 20 kDa band was also present to a lesser extent in unstimulated normal keratinocytes. Interestingly, the larger 30 kDa protein observed in addition to the smaller species in COLO 16 cells (Figure 4.1.3A, lane 2) is absent from the normal keratinocytes. If the smaller species is the result of post-translational processing of the larger form, then there appear to be some differences in the processing ability of normal keratinocytes and the keratinocyte cell line.

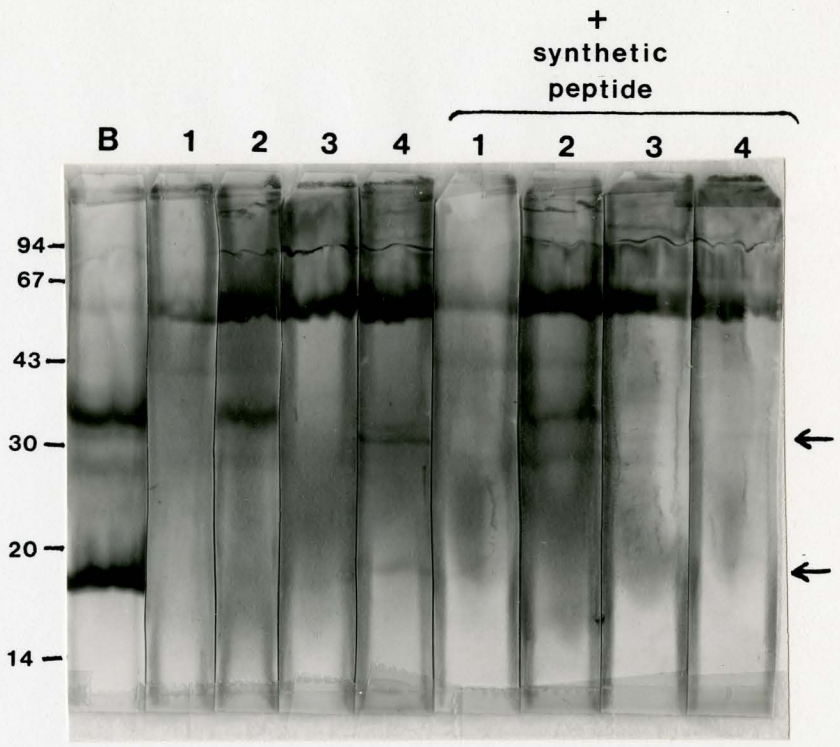


Fig. 4.1.2. A competition experiment showing specific inhibition of K3-reactive protein species on a Western blot of COLO 16 cells. A crude cell lysate of COLO 16 cells was electrophoresed on a 0.4% SDS/ 15% polyacrylamide gel, transferred to nitrocellulose and treated with various antisera either untreated or pretreated with its corresponding synthetic peptide. K1 preimmune serum (1); K1 antiserum (2); K3 preimmune serum (3); K3 antiserum (4); IL-1β antiserum (B). Arrows indicate K3 specific protein bands of interest. M_r values of standard proteins electrophoresed in parallel are indicated on the left.

