IN VITRO ANALYSIS OF POLYOMAVIRUS ENHANCER BINDING PROTEINS

POLYOMAVIRUS ENHANCER BINDING PROTEINS PEA1, PEA2, AND PEA3: FUNCTIONAL ANALYSIS BY *IN VITRO* TRANSCRIPTION

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ABSTRACT

The polyomavirus enhancer consists of functionally redundant DNA sub-elements. One such sub-element, element 2, comprises a region with contiguous binding sites, or motifs, for at least three nuclear factors, designated as PEA1, PEA2, and PEA3. Although little is known of PEA2, PEA1 is presumed to be a murine homolog of human transcription activator protein 1 (AP-1), and PEA3 has recently been shown to be encoded by a member of the Ets family of oncogenes. The contributions of each factor to enhancer function are not understood.

A cell-free system was devised to assay the individual abilities of the DNA motifs recognized by PEA1, PEA2, and PEA3 to confer transcriptional activation upon a minimal promoter. The motifs were cloned and tested as monomers, as multiple tandem copies, and in paired combinations. The results of these *in vitro* studies indicate that the PEA1 motif behaves as a low affinity AP-1 binding site; that PEA1 and PEA3, but not PEA2, activate transcription; and that both the PEA1 and PEA3 motifs act synergistically. Band shift titration experiments demonstrated that neither PEA1 nor PEA3 bound to their DNA motifs co-operatively, indicating that synergistic activation of transcription by these factors is not due to co-operative binding. Finally, additional *in vitro* transcription

experiments suggest that PEA1 and PEA3 may co-operate with each other to stimulate transcription.

A current model proposes that the minimal sub-units of enhancer structure are small (8-10 base pair) DNA motifs, called enhansons, that act synergistically. I propose that the motifs for PEA1 and PEA3, but not PEA2, are enhansons of the polyomavirus enhancer.

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CONTRIBUTIONS BY OTHERS

The band shift assay shown in Figure 18 was performed by Alison Cowie. Synthetic oligonucleotides comprising factor binding sites (Figure 4C) were synthesized by Dinsdale Gooden of the Institute for Molecular Biology and Biotechnology, McMaster University. All other experimental procedures were performed by Carl B. Yong.

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INTRODUCTION

A) Enhancers

i) Introduction

Promoters of eukaryotic genes consist of many cis-acting elements which together determine the strength and specificity of expression of the promoter they constitute. In general, these elements can be divided into two groups: promoter proximal elements which lie within 110 base pairs (bp) upstream of the transcriptional initiation site; and promoter distal elements which are found far from the coding regions of the genes they activate. Members of the first group include the TATA box, CCAAT box, and Sp1 binding sites. Prominent members of the latter group are the enhancers.

Enhancers were first discovered in the small DNA tumor viruses simian virus 40 (SV40) (Banerji et al., 1981; Moreau et al., 1981) and polyomavirus (Py) (deVilliers and Schaffner, 1981). They were operationally defined as cis-acting sequences that could dramatically increase transcription from their natural promoter or from heterologous promoters, independent of orientation and position relative to the transcriptional start site (Banerji et al., 1983). Subsequently, they have been found associated with many viral and cellular genes. Enhancers are believed to be major determinants in the complex patterns of gene regulation.

ii) Functional characteristics

Enhancers exhibit a diversity of functional characteristics. The viral enhancers of SV40, Moloney sarcoma virus (Laimins et al., 1982), and Py (de Villiers et al., 1982) activate transcription in a wide variety of mammalian cells. By contrast, enhancers of the cellular genes for immunoglobulin (Gillies et al., 1983), insulin (Walker et al., 1983), and elastase (Hammer et al., 1987) confer tissue-specificity upon the promoters to which they are linked. Many enhancers are responsive to environmental stimuli such as heavy metal concentration, growth factors, phorbol esters and steroids. Enhancers have now been identified that can be loosely categorized as having either constitutive, temporally regulated, tissue-specific, or inducible activity (reviewed in Marriott and Brady, 1989).

The activity of enhancers may also be modulated by oncogenes (for review, see Kingston et al., 1985). Oncogenes arise from normal cellular genes that have acquired cancer-causing potential by undergoing various alterations. Oncogenes appear to be involved in the control of transcription, replication, and differentiation (for reviews, see Barbacid, 1986; Müller, 1986). Tumorigenesis by nuclear oncogenes may involve their abnormal modulation of cellular enhancer activity.

Several enhancers have been shown to function as

auxiliary elements for DNA replication. Viral origins for DNA replication generally consist of an origin core, which is absolutely required for replication, and one or two auxiliary replication elements which are often dispensable in certain host cells. Enhancers that have been shown to activate replication at their own or heterologous viral origin cores include those of the Harvey sarcoma virus and the immunoglobulin heavy chain gene (Lusky and Botchan, 1986), SV40 (Bergsma et al., 1982; Fromm and Berg, 1982), and Py (Muller et al., 1983; 1988; deVilliers et al., 1984; Campbell and Villareal, 1985; Bennett et al., 1989). These observations suggest that enhancer elements may activate both DNA replication and transcription by a common or very similar mechanism (Hassell et al., 1986; reviewed in De Pamphilis, 1988).

Another interesting aspect of enhancers is their association with sites which are hypersensitive to digestion by endonucleases. DNase I hypersensitive sites have been mapped to the enhancers of SV40 (Saragosti et al., 1980; Herbomel et al., 1981; Jakobovitz et al., 1982), Py (Martin et al., 1989), hepatitis B virus (Akmal et al., 1989), and immunoglobulin genes (Chung et al., 1983; Mills et al., 1983). Electron microscopy has shown that these sites are regions on the viral or cellular chromatin which are devoid of nucleosomes (Jongstra et al., 1984). It has been postulated

that one of the mechanisms by which enhancers act, both as transcription and replication elements, is by displacing or excluding nucleosomes. This is thought to increase accessibility of the chromatin to DNA-binding transcription or initiation factors (reviewed by Elgin, 1988).

iii) Structure and organization

Most enhancers occupy one to two hundred base pairs of DNA. Comparisons of the nucleotide (nt) sequences of many enhancers have revealed some consensus sequences shared by sets of enhancers. These include the SV40 enhancer core sequence 5'-GTGG(A/T)(A/T)(A/T)G-3' (Laimins et al., 1982; Weiher et al. 1983), the octamer sequence 5'-ATGCAAAT-3' (Falkner et al, 1986), and the adenovirus type 5 E1a enhancer core sequence 5'-(A/C)GGAAGTGA(A/C)-3' (Hearing and Schenk, 1983). These sequences are often present in two or three copies within an enhancer. However, no nucleotide sequence has been identified that is common to all enhancers.

Genetic analyses of the prototypic SV40 enhancer revealed that no one sequence, including the SV40 enhancer core motif, is essential for its activity. In one series of experiments, mutant SV40 viruses were constructed by generating point mutations in three distinct regions that impaired both viral growth and enhancer function. Subsequently, revertants of these mutant viruses were isolated

and shown to have acquired small tandem duplications within the enhancer region which were responsible for restored viability (Herr and Gluzman, 1985; Herr and Clarke, 1986). The duplications delimited three different sequence elements of 15-20 bp in length. Multimers of these elements were later shown to display intrinsic enhancer activity when linked to a beta-globin promoter in the absence of other SV40 enhancer sequences (Ondek et al., 1987). These and other experiments led to the realization that the SV40 enhancer is made up of multiple functionally redundant sequence elements, or motifs, and the loss of one can be compensated for by the duplication of another (Herr and Gluzman, 1985; Zenke et al., 1986; Schirm et al., 1987). Analyses of the enhancers in Rous sarcoma virus (Laimins et al., 1984), bovine papillomavirus (Weiher and Botchan, 1984) and Py (Herbomel et al., 1984; Mueller et al., 1984; 1988; Veldman et al., 1985) have led to similar conclusions.

Further mutational analysis of the three SV40 enhancer elements identified by Herr and Gluzman (1985) revealed that the elements themselves are bipartite, consisting of small 8-10 bp sub-units. These sub-units were dubbed "enhansons" because they appear to be the basic units of enhancer structure (Ondek et al., 1988). Individually, enhansons have no or very weak enhancing activity, but act synergistically when paired with a homologous or heterologous enhanson to

create an enhancer element. Enhancer elements, in turn, act in concert to produce the high levels of activity characteristic of naturally occuring enhancers (Figure 1). In contrast with the distance-independent action of enhancers, enhansons are subject to stringent spacing constraints: enhansons must be present as closely juxtaposed pairs, apparently regardless of the DNA's helical periodicity, to generate a functional enhancer element (Ondek et al., 1988). A number of motifs within the SV40 enhancer have now been characterised as enhansons (Fromental et al., 1988; Kanno et al., 1989; Macchi et al., 1989).

iv) Interaction with cellular factors

The cis-acting elements that make up an enhancer operate by interacting with protein factors. This was demonstrated by in vivo competition experiments after transfection of monkey cells with two plasmids, one containing the SV40 enhancer and promoter coupled to a reporter gene, and the other bearing the SV40 enhancer on its own. Co-transfection of increasing quantities of the enhancer-only plasmid reduced expression of the reporter gene (Schöler and Gruss, 1984). These results suggest that cells contain limiting amounts of positively-acting factors whose function requires their binding to enhancer sequences. Subsequently, direct physical interaction of nuclear protein factors with enhancers was demonstrated in

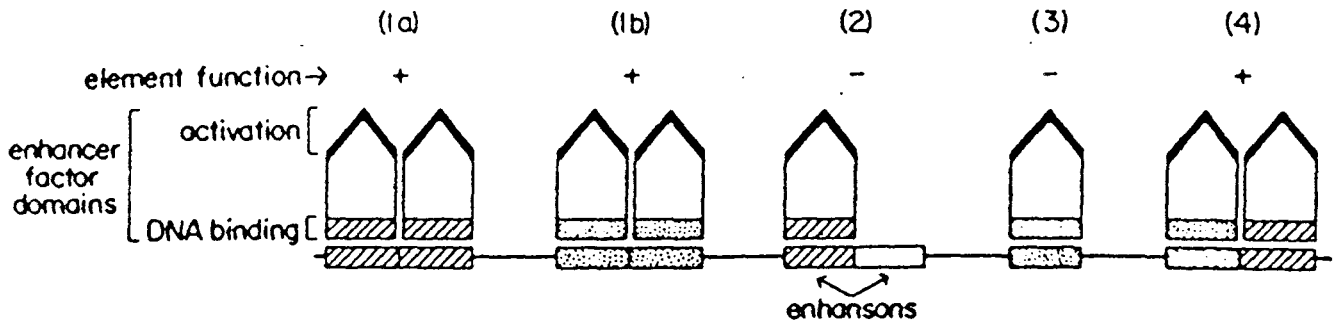
Figure 1. Enhanson model of enhancer structure.

A) Schematic depiction of an enhancer composed of enhancer elements which themselves consist of short DNA sequence motifs called enhansons. Various enhansons are shown as differently shaded boxes along a DNA molecule (line). Enhansons operate by complexing with *trans*-acting factors (shown above enhansons). Putative functional and non-functional complexes are indicated with i) shaded portions representing DNA binding domains that correspond to a specific enhanson sequence, and ii) blackened ∧ shapes representing a half-activation domain (2 and 3) that generates a functional activation domain, ∧∧, by associating with a second enhancer binding factor (1a, 1b, and 4).

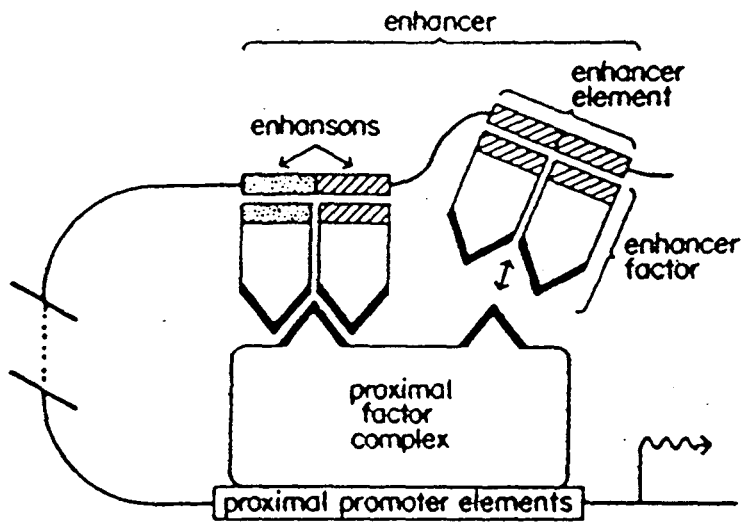
B) Illustration of two functional enhancer element-factor complexes interacting with a factor bound to proximal promoter elements. The structure of the enhancer element complexes is as explained in A). Enhansons must be properly juxtaposed under strict spacing constraints to allow formation of functional activation domains, whereas DNA between successive enhancer elements can be looped out, permitting widely separated elements to maintain their activity.

Adapted from Ondek *et al.*, 1988.

A


(1a)
(1b)
(2)
(3)
(4)
element function→
+
+
−
−
+
enhancer factor domains
activation
DNA binding
enhansons

B


enhancer
enhancer element
enhansons
enhancer factor
proximal factor complex
proximal promoter elements

vivo and in vitro by band shift, DNase protection, and methylation interference assays (Ephrussi et al., 1985; Piette and Yaniv, 1985; Schöler and Gruss, 1985; Sassone-Corsi et al., 1985; Schöler et al., 1986; Sen and Baltimore, 1986; reviewed in Maniatis et al., 1987). In SV40, specific trans-acting nuclear factors were shown to bind in vitro to the same three elements that Herr and Gluzman (1985) had shown by mutational analyses to be required for enhancer activity (Davidson et al., 1986; Zenke et al., 1986).

Enhancers are now believed to be modular structures, each module containing the binding site for a transcription factor(s). Individual modules may be found associated with a number of different enhancers and promoters, but the unique array of subelements which make up a particular enhancer are believed to determine its properties. For example, host range of enhancer activity may depend on the presence of particular modules whose corresponding transcription factors are tissue-specific. In addition to positively-acting modules, several silencer elements have been found in the enhancers of SV40 (Velcich and Ziff, 1985), Py (Rochford et al., 1990) and the immunoglobulin heavy chain gene (Hen et al., 1985; Dougherty et al., 1986).

B) Transcription Factors

i) Introduction

Many enhancer motifs have been characterized as specific binding sites for cellular factors. Frequently, these cellular proteins are also known to bind to promoter proximal elements. It has been suggested that the difference between promoter distal enhancer motifs and promoter proximal elements is quantitative, rather than qualitative (Serfling et al., 1985). In support of this view, Sp1, which binds to proximal promoter elements, has been shown to stimulate transcription at a distance when high concentrations of the factor are provided *in vitro* (Courey et al., 1989).

Some of these *trans*-acting factors have been purified from cell or tissue homogenates, and in a few cases their corresponding genes have been cloned (reviewed in Jones et al., 1988; Johnson and McKnight 1989; Struhl, 1989). These factors generally consist of multiple protein domains: a DNA-binding domain; one or more transcriptional activation domains; and possibly additional domains responsible for dimerization, nuclear localization, other protein-protein interactions and so forth. Often, these domains have been characterized by testing the properties of chimaeric proteins that are constructed, for example, by fusing the transcriptional activation domain of one factor to the DNA-

binding domain of an heterologous factor (Giniger and Ptashne, 1987; Lillie and Green, 1987; Lech et al., 1988; Courey et al., 1989; Stern et al., 1989; Tanaka and Herr, 1990; Wasylyk et al., 1990).

ii) DNA-binding and transcription activating domains

Protein domains that serve to recognize and bind to specific short nucleotide sequences are generally discrete regions 60-100 amino acids in length. Although not all have been verified by X-ray crystallography, a number of conformational structures capable of binding to specific DNA motifs have been identified. These include zinc fingers, homeodomains, and leucine zippers (reviewed in Johnson and McKnight, 1989; Struhl, 1989).

Protein sequences capable of forming zinc fingers are characterised by pairs of cysteine or histidine residues separated by a loop of about twelve amino acids. The cysteine or histidine residues are chelated to a zinc atom in a tetrahedral structure creating a polypeptide loop which makes specific contacts with DNA bases (reviewed in Evans and Hollenberg, 1988). This structure was first recognized in the Xenopus factor TFIIIA which activates RNA polymerase III transcription (Miller et al., 1985), but similar zinc fingers have since been found in factors affecting RNA polymerase II (pol II) transcription in yeast (Hartshorne et al., 1986;

Stillman et al., 1988) , Drosophila (Schuh et al., 1986; Rosenberg et al., 1986; Boulay et al., 1987) and mammals (Huckaby et al., 1987; Kadanoga et al., 1987; Page et al., 1987).

Leucine zippers are more properly categorized as dimerization interfaces. However, it has been demonstrated that factors equipped with leucine zippers must dimerize to form functional DNA-binding domains. Dimer formation may act to bring regions of high basicity (about 30 amino acids long and located immediately adjacent to the zipper structures) into a specific conformation that can bind to DNA (Gentz et al., 1989). The leucine zipper consists of three or four leucine residues spaced at intervals of seven amino acids. Two such regions are thought to interdigitate in parallel by means of hydrophobic interactions (Landschulz et al., 1988). Zipper proteins can form homodimers or heterodimers, and include GCN4 (Hope and Struhl, 1987), C/EBP (Landschulz et al., 1988), CREB (Graves et al., 1986), and the Jun family of proteins with Fos (Gentz et al., 1989; Turner and Tjian, 1989; reviewed in Johnson and McKnight, 1989).

The homeodomain is a tri-alpha-helical structure related to the helix-turn-helix structure revealed by X-ray crystallography of prokaryotic DNA-binding proteins (Pabo and Sauer, 1984). The homeodomain was first noted as a highly conserved region in many genes controlling Drosophila

development (reviewed in Wright et al., 1989). It has now been recognized in a number of mammalian proteins, including Oct-1 (Sturm et al., 1989), Oct-2 (Garcia-Blanco et al., 1989) and Pit-1 (Nelson et al., 1988). Triple alanine substitutions which destroy the ability of this region to form the tri-alpha-helical structure abolish binding in vitro, demonstrating that the homeodomain directly mediates contact with DNA (Stern et al., 1989).

DNA-binding domains are not limited to zinc fingers, leucine zippers, and homeodomains. Binding domains for which no characteristic conformational structure has been described include those of CTF/NF-1 (Santaro et al., 1988), AP-2 (Williams et al., 1988), SRF (Norman et al., 1988), and Ets-1 (Wasylyk et al., 1990).

DNA-binding is necessary but not sufficient for transcriptional activation, which requires the presence of at least one transcriptional activation domain. Activation domains are usually 30-100 amino acids long, and many transcription factors have multiple activation domains (Giniger and Ptashne, 1987; Courey and Tjian, 1988; Hollenberg and Evans, 1988; Treizenberg et al., 1988; Bohmann and Tjian, 1989; Tora et al., 1989). They have not been characterised as well as DNA-binding domains, partly because activation surfaces do not seem to exist as discernible structures. Many have not been systematically examined, and those that have

appear to be characterized only by an abundance of a particular type of amino acid (reviewed in Mitchell and Tjian, 1989).

Activation domains of the yeast factors GAL4 and GCN4 have been studied intensively (Hope and Struhl, 1986; Giniger and Ptashne, 1987; Ma and Ptashne, 1987; reviewed in Ptashne, 1988). Activation function in these proteins resides in negatively-charged regions capable of forming amphipathic alpha-helices. Other factors that appear to activate transcription by acidic alpha-helices include the Herpes viral trans-activator Vmw65 (Treizenberg et al., 1988), the glucocorticoid hormone receptor (Hollenberg and Evans, 1988), the bipartite activator domain of CREB (Yamamoto et al., 1990), and the Jun proteins (Bohmann et al., 1987).

Activation function has also been localized to regions rich in proline (20 to 30%), or glutamine (about 25%). The proline residue is notable for disrupting alpha-helix formation. Proline-rich activation regions are found in CTF (Mermod et al., 1989), Jun (Bohmann and Tjian, 1989) and AP-2 (Williams et al., 1987). Four regions containing approximately 25% glutamine have been shown to mediate transcriptional activation in Sp1 (Courey et al., 1989). Inspection of other transcription factors suggests that glutamine-rich activation domains exist in the Drosophila proteins Antennapedia, Ultrabithorax, and Zeste (Courey and

Tjian, 1988), in the yeast proteins HAP1 and HAP2 (Pfiefer et al., 1989); and in mammalian Jun (Bohmann et al., 1987), and SRF (Norman et al., 1988).

iii) Protein-protein interactions

Activation domains are believed to interact with the general transcription apparatus of the cell, promoting formation or stability of pre-initiation complexes. In transient transfection experiments, over-expression of some factors that bind to enhancers can inhibit transcription from promoters that lack binding sites for those factors (Gill and Ptashne, 1988; Meyer et al., 1989). These observations are consistent with competition in vivo by enhancer-binding factors for a limited pool of soluble factors which are absolutely required for transcription. The formation of a pre-initiation complex on a promoter fragment has been resolved into five intermediate complexes by native gel electrophoresis (Buratowski et al., 1989). These complexes are the result of sequential binding by five protein components (including RNA polymerase II and a factor that binds to the TATA box). It is conceivable that all five proteins, or another as yet unidentified protein, may be targets for interaction with transcription factors.