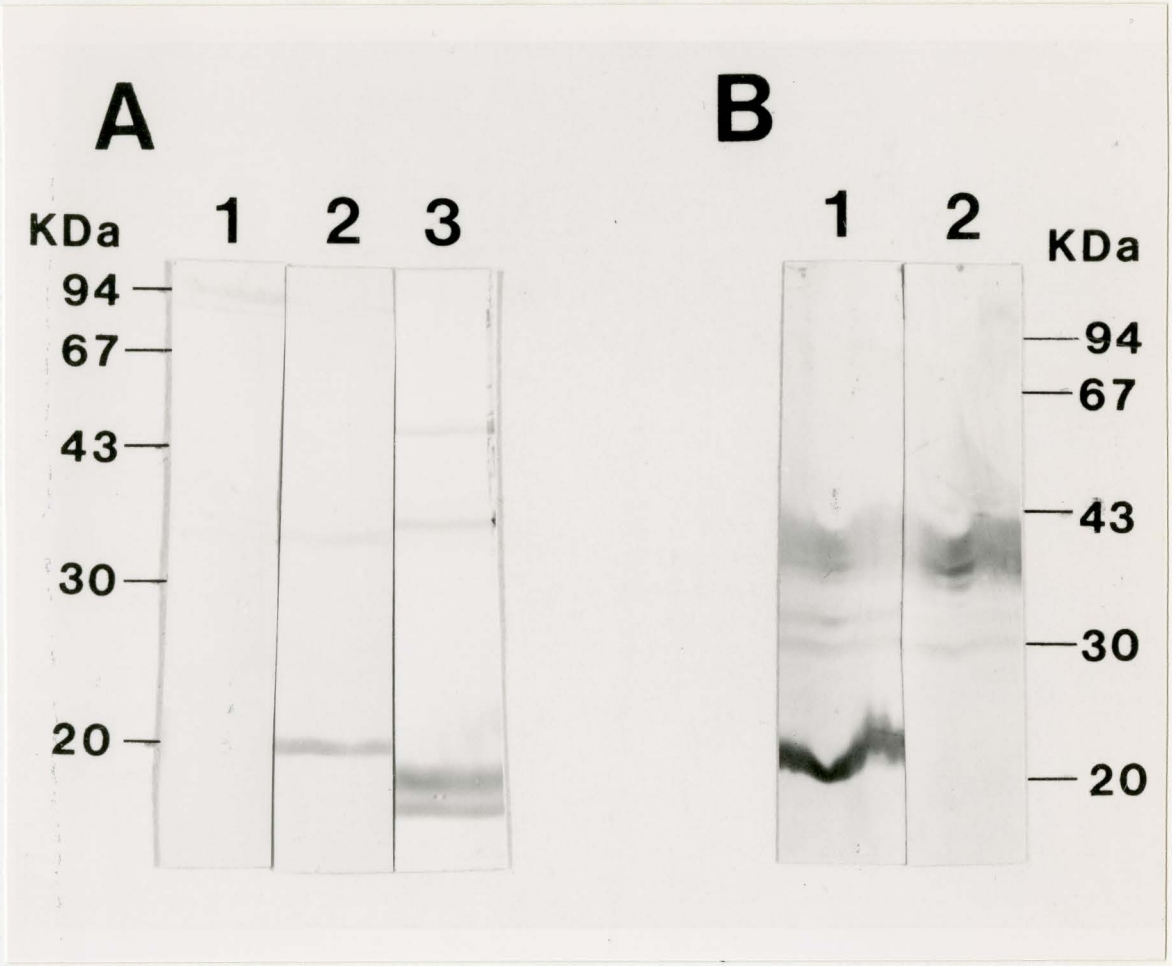


Fig. 4.1.3. Western blot analysis of COLO 16 and PMA-stimulated normal keratinocytes. Crude cell lysates of COLO 16 (A) and PMA-stimulated normal keratinocytes (B) were electrophoresed on 0.4% SDS/ 15% polyacrylamide gels, transferred to nitrocellulose and treated with: preimmune serum (A1, B2), F8 antiserum (A2, B1) or human IL-1β antiserum (A3). M_r values of standard proteins electrophoresed in parallel are indicated on the left and right of the two blots respectively.

5. INVESTIGATIONS INTO POSSIBLE IL-1-LIKE ACTIVITIES OF THE F8 CLONE

5.1 Biological Activity Assays on the Partial cDNA Clone

It was decided that the partial cDNA clone F8 would be tested indirectly for IL-1-like activity using the thymocyte co-stimulator assay. Since a full length clone was not available to directly test for the ability to cause thymocyte proliferation, the partial F8 clone was used to hybrid select mRNA from COLO 16 cells which was then assayed for biological activity. In the initial experiment, the F8 cDNA clone was used and the hybrid-selected RNA was injected into Xenopus oocytes. The resulting supernatant was then fractionated over a TSK 2000 HPLC column and tested in the thymocyte proliferation assay (see Figure 5.1:1) The results showed that there was a peak of activity from the oocyte supernatant in fractions 21-30. As a negative control, the pBR322 vector without the insert was used to hybrid select COLO 16 poly(A)+ RNA. This RNA, when injected into oocytes yielded supernatant which was not active in the thymocyte co-stimulator assay. As a positive control, poly(A)+ RNA from macrophages was used. This was used as a positive control rather than hybrid-selecting RNA from macrophages using an IL-1β probe because it had been noted that the amount of activity isolated from macrophages by this method was usually very low, (personal communication from Dr. Harnish). In addition, the positive control was mainly being used to determine whether the oocyte injection technique and thymocyte assay were functional, whereas the negative control was

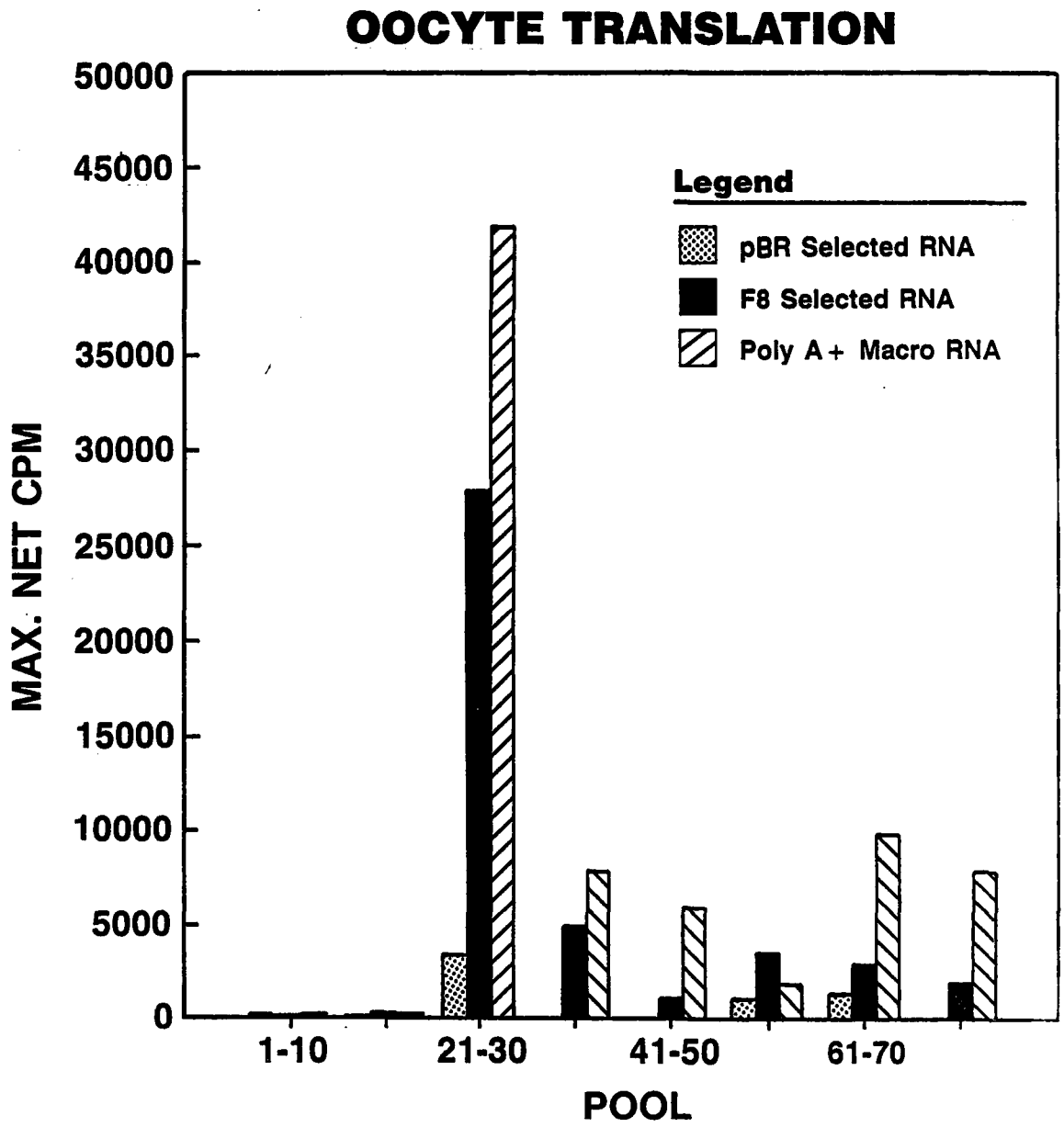


Fig. 5.1.1. Thymocyte proliferation assay on supernatant from oocytes injected with F8 hybrid-selected RNA.

being used to ensure the absence of nonspecific interactions during the hybrid selection process which might give a positive result in the bioactivity assay. Of particular interest was the fact that the activity from the hybrid-selected material eluted in the same fractions as the monocyte-derived activity of the positive control. This suggested that the species were of similar size.

5.2 Bacterial Expression of the Partial F8 cDNA Coding Region

In order to test the partial F8 cDNA clone for biological activity, an attempt was made to express the 398 bp open reading frame in a prokaryotic expression system. The use of a bacterial system would allow not only for a direct method of assaying the F8 clone for biological activity, but also for the production of large amounts of the F8 protein which could be purified and further characterized. For the expression in a prokaryotic host, the expression vector pKK233-2 (Pharmacia) was chosen for the following reasons. Firstly, the vector contained an inducible trp-lac fusion promoter (Amann and Brosius, 1985) which would control constitutive expression of the F8 protein thereby preventing the possible lethal effects from the overexpression of a foreign protein in bacterial cells. Secondly, there had been previous success in our laboratory with the expression of a human cDNA in this vector (Tyers et al, 1988).

Based on the restriction sites available in the pKK233-2 vector and the F8 cDNA insert, the easiest and most direct way of cloning the F8 reading frame into the vector would have been to ligate the entire cDNA clone into the PstI site followed by removal of the fragment

flanked by the NcoI and SspI restriction sites present in the vector and insert respectively. A small portion of the F8 open reading frame at the 5' end would have been removed, but with the use of a custom linker containing the missing F8 sequence flanked by NcoI and SspI restriction sites the F8 5' end could have been ligated into the vector in the correct reading frame for expression. Unfortunately, this scheme could not be used because of the presence of an SspI restriction site within the pKK233-2 vector. As a result, a more complicated approach had to be employed.

The original strategy employed to insert the F8 clone into pKK233-2 in the correct reading frame for expression is outlined in Figure 2.8.1. In this approach, both the vector and the insert had to be modified. The vector had to be prepared in such a way as to generate NcoI and PstI ends. These two sites, although present adjacent to each other and in the correct order, could not be used directly. This was due to their close proximity which prevented a double digestion from being performed. In order to allow for a double digestion, the distance between the two restriction sites was increased by inserting a "filler" sequence into the PstI site. For this purpose, the entire F8 cDNA clone was used as it was conveniently flanked by PstI sites and it was thought that this construct could itself be useful as a negative control in the resulting expression studies with the F8 open reading frame construct. Once the F8 cDNA was cloned into the pKK233-2 vector, a double digest using NcoI and PstI would be feasible and the linearized vector containing the NcoI and PstI ends could be isolated from the restriction digest mixture.

The F8 open reading frame would then be prepared as outlined in Figure 2.8.1. The strategy took advantage of the presence of an SspI restriction site near the start of the F8 open reading frame. The entire F8 cDNA insert contained in pBR322 was first digested with SspI resulting in two fragments approximately 0.9kbp and 4 kbp in size with the larger fragment containing the desired portion of the F8 sequence. The larger fragment was then isolated and a special set of adapters was ligated to both ends. This fragment could then be digested with PstI to generate an F8 open reading frame suitable for ligating directly into the prepared pKK233-2 vector. The special adapter employed would not only provide the neccessary NcoI site for ligation into the vector, but would also regenerate the full open reading frame of the insert and position it in the correct reading frame for expression. This strategy was tried a number of times without success and it was felt that the problems were the result of minimal yield due to the multiple steps and frequent purifications required. A second strategy was therefore devised (see Figure 2.8.2) in an attempt to decrease the number of steps. In the first attempt with this new strategy the only tranformants found to be positive when screened with the IL-1β oligonucleotide were in the incorrect orientation with respect to the F8 insert. In the second attempt, the transformants yielded unexplainable restriction patterns upon digestion with the diagnostic enzymes.

As a result of the difficulties encountered with cloning the F8 open reading frame into pKK233-2 for expression in bacteria, a second vector was chosen. This vector, pRIT2T, allows for an insert to be fused to the IgG binding domains of protein A. The strategy employed

for inserting the F8 open reading frame into pRIT2T is outlined in Figure 2.8.3. On the first attempt with this new vector, despite the fact that only four transformants were obtained, two of these appeared to be in the correct orientation based on restriction analysis. These two clones were sequenced using the F8 sequencing primer AB84 in order to determine the sequence at the junction between the F8 open reading frame and the vector. It was found that one of the clones (designated pRIT2TF8ORF) possessed the F8 insert in the correct reading frame for expression. Expression studies were performed, as outlined in Materials and Methods, on this transformant in addition to the parent plasmid which was used as a control for a non-insert containing vector. Protein lysates were obtained from induced or uninduced cultures of either pRIT2T or pRIT2TF8ORF and analyzed by SDS-PAGE electrophoresis. In an initial experiment, an induced protein A band at approximately 30 kDa was detected in 60 and 90 min induction periods for the parent plasmid pRIT2T. This band was not detected in the uninduced lysate or the 30 min induction period for pRIT2T (see Figure 5.2.1, A). It was also not detected in any of the induced or uninduced pRIT2TF8ORF lysates. This was encouraging as the presence of the F8 insert in the vector would result in the expression of a larger protein species. Unfortunately, the presence of a new larger species or an increase in the abundance of a larger protein band was not detected.