One target for transcription activator domains may be the serine- and threonine-rich carboxy-terminal repeat of the

largest sub-unit of RNA polymerase II. This carboxy tail is highly conserved and has many hydroxyl groups which could facilitate non-specific interactions with activation domains through hydrogen bonding. Removal of the repeat eliminates pol II transcription (Allison et al., 1988; Bartolomei et al., 1988), but, moreover, loss of the repeat can be compensated for by strong transcription activators (Allison et al., 1989). Direct contact of a transcription factor with pol II has been shown by the specific retention of the yeast activator protein GCN4 on pol II-Sepharose affinity columns (Brandl and Struhl, 1989). However, the DNA binding domain of GCN4, not the activation domain, was both necessary and sufficient for this interaction.

Another target for transcription factors may be the TATA box binding factor TFIID (Sawadogo and Roeder, 1985a; Nakajima et al., 1988; Horikoshi et al., 1989). TFIID may be the eukaryotic counterpart for prokaryotic sigma factor (Horikoshi et al., 1989; Sopta et al., 1989; reviewed in Lillie and Green, 1989) which is the most loosely bound component of bacterial RNA polymerase and is required for accurate initiation of transcription in prokaryotes (reviewed in Helmann et al., 1988). Mammalian TFIID appears to be the least abundant and most labile of the general transcription proteins, and its binding is correlated with the first step in initiating transcription (Davison et al., 1983; Fire et

al., 1984). DNase protection studies indicate that transcription factor ATF facilitates assembly of the pre-initiation complex by making contact with TFIID to stabililize its binding to the TATA box (Horikoshi et al., 1988).

Protein-protein interactions are also involved in inter-regulation amongst transcription factors. For example, transcription factor AP-2 binds to SV40 large T antigen, thereby negatively regulating the activity of the latter (Mitchell et al., 1987). Moreover, some trans-acting factors have been shown to lack either an activation or a DNA-binding domain. Instead, these factors form multi-protein complexes in which different members of the complex furnish specific DNA-binding and activation functions (reviewed in Berk and Schmidt, 1990). The paradigm example is trans-activation by the Herpes viral protein Vmw65. Vmw65 is unable to bind DNA on its own, but activates transcription of promoters bearing the motif 5'-TAATGARAT-3'. Vmw65 complexes with the cellular protein Oct I, which confers specific DNA-binding ability (Goding and O'Hare, 1988; Stern et al., 1989). Complexes such as this are thought to allow tissue-specific action of apparently ubiquitous trans-activating factors.

A number of transcription factors have been shown to activate transcription synergistically, i.e. activation by two factors bound to promoter elements which is greater than the sum of the effects of each working alone. Synergistic

activation has been demonstrated both between homologous factors (Lin et al., 1988; Courey et al., 1989) and between heterologous factors (Kakidani and Ptashne, 1988; Lin et al., 1988; Mermod et al., 1988; Tora et al., 1989; Janson and Pettersson, 1990). In some cases, synergistic activation is the result of co-operative binding. Band shift assays were used to show that the factors Sp1 and OTF1 interact to bind DNA with greater affinity as a complex than either factor could on its own, resulting in synergistic transcriptional activation (Janson and Pettersson, 1990).

Synergy occurs between the yeast GAL4 protein and the rat glucocorticoid receptor to activate transcription from a mammalian promoter containing binding sites for both proteins (Kakidani and Ptashne, 1988). Since these factors come from such evolutionarily divergent organisms, it seems unlikely that they contact each other directly, and suggests instead that they both contact a common, evolutionarily conserved target. Synergy between these two factors has been demonstrated <u>in vitro</u> under conditions in which all of their binding sites are saturated (Carey et al., 1990; Lin et al., 1990). In this situation, co-operative binding effects are rendered irrelevant, indicating the existance of a common target for these two diverse transcription factors.

C) Polyomavirus

i) Introduction

Polyomavirus was discovered as a contaminating virus in cell-free filtrates of leukemic AK mouse cells. When injected into newborn mice, the virus induced parotid tumors and so was called the parotid agent (Gross, 1953). Subsequently, this parotid agent was shown capable of inducing a wide range of tumors in mice and named polyomavirus (Stewart, 1958). Polyomavirus is a member of the papovavirus family. It consists of a covalently closed, double-stranded DNA molecule complexed with cellular histones. The viral minichromosome is enclosed within an icosahedral protein capsid composed of three structural proteins, VP1, VP2, and VP3 (for review, see Tooze, 1981).

ii) Lytic cycle

The natural host for polyomavirus is the mouse. Murine cells support the complete viral lytic cycle and are said to be permissive for the virus. Cells of other rodents, such as rats and hamsters, allow replication of the viral DNA but do not yield virus particles and so are termed semi-permissive. Cells from species which do not support viral replication are designated as non-permissive (Basilico et al., 1970).

The sequence of events in the polyomavirus lytic cycle

is reflected in its genomic organization (Fig. 2). The cycle can be divided into early and late phases. The early phase starts with viral adsorption to the host cell and entry by pinocytosis (Borgaux, 1964). After it is transported to the nucleus, the virus particle is uncoated and transcription of the early transcription unit commences (Mattern et al., 1966). The early transcription unit spans nt 173-2912 (numbering according to Soeda et al., 1980) and encodes three proteins: small, middle, and large tumor (T) antigens. The three T antigens appear at 10-12 hours post-infection.

The late phase of the lytic cycle is deemed to begin after the onset of DNA replication. At this point, mRNAs from the late transcription unit accumulate. The late transcription unit comprises nt 5002-2926 and codes for the three capsid proteins. The ratio of late- to early-strand mRNA transcripts rises from 1:10 to about 20:1 (Acheson, 1981) as the nascent viral DNAs are assembled into chromatin and packaged into virions. The lytic cycle is completed within 30-36 hours.

iii) Polyomavirus enhancer

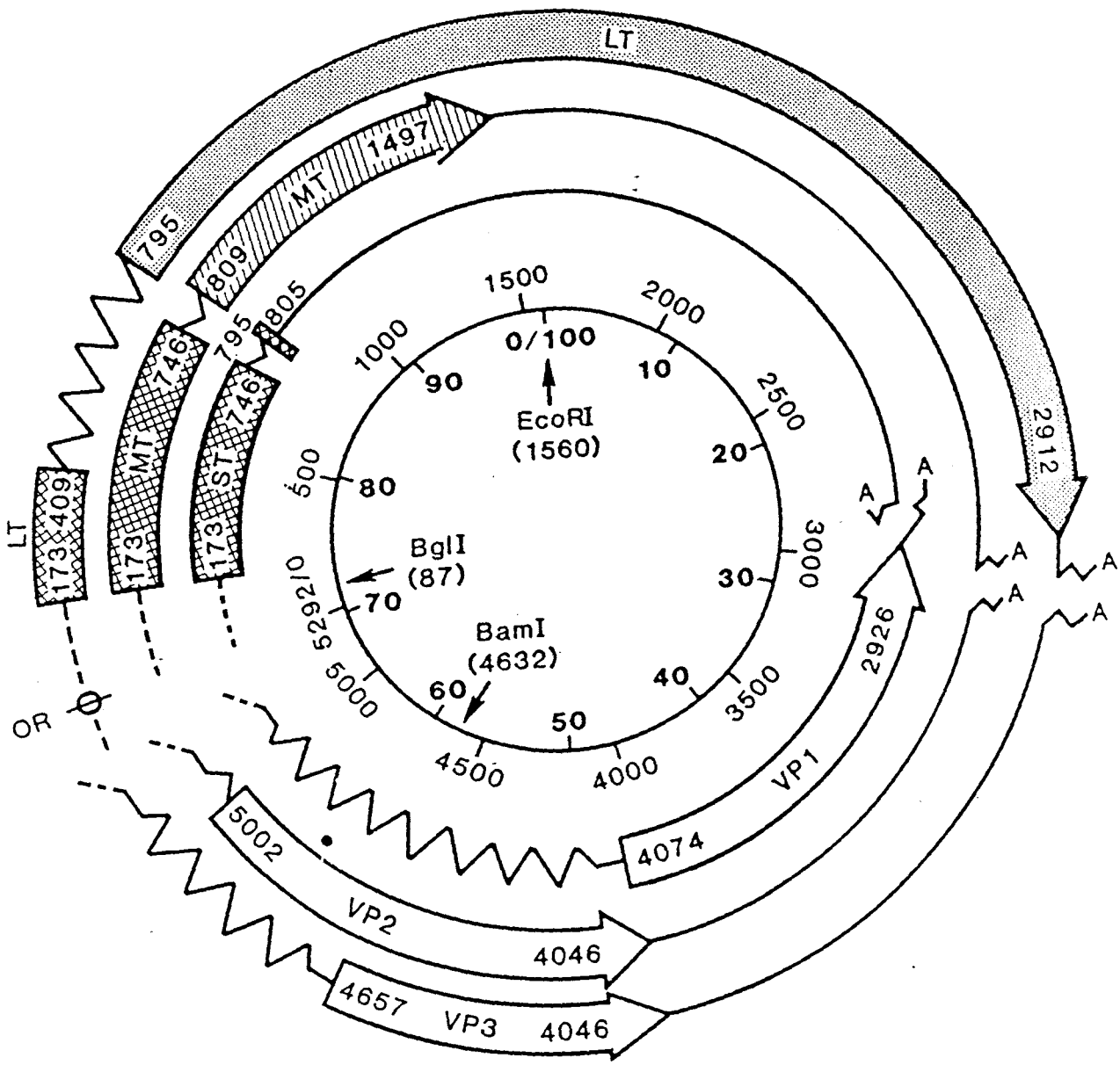
a) Structure

The 460 bp region lying between the early and late transcription units is not known to code for any proteins. This region contains the cis-acting elements required for efficient transcription of both the early and late strands as

Figure 2. Organization of the polyomavirus genome.

The inner circle represents the viral genome with map units and nucleotide co-ordinates (Soeda et al., 1980) given on the inner and outer portions of the circle, respectively. Regions encoding early (clockwise) and late (counter-clockwise) gene products are shown as arrows, with RNA initiation, splice, and polyadenylation sites indicated. The reading frame common to small T antigen (ST) and the amino-terminal regions of middle T (MT) and large T (LT) antigen is denoted by cross-hatching. OR represents the viral origin for DNA replication.

Adapted from Tooze, 1981.


LT
795
MT
1497
809
795
805
MT
746
ST
746
LT
173
409
173
173
OR
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20
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40
50
60
70
80
90
1000
1500
2000
2500
3000
3500
4000
4500
5000
5292/0
500
EcoRI
(1560)
BglI
(87)
BamI
(4632)
2912
A
A
A
A
A
A
2926
VP1
4074
5002
VP2
4046
4657
VP3
4046

well as the sequences necessary for viral DNA replication (Tyndall et al., 1981; Amati, 1985; reviewed in Hassell et al., 1986). The Py enhancer was first identified as a 244 bp fragment within the viral non-coding region (deVilliers and Schaffner, 1981). It activates both transcription from the viral early promoter and replication of the viral DNA (Muller et al., 1983; deVilliers et al., 1984; Mueller et al., 1984; Hassell et al., 1986; Campbell and Villarreal, 1988).

The enhancers of the two prototype Py strains (A2 and A3) do not contain tandemly repeated sequences characteristic of other papovavirus enhancers, such as the 72 bp repeats of SV40 (Benoist et al., 1981), the 69 bp repeats of BK virus (Rosenthal et al., 1983), or the 62 bp repeats of lymphotropic papovavirus (Mosthaf et al., 1985). However, five other Py strains (P16, Toronto large plaque, MV, Ts 48, and NG59R) have been shown to harbor tandem duplications that contain a conserved 24 bp sequence found between nt 5114-5137 (Ruley and Fried, 1983). As was the case with the SV40 enhancer, genetic analyses of the Py enhancer in the context of the viral genome failed to reveal any particular sequence motif essential for activity, although many deletions were found that impaired activity (Tang et al., 1987).

Herbomel et al., (1984) tested the activities of Py enhancer restriction fragments in transient assays. These authors concluded that the Py enhancer consists of two

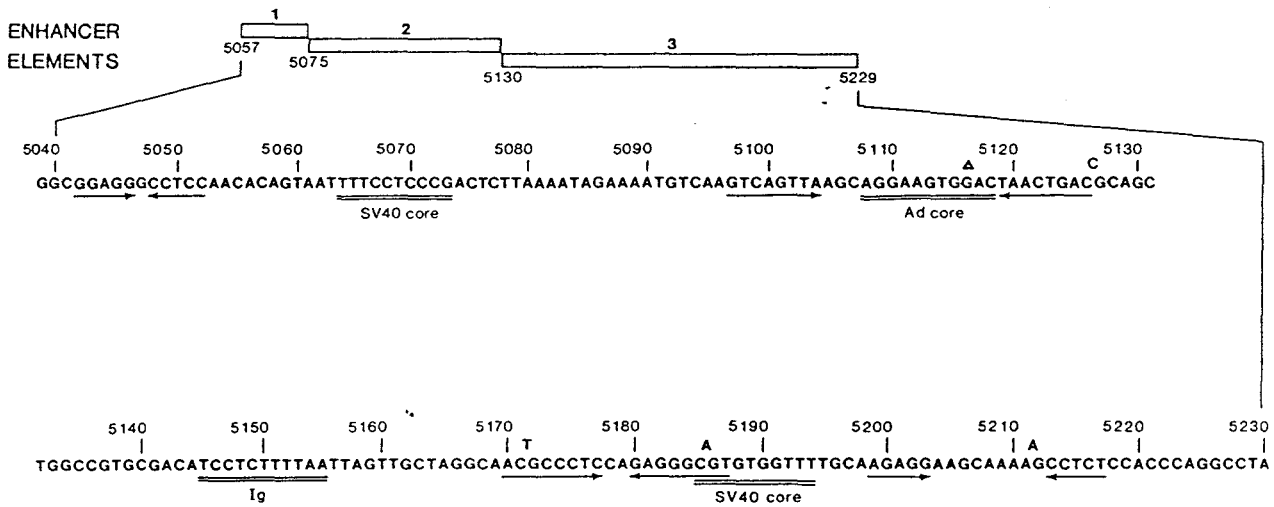
separable enhancers, designated A and B, which differed from each other by their activities in murine fibroblasts and embryonal carcinoma cells. Subsequently, other groups divided the Py enhancer into three (Muller et al., 1983; Mueller et al., 1988) and four (Veldman et al., 1985; Tang et al., 1987) sub-elements that can individually activate DNA replication and transcription. These results indicate that the Py enhancer, like other enhancers, is a mosaic of functionally redundant elements.

The sequences specifically required for transcription enhancement have been defined by deletion mutagenesis (Mueller et al., 1988). These authors tested the ability of various sections of the Py enhancer to activate transcription of a reporter gene in transfected murine 3T3 fibroblasts. They found that the Py enhancer comprises about 172 bp and could be divided into three domains which they named elements 1, 2 and 3 (Fig. 3; Mueller et al., 1984; 1988). These domains could replace each other functionally, and combinations of any two of them functioned almost as well as the complete enhancer. Elements 1, 2, and 3 have not, however, been shown to have intrinsic enhancer activity as isolated elements. Moreover, multiple sequence motifs are present within each element whose deletion or mutation affected the function of the elements to varying degrees (Mueller et al., 1988). This suggests that elements 1, 2, and 3 are not the minimal units

Figure 3. Structure of the polyomavirus enhancer.

Numbered boxes depict functionally redundant enhancer elements. The DNA sequence of the enhancer is shown below the boxes in two segments. Inverted repeats are indicated by arrows, and immunoglobulin (Ig), adenovirus (Ad), simian virus 40 (SV40) core sequence homologies are marked by double underlines. Numbering is according to Soeda et al., (1980). Nucleotide letters listed above the DNA sequence indicate insertions, whereas Δ indicates a nucleotide not present, in the strain of polyomavirus used in defining elements 1, 2, and 3.

Adapted from Mueller et al., 1988.


ENHANCER
ELEMENTS
1
2
3
5057
5075
5130
5229
5040
5050
5060
5070
5080
5090
5100
5110
5120
5130
GGCGGAGGGCCTCCAACACAGTAATTTTCCTCCCGACTCTTAAAATAGAAAATGTCAAGTCAGTTAAGCAGGAAGTGGACTAACTGACGCAGC
SV40 core
Ad core
5140
5150
5160
5170
5180
5190
5200
5210
5220
5230
TGGCCGTGCGACATCCTCTTTTAATTAGTTGCTAGGCAACGCCCTCCAGAGGGCGTGTGGTTTTGCAAGAGGAAGCAAAAGCCTCTCCACCCAGGCCTA
Ig
SV40 core

of Py enhancer structure.

Many of the sequences of the Py enhancer responsible for transcriptional enhancer function overlap with elements required for viral replication (Muller et al., 1983; Mueller et al., 1984). Mutations in the Py enhancer generated *in vitro* by treatment with sodium bisulfite were shown to have qualitatively similar effects on both replication and transcription in transient assays (Tang et al., 1987). These investigators also isolated a revertant virus in which a single nucleotide change in a mutant enhancer restored both viral replication and transcription. These observations indicate that the Py enhancer activates replication and transcription by a common mechanism (Hassell et al., 1986).

b) Regulation by *trans*-acting factors

Infecting or transfected wild-type Py genomes are unable to express early genes or replicate in undifferentiated embryonal carcinoma (EC) cells and other cells that are able to differentiate *in vitro*. Uncoated Py genomes could, however, be recovered from the nuclei of infected undifferentiated EC cells, suggesting that the block occurs at the level of transcription (Swartzendruber et al., 1977). Early evidence that the Py enhancer functions through *trans*-acting proteins came from experiments in which non-permissive, undifferentiated EC cells that had been pre-infected with Py

were fused to permissive differentiated EC cells. Viral proteins were expressed in the resulting heterokaryons, suggesting that permissive cells contain a positively-acting diffusible factor (Boccara and Kelly, 1978).

Semi-permissive cell systems have now been used to isolate a number of mutant polyomaviruses that have overcome cellular blocks to the viral lytic cycle (Katinka et al., 1980; Tanaka et al., 1982; De Simone et al., 1985; Maione et al., 1985). All of these mutants have been found to contain rearrangements and/or point mutations in the viral enhancer (Amati et al., 1985). In many cases, these changes in the enhancer sequences have been shown to generate novel binding sites for cellular factors. The F9.1 mutant, selected for its viability in undifferentiated F9 EC cells, has a single base-pair transition at nt 5233 in its enhancer (Fujimura et al., 1981). This mutation creates a novel sequence motif that is recognized by a factor present in the host cell (Kovesdi et al., 1988). The newly created motif is responsible for the function of the B enhancer in F9 cells and acts synergistically with two adjacent DNA motifs to achieve the new specificity of activity (Tseng and Fujimura, 1988). Another mutant, PyNB11/1, capable of growing in neuroblastoma cells, harbors a 91 bp tandem duplication in the enhancer region (Caruso et al., 1986). It has been shown that the PyNB11/1 duplication generates a novel binding motif for a

nuclear factor, NF-D (Caruso et al., 1990). Similarly, other host range mutants PyFL78 (De Simone et al., 1985), PyEC PCC4-5000 (Melin et al., 1985) and the Py Toronto strain (Ruley and Fried, 1983) all harbor enhancer rearrangements generating novel binding sites for the transcription factor AP-1. These observations clearly indicate that the array of factor-binding motifs in the Py enhancer determines its potential to function in a given host cell.

The Py enhancer has also been demonstrated to control tropism of viral replication in infected mice (Rochford et al., 1990). These authors have identified sequence elements in the Py enhancer which specify permissiveness to replicate in particular organs, including the mouse kidney, pancreas, and heart. Interestingly, these elements all appear to function as organ-specific repressors of viral enhancer function rather than as positively-acting elements. Earlier, Cremisi and Babinet (1986) reported evidence of negative regulators of Py enhancer function. They found that brief treatment of non-permissive PCC4 EC cells with the protein synthesis inhibitor cycloheximide allowed these cells to express Py large T antigen, suggesting the presence of a labile repressor of the enhancer in untreated cells.

These results are consistent with the hypothesis that activity of the viral enhancer is controlled by the presence or absence of cellular protein factors recognizing specific

DNA motifs in the enhancer. A number of factors have been described that bind specifically to sequence motifs within the Py enhancer (Piette et al., 1985; Bohnlein and Gruss, 1986; Ostapchuk et al., 1986; Piette and Yaniv, 1986; 1987; Johnson et al., 1987; Sassone-Corsi et al., 1987; Martin et al., 1988; reviewed in Jones et al., 1988).