In an attempt to ascertain whether a fusion protein was indeed being expressed, several variables were changed: more sample was loaded and cells were induced for longer periods of time (120 and 150 min). When lysates were again analyzed by electrophoresis, the absence of the

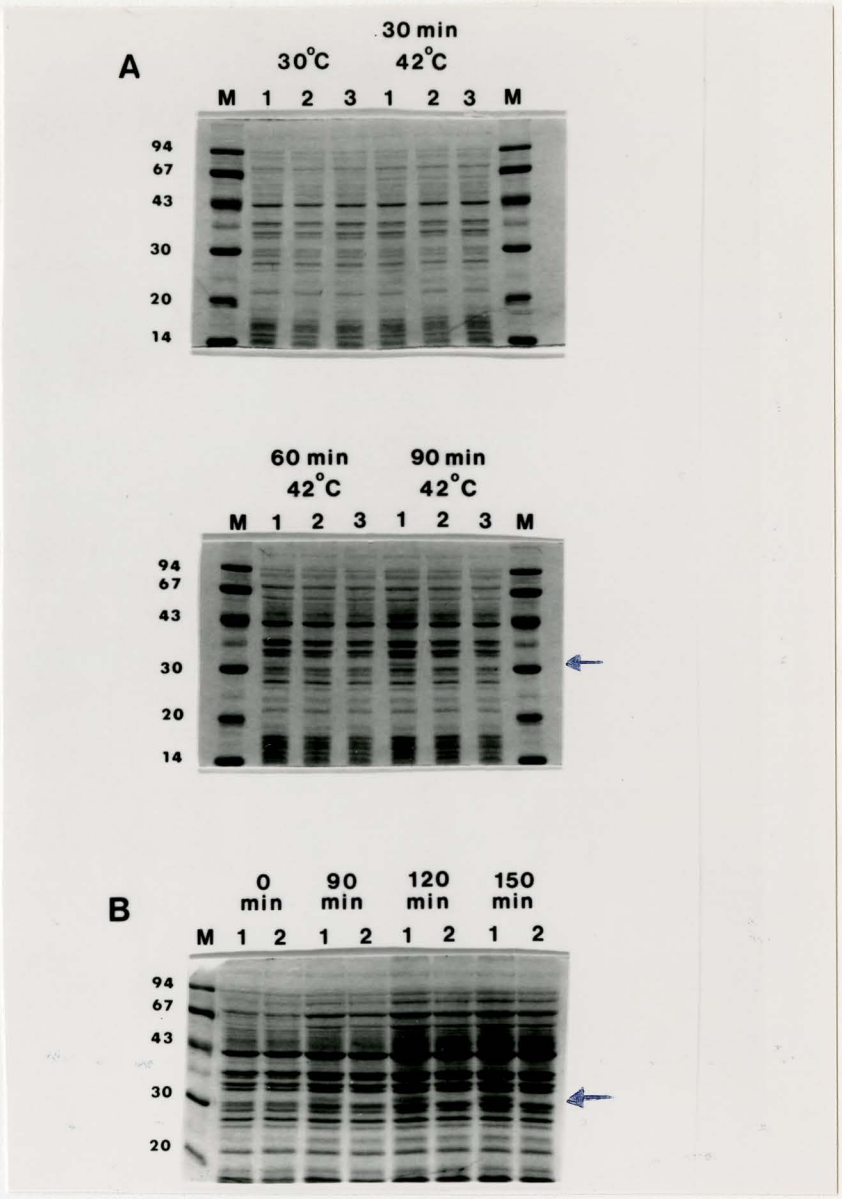


Fig. 5.2.1. Analysis of proteins produced by bacteria transformed with pRIT2T containing the F8 open reading frame. (A) Protein lysates from either uninduced (30°C) or induced (42°C) cultures of the parent plasmid (lane 1) and two independent F8-containing clones (lanes 2 and 3) were electrophoresed and stained for total protein as described in section 2.7. The induced protein A band from the parent vector (60 and 90 min induction points, lane 1) is indicated by the arrow. M_r values of standard proteins electrophoresed in parallel are indicated to the left and right. (B) Protein lysates from the parent vector (lane 1) and an F8-containing clone (lane 2) were induced at 42°C for longer periods of time. The induced protein A band from the parent vector is indicated by the arrow. M_r values of standard proteins electrophoresed in parallel are indicated on the left.

protein A species from the pRIT2TF8ORF construct was again evident, however, no fusion protein could be detected (see Figure 5.2.1, B). A Western blot was then performed on a duplicate of this gel and reacted with antisera to the F8 synthetic peptide K3 followed by a protein A-horseradish peroxidase (HRP) conjugate. Development of this Western blot revealed the presence of a number of reactive protein species (see Figure 5.2.2). The protein A band was clearly visible in the pRIT2T lysates at approximately 30 kDa. The pRIT2TF8ORF lysates contained a large number of reactive bands. It should be noted that the protein species detected in these lysates may not represent F8 specific proteins for even though an F8 specific antisera was used as the primary antibody, the fact that the expressed protein is fused to protein A means that any IgG fraction would bind to it. As with the Coomassie brilliant blue stained gels, the prevalent protein A band seen in the parent vector lysates was not observed in the pRIT2TF8ORF lysates. What could not be seen in the former gels, however, was the presence of a number of larger protein bands possibly corresponding to the F8-protein A fusion protein. The presence of such a large number of bands in these lysates was puzzling and was postulated to be the result of either degradation, nonspecific binding of the protein A-HRP conjugate or inefficient translation of the F8-protein A fusion protein resulting in multiple pauses and stops by the translation machinery. The possibility of degradation did not seem likely since it is usually characterized by a smear rather than distinct bands as observed in Figure 5.2.2. The possibility of nonspecificity was further investigated as follows: it was thought that if the bands were due to nonspecific interactions of