Like other viral enhancers, the Py enhancer is modulated by the activities of several oncogene products. For example, the enhancer is normally almost silent in mouse myeloma cells, but can be stimulated to a high degree of activity by the expression of the proto-oncogene c-Ha-ras (Wasylyk, C. et al., 1987). The c-Ha-ras product, p21, is associated with the cell membrane and is involved in cellular signal transduction (Levinson, 1986). The enhancer can also be repressed in HeLa or L cells by E1a, the transforming protein of adenoviruses (Borelli et al., 1984).

D) Element 2 of the Polyomavirus Enhancer

i) Sequence motifs

Element 2 contains an adenovirus enhancer core sequence flanked by an inverted repeat (Fig. 3). The two inverted repeat sequences are not functionally equivalent; deletion of the late region proximal repeat (nt 5096-5104) had a

negligible effect on enhancer activity whereas deletion of the early region proximal repeat abolished activity (Mueller et al., 1988). Furthermore, it was shown that both the adenovirus core and the inverted repeat on its right must be present for element 2 activity; deletion of either one impaired the ability of element 2 to activate transcription in conjunction with either of elements 1 or 3. (Mueller et al., 1988). These observations suggest that element 2 is itself composed of still smaller units which embody the minimal units of enhancer structure, such as the enhansons in SV40 (Ondek et al., 1988).

A 26 bp fragment of element 2 (nt 5108-5130) has been shown to activate both transcription and replication when multimerized, though not as a monomer, after transfection of murine 3T3 or human HeLa cells (Veldman et al., 1985). The boundaries of this fragment are identical to those of the alpha-core replication element described by Muller et al., (1988). One copy of the alpha-core is able to activate replication of a plasmid bearing the Py origin of DNA replication (nt 5265-90) after transfection of murine cells (in the presence of virally encoded large T antigen) (M. McWilliams, unpublished results).

The alpha-core comprises both the adenovirus core consensus sequence and the inverted repeat on its right. Moreover, this segment of the Py enhancer has been shown to contain a ras-responsive element (RRE) (Imler et al., 1988).

These authors showed that the RRE was also responsible for mediating enhancer activation by the tumor promoter 12-O-tetradecanoylphorbol-13-acetate (TPA) and serum components in transient assays. Further analysis revealed that the RRE comprised two motifs which were independently capable of mediating enhancer activation by the ras oncoprotein (Imler et al., 1988; Satake et al., 1988; Yamaguchi et al., 1989). The two motifs have subsequently been characterized as binding sites for the nuclear factors PEA1 and PEA3.

Three distinct factors have now been shown to interact with sequences within element 2. These have been designated as PEA1, PEA2 (Piette and Yaniv, 1987), and PEA3 (Martin et al., 1988; also called PEBP5: Murakami et al., 1990). The three factors bind to adjoining sites within the alpha-core region (Fig. 4A); to date, no factors have been identified that bind to element 2 upstream of the 5' boundary of the alpha-core, nt 5108.

ii) PEA1

PEA1 was initially detected as a binding activity in nuclear extracts of murine 3T6 cells. PEA1 binds to a motif within element 2, 5'-TGACTAA-3'(nt 5114-5130 of the Py A2 strain), as well as to sites within the SV40 and c-fos enhancers (Piette and Yaniv, 1987; Satake et al., 1988). PEA1 has been studied indirectly in vitro by determining how the presence

of the PEA1 binding motif influences transcription or replication in transient assays. These assays indicate that PEA1 activates both transcription (Imler et al., 1988; Mueller et al., 1988; Satake et al., 1988; Wasylyk et al., 1988; Wasylyk, C. et al., 1988; Yamaguchi et al., 1989) and replication (Muller et al., 1988; Murakami et al., 1990).

The PEA1 motif differs by a single nucleotide from the high-affinity binding site for human transcription activator protein 1 (AP-1), 5'-TGACTCA-3' (Lee et al., 1987). Both motifs confer transcriptional activation that can be induced to higher levels by serum, TPA and the oncoprotein $p21^{Ha\text{-}ras}$ (Angel et al., 1987; Lee et al., 1987; Ryder and Nathans, 1988; Imler et al., 1988; Yamaguchi et al., 1989). Although it has not been formally demonstrated, it is probable that these two sequences are bound by the same factor, and PEA1 is presumed to be a murine homolog of AP-1 (Kryszke et al., 1987; Piette et al., 1988).

The AP-1 motif is recognized by the Jun family of proteins, which include the products of the c-jun, jun-B, and jun-D genes. These proteins bind as homodimers or as heterodimers with the c-fos gene product by means of a leucine zipper (reviewed in Kouzarides and Ziff, 1989), and the particular members of the heterodimer determine its DNA binding affinity (Halazonetis et al., 1988). Although the transcriptional activation domains of the Jun/Fos heterodimer

have not been thoroughly defined, an acidic proline-rich activation domain has been identified near the carboxy-terminus of the c-Jun protein (Bohmann and Tjian, 1989). Moreover, each species of Jun protein may have unique roles in cell regulation. All three have distinct patterns of induction by serum components and mRNA expression (Ryder et al., 1989; Hirai et al., 1989), and Jun-B appears to be a negative regulator of c-Jun activity (Chiu et al., 1989; Schutte et al., 1989).

PEA1 is critical for enhancer activity in vivo. Mutant polyomaviruses containing point mutations that abolish PEA1 binding in element 2 are defective in replication and transcription. Single nucleotide reversions that restore PEA1 binding in these mutants simultaneously restore enhancer function (Martin et al., 1988). These authors also found that binding of PEA1 and enhancer function in wild-type and revertant viruses correlates with the formation of a DNase I hypersensitive site (HS) at nt 5125 in viral minichomosomes isolated from the nuclei of infected mouse cells. Furthermore, part of the PEA1 motif, 5'-TGACT-3', is commonly duplicated in various wild-type strains of Py, suggesting that the PEA1 motif confers some advantage for virus viability (Ruley and Fried, 1983).

Active PEA1 is either not present or present in very low amounts in undifferentiated F9 cells (Kryszke et al.,

1987; Wasylyk, C. et al., 1988). However, treatment with retinoic acid induces these cells to differentiate into parietal endoderm, and differentiation is accompanied by the appearance of PEA1 binding activity (Kryszke et al., 1987). PEA1 also binds to the enhancer-like sequences of the murine MHC gene H-2K, a gene which is only expressed after F9 differentiation (Kryzske et al., 1987), suggesting that PEA1 is important in the control of gene expression during early embryonic development.

PEA1 binding activity can be induced in quiescent NIH 3T3 cells by transformation with SV40 (Piette et al., 1988). Moreover, PEA1 transcriptional activity is modulated by the expression of a variety of oncogenes. Oncogenes with common properties can be placed into functional groups. One such classification is into immortalizing and transforming oncogenes. Immortalization is the ability to convert cell lines which normally senesce and perish into lines that can be passaged indefinitely. Transformation confers anchorage-independent growth. Transforming oncogenes are generally weak in their ability to immortalize cells and immortalizing oncogenes generally have weak transforming activity. Wasylyk, C. et al., (1988) linked four copies of an oligonucleotide bearing the PEA1 motif to the beta-globin gene and measured beta-globin transcripts in cells transfected with these constructs. Co-transfection of plasmid expression vectors of

five transforming oncogenes (c-Ha-ras, v-src, Py middle T, v-mos, and c-fos) activated PEA1, whereas expression of immortalizing oncogenes, (Py large T, myc, SV40 large T, and E1a) did not affect PEA1 activity (Imler et al., 1988; Wasylyk, C. et al., 1988). Constitutive activation of PEA1 may be a critical event in transformation by oncogenes.

PEA1 is thought to be an AP-1 homolog, and the AP-1 motif is recognized by Jun/Fos heterodimers. jun itself has an oncogenic counterpart, v-jun, found in the genome of avian sarcoma virus 17 (Reviewed in Vogt and Tjian, 1988). Comparison of v-Jun and c-Jun by in vitro transcription assays has revealed that v-Jun is a much stronger transcription activator than the proto-oncogene product. The viral protein may have greater activity because it lacks an amino-terminal negative-regulating region that is present in c-Jun (Bohmann and Tjian, 1989).

iii) PEA2

PEA2 was first detected as a binding activity in 3T3 nuclear extracts. PEA2 binds independent of PEA1, and protects the sequence 5'-GACCGCA-3' from digestion by DNase I. The PEA2 motif lies immediately 3' of, and adjoining to, the PEA1 binding site in element 2 (Fig. 4A; Piette and Yaniv, 1987; Satake et al., 1988). Martin et al., (1988) have shown by DNase I footprinting experiments that PEA2 binding is either

enhanced or stabilised by PEA1.

There is no consensus as to the role of PEA2. The enhancer activity of element 2 in murine 3T3 cells is substantially reduced when the PEA2 binding site is deleted from an element 1 plus 2 construct, suggesting that PEA2 is a transcription activator (Mueller et al., 1988). Deletion of the PEA2 motif abolished activity of the Py alpha-core in replication assays using 3T3 cells, indicating that PEA2 is an activator of DNA replication (Muller et al., 1988).

By contrast, another group has found that a synthetic multimer comprising only the PEA2 binding motif was incapable of activating replication in C127 cells (Murakami et al., 1990). Furthermore, Wasylyk et al., (1988) have reported that constructs bearing PEA2 sites juxtaposed to mutated PEA1 motifs had no effect on expression of reporter gene mRNA in mouse L cells. Moreover, constructs bearing the entire alpha-core displayed lower activity than equivalent constructs bearing point mutations that block PEA2 binding. This difference in activities was eliminated after treating the cells with cyclohexamide. Repression of enhancer activity in undifferentiated F9 cells was also relieved when PEA2 binding was abolished, and decreased slightly when these cells differentiated. These results are consistent with the hypothesis that PEA2 is not an independent positively-acting factor, and, instead, is a labile, developmentally-regulated

repressor of PEA1.

iv) PEA3

The presence of a third protein that binds to the Py alpha-core was first suggested when mutant Py viruses were isolated that were defective in replication and transcription, but in which binding of PEA1 and PEA2 were not affected in vitro. These authors noticed a DNase I hypersensitive site at nt 5136 in the genome of the wild-type A3 Py strain (corresponding to nt 5112 in the A2 strain) that was absent in a mutant virus but reappeared in a revertant. This suggested that HS 5136 was induced by a factor interacting with the sequence, 5'-AGGAAG-3', that had been deleted in the mutant. Using an oligonucleotide probe comprising the deleted sequence, a third binding activity, PEA3, was detected in both 3T6 murine nuclei and human 293 nuclei (Martin et al., 1988).

The PEA3 binding site is contained within the conserved enhancer motif known as the adenovirus E1a enhancer core (Hearing and Shenk, 1983; Satake et al., 1988). The PEA3 motif is also present in the human papovarius BK and human beta-interferon enhancers (Yamaguchi et al., 1989). Like PEA1, the PEA3 motif increases transcription from heterologous promoters, and this activity can be stimulated by serum components and TPA (Martin et al., 1988; Satake et al., 1988; Wasylyk, C. et al., 1989; Yamaguchi et al., 1989).

PEA3 transcriptional activity can be stimulated by expression of a number of oncogenes whose products are not localized to the nucleus (v-*src*, Py middle T, Ha-*ras*, v-*mos*, v-*raf*); whereas its activity is not affected by the oncoproteins SV40 large T, Py large T, Myc, E1a, BPV E2 and E5, Fos, or Jun, all of which are localized to the cell nucleus (Wasylyk et al., 1989).

The PEA3 motif is recognized by the Ets family of proteins (Wasylyk et al., 1990; Xin et al., manuscript in preparation). *In vitro* synthesized avian c-Ets-1 binds specifically to the PEA3 motif, and co-transfection of c-*ets*-1 expression vectors activates transcription of reporter genes from promoters bearing PEA3 motifs in transient assays (Wasylyk et al., 1990). Recently, Xin et al., (manuscript in preparation) isolated a partial cDNA encoding a polypeptide that specifically interacts with the PEA3 binding motif, 5'-AGGAAG-3'. The predicted amino acid sequence of this factor, named PEA3, indicates that it is distinct from, but related to, c-Ets-1, and is highly homologous to Ets-1 in the region corresponding to its DNA binding domain.

The c-Ets proteins are encoded by a family of genes with sequence identity to the v-*ets* oncogene of avian erythroblastosis virus, E26. E26 causes erythroblastic and myeloblastic leukemias in chickens (Moscovici et al., 1981; Radke et al., 1982). v-*ets* has two cellular homologs, *ets*-1

and ets-2 (Watson et al., 1985;1986), and is related to the erg (Rao et al., 1987; Reddy et al., 1987), and elk genes (Rao et al., 1989). The c-ets-1 gene product is believed to have a role in lymphoid cell activation (Pognonec et al., 1988), whereas the product of c-ets-2 may be involved in cell proliferation (Bhat et al., 1987). Both Ets proteins are phosphorylated and localized to the nucleus (Boulukos et al., 1988; 1989; Fujiwara et al., 1988). It is probable that the Ets proteins are all DNA-binding transcription factors with transcriptional activation domains (Wasylyk et al., 1990; Gutman and Wasylyk, 1990). The DNA-binding domain is located in the carboxy-terminal portion of the protein that is basic, highly conserved between different ets gene family members, but bears no resemblance to any described DNA-binding structures. The transcriptional activation domain(s) of the Ets proteins remains to be characterized.

v) Interaction of proteins binding to element 2

PEA1, PEA2, and PEA3 can bind to their respective motifs independent of each other in vitro (Piette and Yaniv, 1987; Wasylyk et al., 1989; Yong et al., manuscript in preparation), although the binding of PEA2 may be enhanced by PEA1 (Martin et al., 1988). Analyses of mutant polyomaviruses have revealed that binding sites for all three factors are required for optimal enhancer function. The Py enhancer comprises a second

possible PEA3 binding motif located at nt 5228-5233. A mutant polyomavirus has been isolated with a functionally impaired enhancer in which PEA1 and PEA2 sites are intact, but both PEA3 sites are altered. Comparison of revertants suggests that enhancers with intact PEA1 and PEA2 sites are only functional if one of the two PEA3 sites are present (Tang et al., 1987; Martin et al., 1988). However, it is not known how each these factors contributes to activate (or repress) transcription.

E) Functional Assays for Enhancer Elements

i) Transient in vivo assays

Functional mapping of enhancer sequences has largely been accomplished by the use of transient in vivo assays. For transient assays, segments of the enhancer are linked to an heterologous eukaryotic promoter driving transcription of a reporter gene in a recombinant plasmid. The reporter gene codes for an enzyme whose activity can be easily measured, such as bacterial chloramphenicol acetyl transferase (CAT) (Gorman et al., 1982; Laimins et al., 1982), or an mRNA that can be detected by RNase protection or primer extension analysis, such as beta-globin mRNA (Treisman et al., 1983). Activity of the cloned enhancer sequence is determined by its effect on expression of the reporter gene in cultured cells

after they have been transfected with the recombinant DNA.

Transient assays have also been developed that measure the ability of enhancer elements to activate DNA replication from a viral origin. These are based on the fact that certain restriction endonucleases, such as Dpn I, distinguish between methylated and unmethylated bases in the sequences they recognize. Replication assays employ plasmids bearing viral (mammalian) origins of replication coupled to the enhancer element whose function is to be tested. The plasmids are prepared from bacteria, in which Dpn I sites are methylated and cleavable by the enzyme, and transfected into cultured mammalian cells. Any plasmid DNA which is then replicated at least once in mammalian cells is not methylated, and therefore resistant to cleavage by Dpn I. The amount of Dpn I resistant DNA which can subsequently be recovered provides a measure of the ability of the enhancer element to activate DNA replication (Peden et al., 1980; Muller et al., 1983).

ii) In vitro transcription systems

An understanding at the biochemical level of the mechanisms through which initiation of transcription is controlled depends on the ability to study these processes in vitro. Cell-free systems employing soluble whole-cell extracts capable of transcribing exogenously added DNA templates were first developed more than a decade ago (Weil et al., 1979;

Manley et al., 1980; 1983). Subsequently, procedures to prepare transcriptionally active extracts have been optimized to take advantage of the nuclear localization of transcription factors and improve extract activity (Dignam et al., 1983; Gorski et al., 1986; Lee et al., 1988; Shapiro et al., 1988; Zerivitz and Akusjarvi, 1989).

Nuclear extracts have now been employed to investigate a variety of phenomena. A few examples include: RNA processing (Padgett et al., 1983; Hernandez and Keller, 1983; Krainer et al., 1984); tissue-specific activity of the mouse albumin promoter using extracts prepared from organ tissue (Gorski et al., 1986); cell-type-specific transcription of the rat prolactin gene (Cao et al., 1987); and temporal patterns of transcription using Drosophila stage-specific embryonic extracts (Heberlein and Tjian, 1988).

In vivo, enhancers can stimulate transcription up to a thousand-fold when inserted downstream as well as upstream of the transcribed DNA sequences, and can exert their effects from up to several kilobase pairs away from the transcription start site (Moreau et al., 1981; Banerji et al., 1981; Fromm and Berg, 1982; Wasylyk et al., 1983). Stimulation of transcription in vitro from heterologous promoters was first described for the SV40 enhancer under conditions where no chromatin reconstitution takes place (Sassone-Corsi et al., 1984). Sergeant et al., (1984) also reported stimulation of

transcription *in vitro* of DNA templates when partially or completely nucleosome free. However, in these and other cell-free systems (Sassone-Corsi et al., 1984; 1985; Wildeman et al., 1984; Sergeant et al., 1984; Schöler and Gruss, 1985; Stillman et al., 1985; Li et al., 1986; Westin et al., 1987), replication and transcription are only moderately dependent on enhancer sequences, producing at best ten-fold increases in activity. Moreover, significant distance-independent enhancer activity has yet to be demonstrated *in vitro*.

The difficulties encountered in attempts to mimic enhancer functions *in vitro* are most likely due to differences from conditions that exist within the cell. Aside from obvious discrepancies in DNA template configurations and composition of the aqueous environment, *in vitro* reactions are performed in the absence of an organizing nuclear matrix and plasma membrane. Nuclear organization could be essential for enhancer activity. For example, one model proposed for the mechanism of enhancer function suggests that factors interact with enhancer sequences to direct the surrounding regions of chromatin to the nuclear matrix or some other nuclear compartment which is enriched with transcription factors (Serfling et al., 1985).

F) Experimental Rationale

At the time these experiments were begun, the polyomavirus enhancer had been mapped as functionally redundant sub-elements. In particular regard to Py enhancer element 2, results from deletion analyses suggested that element 2 was composed of still smaller functional units. Moreover, sequence-specific binding proteins that recognize DNA motifs within element 2 had recently been identified, but whose functions were not known. This study was undertaken in an attempt to determine whether these motifs were functional units of element 2, and how enhancer-binding factors interact with these units to activate transcription.

In view of the modest enhancer effects reported in attempts to test the activity of entire enhancers in vitro, I elected instead to study the Py enhancer by testing the activities of small enhancer motifs that were known targets of sequence-specific binding proteins. First, I describe a straightforward system that allowed me to investigate the effects of individual enhancer sequence motifs on transcription in vitro. Next, I describe the use of the system to study the structure of the polyomavirus enhancer alpha-core. The activities conferred by sequence motifs of the alpha-core, cloned in various combinatorial arrangements, were used to define functional units of this enhancer segment, as

well as to investigate the roles of the cognate protein factors that bind to them. Finally, I discuss how the results of this study relate to a current model of enhancer organisation.

MATERIALS AND METHODS

A) Construction of Recombinant Plasmids

E. coli strain DH1 or DH5 were made competent for DNA-uptake by a modification of the $CaCl_2$/RbCl method (Maniatis et al., 1982). Large scale plasmid preparations were done by a modification of the alkaline lysis method (Maniatis et al., 1982; Frank Graham, personal communication). Plasmids for use as templates for in vitro transcription were purified on two successive CsCl gradients (Maniatis et al., 1982).

Procedures for manipulating DNA were performed as described in Maniatis et al., (1982). Restriction endonucleases and modifying enzymes were used in accordance with conditions specified by their manufacturers (Boehringer, Amersham, Pharmacia). Sequences of recombinant plasmids were verified by means of a DNA sequencing kit employing the dideoxy method (Sequenase, United States Biochemical).

The plasmids $p(C_2AT)_{19}$ and $pML(C_2AT)_{19}$ containing the G-less cassette were gifts of M. Sawadogo and R. Roeder. Recombinant plasmids that served as templates for in vitro transcription were based on $p(C_2AT)_{19}$. The plasmid containing the minimal promoter, p371 (Fig. 1B), was constructed by inserting the complementary oligonucleotides

5'-GATCTCGAGCTCGGGGGGCTATAAAAGGGG-3' and

3'-AGCACGAGCCCCCCGATATTTTCCCCCTAG-5'

(gifts of J. Smiley) into the Sac I site of $p(C_2AT)_{19}$.

Plasmid p300 was derived from p371 as follows. p371 was cut with Bam HI and treated with Bal 31 nuclease. Bam HI linkers were ligated to the ends of the digested DNAs, which were then circularized with T4 DNA ligase and used to transform E. coli. Plasmids bearing deletions in the 3' end of the G-less cassette were isolated. DNA fragments comprising the synthetic TATA sequence and the shortened G-less cassette (Xho I to Bam HI) were isolated and ligated to the vector DNA fragment (Xho I to Bam HI) of p371. The resulting plasmid p300 contains a G-less cassette of approximately 300 bp.