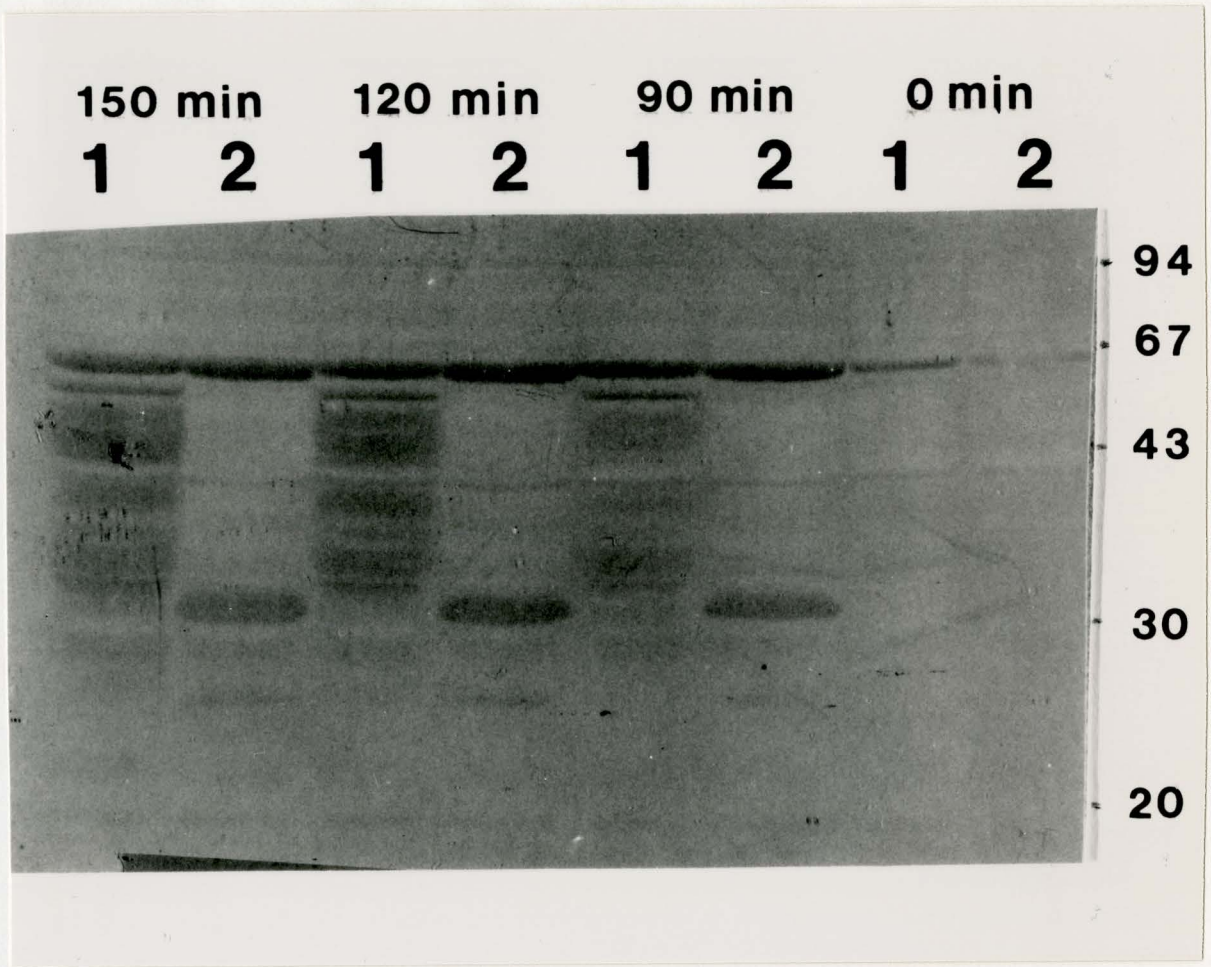


Fig. 5.2.2. Western analysis of F8 bacterial expression studies using the vector pRIT2T. Cultures of the parent plasmid (2) and an F8-containing clone (1) were induced for varying lengths of time at 42°C. Protein lysates of these cultures were then electrophoresed, transferred to nitrocellulose and reacted with K3 antiserum. M_r values of standard proteins electrophoresed in parallel are indicated on the right.

the protein A-HRP conjugate, then incubation of the Western blot with this conjugate without prior incubation with a primary antisera would produce a similar pattern. However, without prior incubation with the F8 antisera, no reactive species are detected. It also seemed unlikely that the protein bands detected were nonspecific due to the fact that they appeared to be inducible, being absent in the uninduced lysate while in the induced cultures, their abundance seemed to be directly related to the length of time of the induction period. This, however, was only a qualitative observation since the amount of protein loaded for each sample was not normalized. The presence of a large species at approximately 67kDa (see Figure 5.2.2) was also postulated to be the result of nonspecificity due to the fact that it was present in both uninduced and induced lysates and appeared in both the parent vector and the F8 construct. As with the multiple bands, however, this large species was not detected when blots were incubated only with the protein A-HRP conjugate. Although this large protein band present in all samples and the multiple bands in the pRIT2TF8ORF lysates on Western blots were concluded to not be the result of nonspecific binding of the conjugate, an explanation as to their presence could not be resolved. Another possibility was that the F8 antisera was nonspecific and therefore reacting with bacterial proteins in the lysate. This did not seem to be the case, however, since the primary antisera was reacted with the blots in the presence of a control bacterial lysate (from cells which do not contain plasmid).

Due to the problems with the above vector construct, attempts were again made to insert the F8 partial cDNA into pKK233-2. It was

felt that if it were possible to achieve success, then the production of F8 protein could not only be used to directly study its biological activities, but could also be employed to affinity purify the F8 antisera in an attempt to eliminate the problems of specificity previously encountered with the antisera. A new cloning scheme was devised for inserting the F8 open reading frame into pKK233-2, however, it was also unsuccessful in yielding transformants containing the desired construct.

In an attempt to determine whether the F8 insert itself was the cause of the problem, its ability to ligate to pGEM containing NcoI and PstI sites was tested. On the first attempt, 25% of the recombinants contained the F8 insert. The ease with which the F8 fragment was inserted into this other plasmid ruled out the possibility that the F8 fragment was the problem. The next probable cause of the problem was then thought to be the pKK233-2 vector itself; perhaps it had suffered a point mutation making it impossible to clone into. A second source of the plasmid was obtained which was known to have worked in a previous case (Tyers et al, 1988). Using F8 insert and pKK233-2 plasmid (both individually used in successful cloning experiments), an attempt was made to insert the F8 open reading frame into the vector using the previous method outlined in Figure 2.8.1. A total of 108 transformants were screened by colony hybridization with the IL-1β oligonucleotide yielding 8 positives. DNA was isolated from these and tested for the presence of an NcoI/PstI fragment of approximately 400 bp. All 8 clones contained an insert, although the size was closer to 500 bp than 400 bp. Protein lysates were prepared from cultures of these clones and analyzed

by SDS-PAGE as outlined in Materials and Methods. None of the transformants appeared to produce a recombinant protein. The junction between the insert and the vector was then sequenced for each clone to determine whether the inserts were indeed in the correct reading frame. Sequencing results showed that none of the inserts were in the correct reading frame which explained the results of the SDS-PAGE analysis. The absence of any F8 inserts in the correct reading frame within the pKK233-2 vector was postulated to be the result of selection pressure against those transformants which contain the F8 insert in the proper reading frame for expression. This could be possible if the overexpression of the F8 protein is deleterious to the cells since there is leaky expression from the pKK233-2 vector in the absence of the inducer IPTG (Tyers et al, 1988). As a result of these findings, it was concluded that the pKK233-2 vector was unsuitable for expression of the F8 open reading frame and that a vector which enabled more strict control of its promoter would be necessary to continue this part of the project.