Oligonucleotides were synthesized comprising the high-affinity AP-1 binding motif, and the known binding sites of PEA1, PEA2, and PEA3, as they appear in Element 2 of the Py enhancer (see Figure 4C for sequences). Mutant versions bearing base substitutions that block factor binding (Piette and Yaniv, 1987; Wasylyk, et al., 1989; Satake et al., 1989) were also made. Plasmids were generated by cloning these oligonucleotides into the Bgl II site of p371 and named according to the nature of the oligonucleotide with a subscript denoting the number of copies inserted. Copy number and orientation were confirmed by sequencing.

To construct $p(SP1)_6371$, the 21 bp repeat sequence of the SV40 promoter was isolated from pUC A_0 (Zenke et al., 1986) as a Bam HI to Nco I fragment. After filling the recessed ends and ligation of Bgl II linkers, the fragment was

cloned in the early orientation into the Bgl II site of p371.

B) Cell Culture and Extract Preparation

FM3A murine mammary epithelial cells were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum and antibiotics (20 ug/ml gentamycin and 1 ug/ml Fungizone). Cells were grown in suspension culture at 37 ^{0}C to a density of 1 x 10^{6} cells/ml.

Nuclear extracts to be used for transcription assays were prepared according to Shapiro et al., (1988). The hypotonic, sucrose restore, nuclear resuspension, and nuclear dialysis buffers used are as described in Shapiro et al., (1988). Rapidly dividing cells were harvested by centrifugation at 4500 x g (4000 rpm, Sorvall H6000A rotor) for 20 min at 4 ^{0}C, washed twice in cold phosphate buffered saline (10mM sodium phosphate pH 7.4 150mM NaCl), and pelleted in a 15-ml graduated centrifuge tube by centrifugation at 300 x g (1000 rpm, Sorvall H6000A rotor) for 10 min at 4 ^{0}C. The cells were resuspended in 5 packed cell volumes (PCV) of hypotonic buffer and allowed to swell for 10 min on ice. The swollen cells were pelleted by centrifugation at 1000 x g (2000 rpm, Sorvall H6000A rotor) for 10 min at 4 ^{0}C and the cells resuspended in 3-4 PCV of fresh hypotonic buffer. The

cells were broken with 10 strokes in a Ten Broek tissue grinder (>95% cell breakage). 0.1 vol of sucrose restore buffer was quickly added and the homogenate mixed with two more strokes in the tissue grinder. The homogenate was immediately centrifuged for 30 sec at 10,000 rpm in a Sorvall HB-4 rotor (16,000 x g) at 4 ^{0}C. The supernatant was removed by aspiration. The nuclear pellet resuspended in 4 ml of nuclear resuspension buffer per 1 x 10^{9} cells and transferred to a straight wall centrifuge tube (Beckman Ultra Clear). The extract was agitated on a vertical rotator for 30 min at 4 ^{0}C to ensure complete lysis of nuclei, and then cleared by centrifugation at 2 ^{0}C for 1 h at 145,000 x g (39,000 rpm, Beckman Ti-50 rotor). Solid ammonium sulfate (0.33 g/ml) was added to the supernatant, which was then agitated on a vertical rotator for 20 min at 4 ^{0}C after the ammonium sulfate was dissolved to ensure complete precipitation of protein. The precipitate was collected by centrifugation at 85,000 x g for 20 min at 2 ^{0}C (30,000 rpm, Beckman Ti-50 rotor). The pellet was redissolved in 0.8 ml of nuclear dialysis buffer per 10^{9} cells, and dialyzed twice for 90 min each against >500 volumes of nuclear dialysis buffer at 4 ^{0}C. The extract was quick frozen in small aliquots and stored at -85 ^{0}C without significant loss of activity for at least three months.

Protein concentration of the nuclear extracts was determined by means of a colorimetric assay (Bio-Rad) using

bovine serum albumin as a standard. Typically, 3 x 10^9 cells yielded 3 mls of nuclear extract at concentrations of 8-10 mg/ml protein.

C) In Vitro Transcription

An elegant system to study transcription in vitro was devised by Sawadogo et al., (1985a) using the vector $p(C_2AT)_{19}$. This vector obviates the need for truncated templates used in most in vitro transcription assays, and provides an attractive alternative to the use of more laborious methods of detecting accurately initiated transcripts from supercoiled DNA templates. $p(C_2AT)_{19}$ contains a "G-less cassette" composed of 380 A,C, and T residues on the copied strand. A promoter fragment cloned upstream of the G-less cassette creates a template which can be transcribed in vitro in the absence of GTP. A specific transcript is produced whose 5' and 3' ends are determined by the cap site and the first G residue flanking the cassette. Since the transcribed RNA product contains no G residues, transcription can be performed in the presence of the chain terminator analog 3'-O-methyl GTP and RNase T1, thereby effectively eliminating improperly initiated transcripts. The G-less cassette vector has been used to study a number of transcriptional phenomena, including factors

interacting with the adenovirus major late promoter (Sawadogo and Roeder, 1985b), tissue-specific transcription from the mouse albumin promoter (Gorski, et al., 1986), and optimization of nuclear extracts for transcription (Shapiro et al., 1988).

Transcription reactions were performed according to Sawadogo and Roeder (1985a) with minor modifications. Reactions were in a volume of 20 ul containing 200 ng of supercoiled test template DNA, 200 ng p300 (control template), 200uM ATP, CTP, 3'-O-methyl-GTP, and 20uM UTP, 10uCi [alpha-^{32} P]UTP (3000 Ci/mmol, Amersham), and 4 to 6ul of nuclear extract (final concentration 2mg/ml protein) in a buffer of 20mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) pH 7.9, 50mM KCl, 2mM dithiothreitol (DTT), 0.2mM ethylene glycol-bis(beta-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA), 4mM $MgCl_2$, and 4mM spermidine. After a 30 min incubation at 30^0 C, unlabeled UTP was added to a final concentration of 200uM and the reactions incubated for another 15 min at 30^0 C. 15U of RNase T1 were added and incubation continued for 15 min at 30^0 C. The reactions were terminated with 380ul of STOP buffer (50mM Tris pH 7.4, 10mM EDTA, 0.5% sodium dodecyl sulfate (SDS) and 25ug/ml tRNA). Samples were extracted twice with phenol/chloroform (1:1), once again with chloroform,and precipitated with a mixture of 50ul sodium acetate 3M pH 5.2 and 950ul ethanol. Pellets were

washed once in absolute ethanol, air-dried, and dissolved in 10ul of loading dye (90% formamide, 0.01% bromphenol blue, 0.01% xylene cyanol in TBE buffer). Samples were electrophoresed through 4% polyacrylamide/8M urea gels in TBE buffer (89mM Tris-borate, 89mM boric acid, and 2mM EDTA). Gels were dried and exposed to Kodak XAR-5 film with intensifying screens at -85 ^{0}C. Densitometric scanning was done on an LKB Ultroscan enhanced laser densitometer.

RNA molecular weight markers were generated by *in vitro* transcription using SP6 RNA polymerase (Boehringer) according to Melton et al., (1984). DNA templates were prepared from the plasmid pSP64dl165 (a gift of D. Cook) linearized at appropriate restriction sites.

D) Band Shift Assays

The band shift assay, also known as the electrophoretic mobility shift or gel retardation assay, is based on the fact that protein-DNA complexes can be resolved from faster migrating uncomplexed DNA by electrophoresis through polyacrylamide gels in low ionic strength buffers (Fried and Crothers, 1981; Garner and Revzin, 1981). It was initially developed to study equilibrium kinetics of prokaryotic DNA-binding factors, but has since been used to detect eukaryotic

proteins that were believed to recognize specific DNA sequences.

Binding reactions were carried out in a final volume of 20ul containing 0.2ng of end-labeled DNA probe, 2ug of poly(dIdC) in 10mM HEPES pH 7.9, 50mM NaCl, 0.1mM EDTA, 4mM $MgCl_2$, 2mM DTT, 2mM spermidine, and 0.5-9 mg/ml nuclear extract (see figures for particular concentrations used with each experiment). The reaction was terminated by addition of 5ul of a 10% glycerol, 1% orange G dye mixture. The samples were applied to 5% polyacrylamide gels (40:1 acrylamide: bis-acrylamide) in 0.25X TBE and electrophoresed in the same buffer with recirculation until the orange G dye was near the bottom of the gel. Gels were dried prior to autoradiography.

To generate DNA for use as probes, oligonucleotides were cloned as single copies into the Bgl II site of the plasmid p371. Plasmid DNA was digested with Eco RI and Xho I to release fragments comprising the cloned oligonucleotide with some flanking polylinker sequences. The restriction digests were treated with calf intestinal alkaline phosphatase, end-labeled with T4 polynucleotide kinase and [gamma-^{32}P]ATP (5000 Ci/mmol, Amersham) and electrophoresed through 12% native polyacrylamide gels in TBE. Probe fragments were eluted from gel slices by the crush-soak method (Maxam and Gilbert, 1980). Briefly, probe fragments were localized on the wet gel by autoradiography (2 min exposure) and

excised. The excised fragments were crushed and incubated in 400ul of elution buffer (500mM ammonium acetate, 1 mM EDTA, 0.1% SDS) at 37 ^{0}C overnight. Polyacrylamide particles were removed by passage of the supernatant through 1MM Whatman filter paper. The purified probe fragments were then precipitated with 800ul ethanol, pelleted, and resuspended at 0.1 ng/ul in an appropriate volume of TEN_{100} (10mM Tris pH 8.0, 1mM EDTA, 100mM NaCl).

RESULTS

A) A Minimal Promoter Template for *In Vitro* Transcription

I wished to generate a plasmid based on the vector $p(C_2AT)_{19}$ (Sawadogo and Roeder, 1985a) which would allow me to test transcriptional activity conferred by individual enhancer motifs. Reasoning that strong promoters might easily mask small effects of such motifs, I sought a "minimal promoter" to direct transcription of the G-less cassette in $p(C_2AT)_{19}$. The TATA, or Goldberg-Hogness box, is thought to specify the precise starting point for transcription *in vivo* because its removal results in a heterogeneity of start sites from various promoters (Corden et al., 1980; Breathnach and Chambon, 1981; Wasylyk et al., 1983). It has been shown that a TATA box is the only element required to accurately direct initiation of transcription by RNA polymerase II (Homa et al., 1988, J. Smiley, personal communication). The use of a simple TATA sequence to direct transcription might eliminate the influence of transcription factors binding to the promoter other than those specifically recognizing the motif(s) under study. The TATA element is bound by the factor TFIID (Sawadogo and Roeder, 1985b; Horikoshi et al., 1989), and this binding is correlated with the first step in the initiation of transcription (Davison et al., 1983; Fire et al., 1984).