5.3 Mammalian Expression Studies

The F8 partial cDNA was transfected into COS-1 cells by Dr. R.C. McKenzie in order to express the F8 coding region and assay for biological activity (see methods and results in Appendix). Two different assays (thymocyte co-stimulation, D10 stimulation) were used for testing F8-transfected COS-1 cells for IL-1-like activity.

5.3.1 Thymocyte Co-stimulation Assay

The thymocyte co-stimulation assay has become a classical test for IL-1-like activity. Unfortunately, a number of molecules other than interleukin-1 will also result in stimulation of murine thymocytes treated with sub-optimal concentrations of mitogen as in this assay. These include, along with IL-1, interleukins 2, 4 and 6 as well as tumor necrosis factor (TNF) and LPS itself (Uyttenhove et al, 1988; Ranges et al, 1988). In related studies, IL-2, IL-4, IL-6 and TNF message was not detected by Northern analysis of COLO 16 cells (R.C. McKenzie, personal communication). These findings diminish the possibility that any activity that might be observed in this assay could be due to one of these other factors.

The F8 open reading frame was inserted into the mammalian expression vector pXM (Yang et al, 1986) to test for biological activity. Plasmid pXM without insert was used as a negative control, while the human IL-1β cDNA in an Okayama and Berg expression vector pcD1218 (Auron et al, 1984) was used as a positive control for IL-1 expression. Initially, difficulties were encountered in obtaining consistent activity in the thymocyte co-stimulator assay from the crude supernatant of transfected COS cells. This was deduced to be due to interference by an IL-1 inhibitor (McKenzie et al, 1990b) present in the fetal bovine serum of the media. This problem was later circumvented by maintaining the transfected COS cells in 2% FBS/DMEM. An assay of crude cell-conditioned media from a typical transfection experiment is shown in Figure A.1 (data from R.C. McKenzie). The activity present in the F8-transfected cells was routinely much less than the activity seen from

the supernatants of COS cells transfected with the IL-1β vector pcD1218. However, the activity was significant when compared to the pXM negative control.

5.3.2 D10 Stimulation Assay

Another assay, employing the helper T cell line D10 G4.1, was also used as an assay for IL-1 activity. This assay has an increased specificity for IL-1 over that of the thymocyte assay as it does not respond to either IL-6 or TNF. It does, however, share the disadvantage of the thymocyte co-stimulation assay in that it also responds to IL-2 and IL-4 (Gearing and Thorpe, 1989). As mentioned in section 5.3.1, however, these species have not been detected in the human keratinocyte cell line COLO 16. Therefore it was assumed that any stimulatory activity observed in this assay was due to IL-1 or IL-1-like factor(s). In Figure A.2 (data from R.C. McKenzie) it can be seen that significant IL-1 activity, representing approximately 50% of that obtained with pCD1218, was detected in the F8-transfected cell supernatants.

The results of these mammalian expression studies coupled with the earlier results obtained from the indirect expression studies in Xenopus oocytes indicate that the open reading frame in the partial F8 cDNA clone may encode at least one IL-1-like activity, namely thymocyte proliferation. Although these experiments can provide some evidence for an IL-1-like activity being attributed to the F8 partial cDNA, they do not rule out other possible explanations. It could be that the bioactivity observed with this clone is due solely to the small

region of sequence identity between IL-1β and the F8 cDNA at the 3' end. If this were the case, however, this finding would still be significant as it would provide new information on the specific sequences of the IL-1β molecule which may be important for biological activity. Once enough independent information is gleaned on the possible regions of the IL-1β sequence involved in biological function, it may be possible to dissect the molecular nature of the diverse activities attributed to this protein.

In an attempt to quantitate the contribution of F8 to the overall ETAF activity in COLO 16 cells, antisera against F8, IL-1α and IL-1β were used in various combinations to neutralize thymocyte proliferation activity. This work was performed by R.C. McKenzie and is presented in Arsenault et al, 1992. These studies demonstrated that the F8 peptide antiserum inhibited a small but significant component of the total IL-1 activity present in COLO supernatant. The amount of inhibition observed with the F8 antisera varied from 10 to 30% but was significant when compared to the samples treated with preimmune sera, the latter of which actually caused a slight increase in the total bioactivity present in the COLO conditioned media. The inhibition by IL-1α and IL-1β was potentiated when K3 antiserum was added compared to similar samples treated with preimmune sera. This suggests that the F8 protein does indeed contribute a small portion of the IL-1 activity seen in COLO 16 cells.

6. CONCLUSIONS

As a result of this work, IL-1β homologous sequences present in the human keratinocyte cell line COLO 16 can be attributed to at least two sequences: what appears to be keratinocyte IL-1β (C22), and a unique sequence which has minimal homology to IL-1β (F8). A major part of this thesis was involved in the cloning and sequencing of these two species.

The cloning and sequencing of a partial cDNA clone for keratinocyte IL-1β reveals that it is virtually identical to that of monocyte-derived IL-1β (excluding the 262 nucleotides missing at the 5' end of the partial cDNA clone). Since C22 is not a full length cDNA, it is possible that keratinocyte IL-1β may differ from monocyte-derived IL-1β at the 5' end. This seems unlikely, however, as only one copy of the IL-1β sequence is reported to be present in the human genome (Clark et al, 1986).

The isolation of a unique sequence from COLO 16 cells with only a small portion of homology to monocyte-derived IL-1β and which appears to possess IL-1-like activities, suggests that keratinocyte IL-1 activity may be due to a combination of factors including IL-1α, IL-1β and now F8. The percentage of ETAF activity in COLO cells that can be attributed to F8 is rather small and leads to the question of possible biological significance. Although the thymocyte proliferation activity of this unique species has been emphasized, it is possible that the

major physiological role of F8 is something quite different and as of yet to be determined.

The presence of two protein species which are immunoreactive with antisera generated against a synthetic peptide based on the F8 sequence suggest that the F8 protein exists in an unprocessed and processed form. The varying amounts of abundance of the proteins may reflect sensitivity to the state of the cell. It also appears that the ratio of these two proteins is different when comparing COLO 16 cells to normal human keratinocytes. It would be interesting to study such a system in order to glean information on the mechanisms governing protein processing.

Just recently, it has been discovered that the F8 sequence has homology to the cDNA for the bovine rod photoreceptor cyclic GMP-gated ion channel (Kaupp et al, 1989). The reason that this homology was not found prior to this was due to the fact this sequence was not present in the databases used in the searches involving the F8 sequence. Although the homology to this bovine ion channel is quite significant, (approximately 75%), the transcript size reported for this molecule (3.2 kb) is much larger than that observed for F8.

This bovine ion channel sequence has regions which are rich in lysine residues as does the F8 sequence. In addition, both sequences contain the consensus sequence for the GTP-binding site (Asn-Lys-X-Asp, where X represents any amino acid, Dever et al, 1987). In the case of the F8 sequence, this putative binding site is flanked on both sides by regions rich in charged amino acids which would theoretically position this region on the outer surface of the protein. In light of these

findings, it could be postulated that the activity in the biological assays involving F8-transfected COS cells was somehow the result of an ion channel function rather than a cytokine function. Perhaps expression of the F8 clone in COS cells caused alterations in cell membrane permeability resulting in stimulation of the COS cells to produce a factor which subsequently gave rise to the activity seen in the thymocyte co-stimulation and D10 assays. A possible link between an ion channel and the F8 clone requires the elucidation of the full length F8 cDNA sequence.

NOTE ADDED IN PROOF

The human equivalent of the bovine ion channel has been recently cloned and is reported to be identical to the F8 sequence except for two nucleotide changes, one each in the coding and noncoding region of the F8 sequence (personal communication from U.B. Kaupp to R.C. McKenzie). It is difficult to rationalize the presence of a sequence which is transcribed in a human keratinocyte cell line which would also be a functional component of the human eye. It has been suggested by Kaupp that the initial library used to isolate the F8 sequence was contaminated with genomic DNA. If this were the case, then it would suggest that the F8 clone was not derived from an mRNA species. This can be argued against, however, in light of the fact that a partial genomic sequence has been isolated which has 100% homology to the F8 cDNA sequence, in which there is an abrupt end to the homology between the cDNA sequence and the genomic sequence. This abrupt end in the sequence homology coincides with a splice site consensus sequence. This provides strong evidence that the F8 cDNA was derived from an actual spliced message and is not the result of a cloning artefact due to genomic contamination. There are also some discrepancies between the size of the transcript observed in the case of the human ion channel (3.3 kb), and the 1.6 kb transcript observed in COLO 16 cells. (personal communication from Kaupp to R.C. McKenzie) The genomic Southern patterns of the two cDNAs are also completely different (personal communication from Kaupp to R.C. McKenzie). In addition, the protein species observed is much larger for the ion channel (M_r 63 kDa) than

either species observed with the K3 antisera in COLO cells. All of these differences seem to rule out the possibility that F8 and the ion channel sequence are the same molecule. They may be related in that they are derived from the same gene but are the result of differential splicing. It could be that these two cDNA species that were isolated by myself and Kaupp were derived from two separate mRNAs as the result of differential splicing events. The isolation of the entire F8 sequence would prove whether or not this was the case. Unfortunately, the entire F8 cDNA sequence was never isolated during the course of this work, although numerous attempts were tried. This finding, however, does raise some serious questions as to whether F8 is indeed a unique keratinocyte cytokine. The results of R.C. McKenzie's mammalian expression experiments demonstrate a shared biological function between the F8 open reading frame and IL-1 as determined by the thymocyte and D10 stimulation assays. This does not prove that F8 is a member of the IL-1 family or even a cytokine. It does, however, show that this sequence is worthy of further study in order to ascertain its possible biological role in the keratinocyte.

APPPENDIX

EXPRESSION OF THE F8 PARTIAL cDNA IN MAMMALIAN CELLS

These studies were undertaken concurrently with my work by Dr. R.C. McKenzie. In brief, COS cells were transfected with 25 µg of plasmid by the DEAE dextran method and assayed for transient expression as described, (Rosenwasser et al, 1986) with the following modifications: after 3 h incubation with transfection mix containing 50 µg/ml chloroquin, cells were subjected to a one minute shock for 3 days prior to assay of the supernatant for IL-1 activity.

Conditioned supernatants from transfected COS cells were fractionated by HPLC over a TSK-2000 gel filtration column connected to a Gilson HPLC system (Gilson, France). The column was eluted with 10 mM Tris-HCl pH 7.0, 150 mM NaCl at a flow rate of 1 ml/min. Fractions were assayed immediately for IL-1 activity. The thymocyte co-stimulator assay which uses the increased proliferation of mitogen-stimulated murine thmocytes, and an assay using the antigen-specific murine T-Helper cell line D10.G4.1 were performed as described (Sauder et al, 1982; Kaye et al, 1983). The results of these experiments are shown in Figures A.1 and A.2.

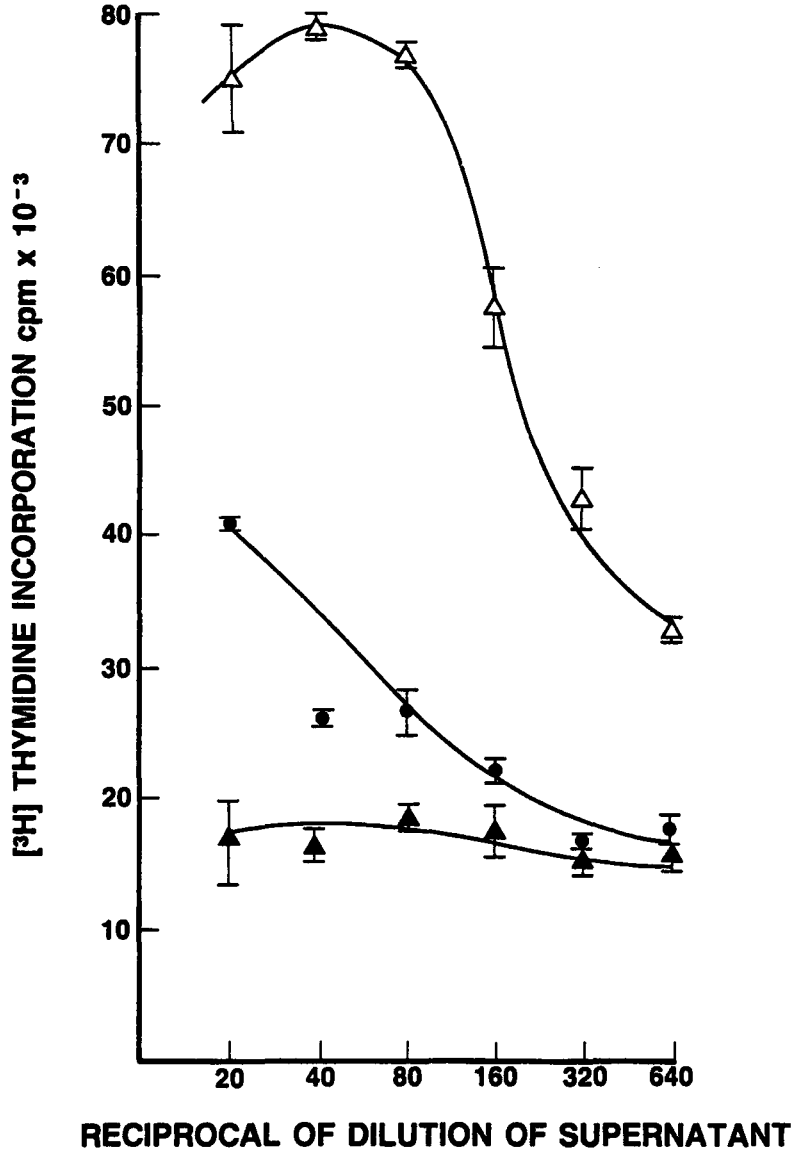


Fig. A.1. IL-1 activity of supernatants from COS cells in the thymocyte co-stimulator assay. COS cells were transfected with pcD1218 (Δ), pXMF8 (●) and pXM (▲).

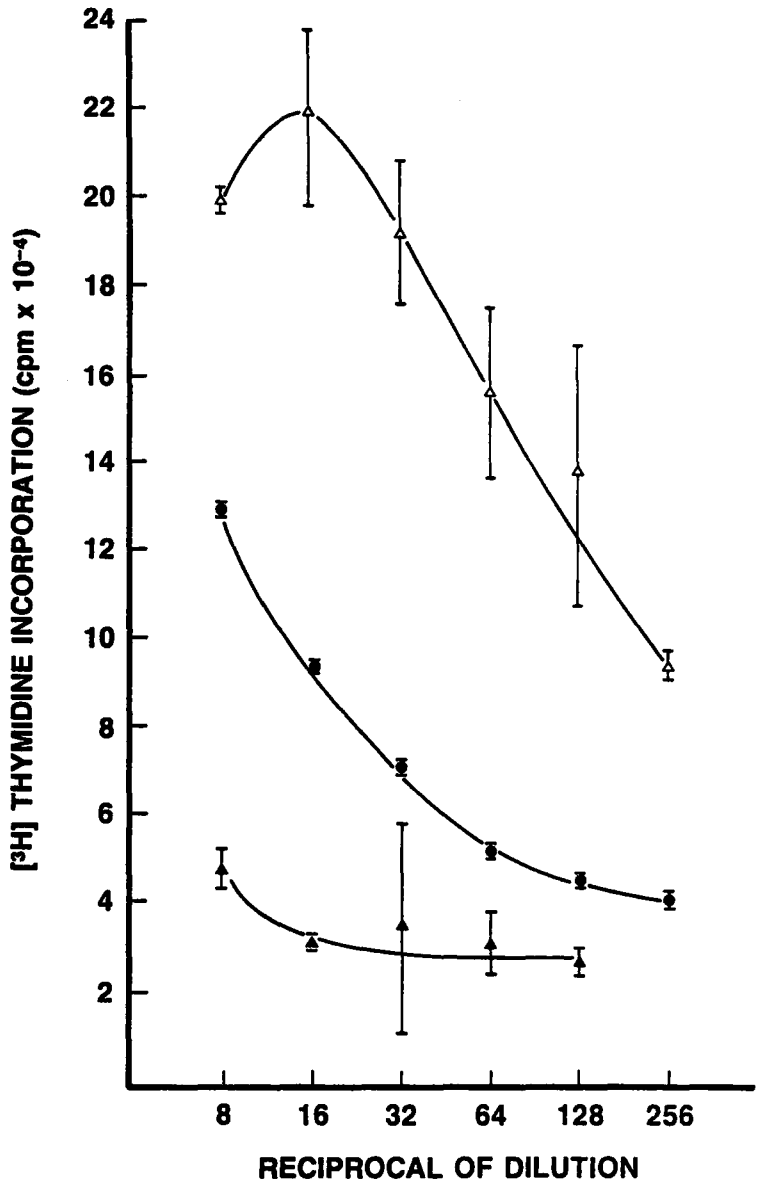


Fig. A.2. IL-1 activity of supernatants from COS cells in the D10.G4.1 T-cell assay. COS cells were transfected with pcD1218 (Δ), pXMF8 (●) and pXM (▲).

APPENDIX B

SEQUENCES OF OLIGONUCLEOTIDES

oligo	sequence	ends	reference
hIL-1β	5'-TCGCTTTTCCATCTTCTTCTTTGG-3'	728 705	Bell et al, 1987
mIL-1α	5'-CAGCTCTGGATAAGCAGC-3'	794 777	Lomedico et al, 1984
pBR AB36	5'-AAACGACGAGCGT-3'	3639 3627	
F8 AB64	5'-GACAATGTCAAGAT-3'	224 237	Arsenault et al, 1992
AB84	5'-ACATTGGGCATGGT-3'	386 373	Arsenault et al, 1992
C22 AB287	5'-GGTGCTCAGGTCATT-3'	99 85	McKenzie et al, 1990
AB288	5'-AGAGAGCTGACTGTC-3'	823 809	McKenzie et al, 1990
M13 Universal	5'-GTAAAACGACGGCCAGT-3'		
M13 Reverse Universal	5'-CAGGAAACAGCTATGAC-3'		
NcoI/SspI Adapter	5'-CATGAAACTATCCATGAAGAACAAT-3' 3'-TTTGATAGGTACTTCTTGTTA-5'		

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