I chose a synthetic 30 bp DNA fragment comprising only a consensus TATA sequence (corresponding to that of the

adenovirus 2 major late promoter) and linker sequences as a basal promoter for the G-less cassette vector (see Materials and Methods for sequence). This fragment was cloned in the proper orientation into the Sac I site of $p(C_2AT)_{19}$ to generate the plasmid p371 (Fig. 4B).

For use as an internal control in transcription reactions, I constructed plasmid p300 which was derived from p371 by digestion of the 3' end of the G-less cassette using Bal 31 nuclease. p300 and p371 are identical except for the lengths of their G-less cassettes.

Transcription of p371 is presumed to initiate at an A residue about 25 bp downstream of the center of the TATA box, generating a 371 nt G-less transcript. Transcription of p371 in vitro by the cell-free extract produced a discrete RNA species of the expected size (Fig. 5, lane 1). Transcripts defined by the TATA box in p300 yielded an RNA of approximately 300 nt. (Fig. 5, lane 2). For unknown reasons, p371 was consistently transcribed about 1.5 times better than p300 using different plasmid preparations (Fig. 5, compare lanes 1 and 2). This may result from the simple fact that, assuming uniform labelling per unit length, transcripts from p300 contain fewer total radioactive counts than the longer transcripts produced from p371. Regardless of the explanation, induction ratios given in subsequent experiments are measured relative to the level of transcription of p371, but the

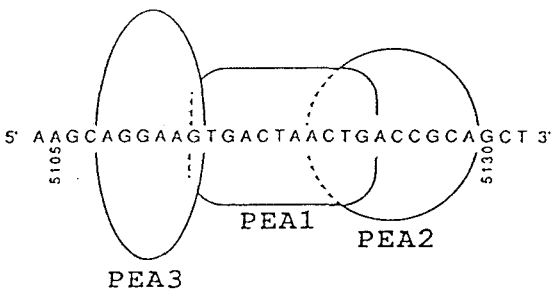
Figure 4. Nucleotide sequences

A) DNA sequence of the wild-type polyomavirus enhancer alpha-core region with binding by nuclear factors PEA3, PEA1, and PEA2 depicted schematically. Numbering according to Soeda et al., 1980.

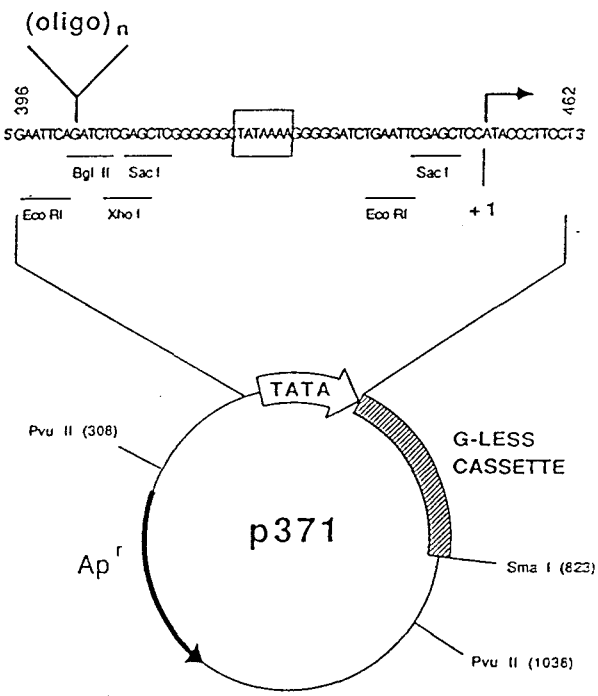
B) Structure of the minimal promoter template p371. The promoter sequence is shown with selected restriction sites, TATA element (boxed), and putative transcriptional start site (+1). Oligonucleotides comprising DNA motifs to be tested for transcriptional activity in vitro are inserted at the unique Bgl II site. The G-less cassette is depicted as a hatched segment.

C) Sequences of the double-stranded oligomers comprising motifs found in the Py enhancer alpha-core that were cloned into the vector p371. The oligomers were named according to the nature of the motif(s) they comprised (left hand column). Cognate protein factors are shown schematically as in A), and the sequences they protect from DNase I digestion are underlined. Lower case nucleotide letters denote linker sequences; point mutations that block factor binding are boxed.

A



B



C

OLIGONUCLEOTIDES

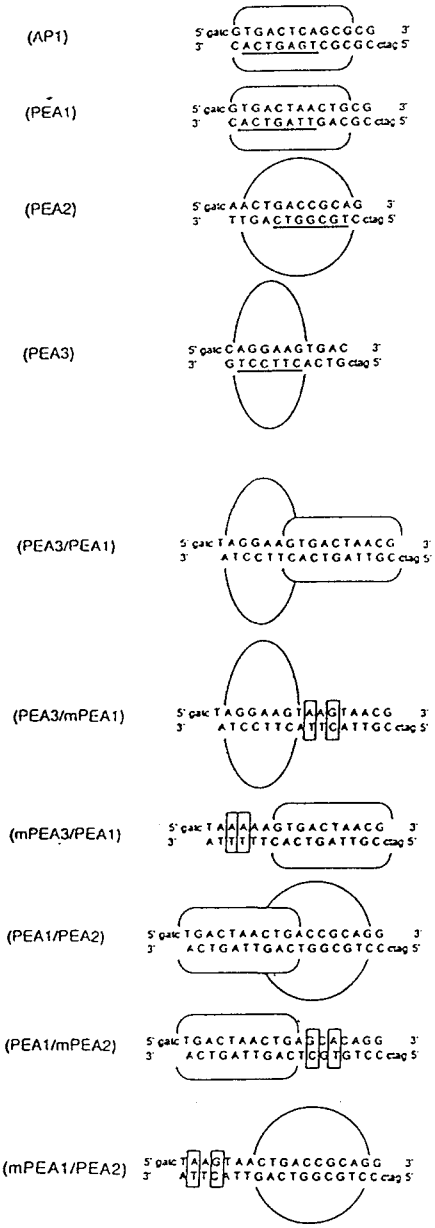
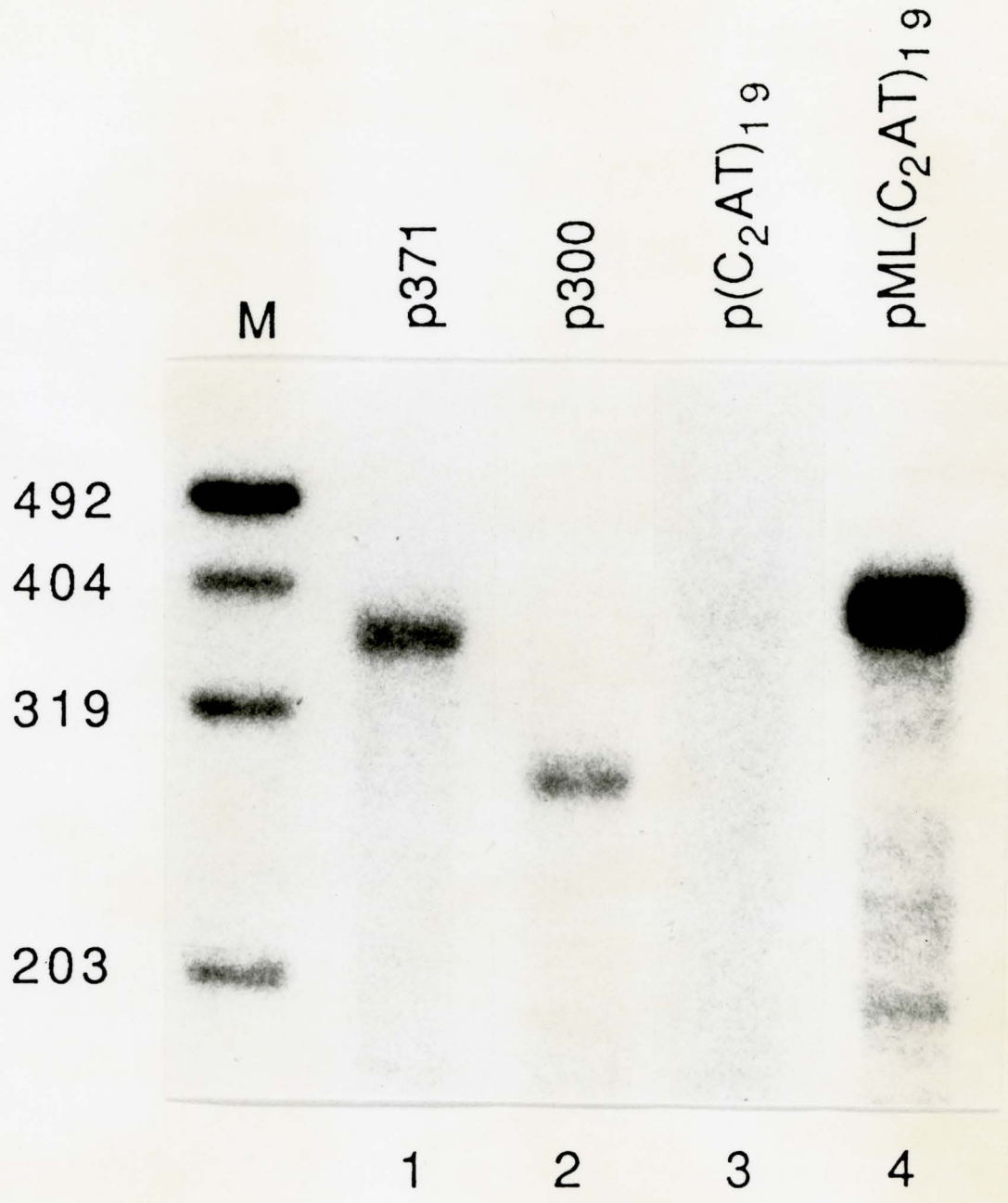


Figure 5. Transcription of exogenously added templates by the FM3A nuclear extract.

Plasmids used as templates for transcription reactions are indicated above each lane and comprise a G-less cassette driven by either the minimal promoter (lanes 1 and 2), no promoter (lane 3), or the entire adenovirus 2 major late promoter (lane 4). The *in vitro* transcription products were analyzed as described in Materials and Methods. M: RNA markers of sizes (in nucleotides) as indicated.


M
p371
p300
p(C2AT)19
pML(C2AT)19
492
404
319
203
1
2
3
4

densitometry values in each lane were adjusted according to the internal standard, p300, prior to calculating these ratios.

No transcripts were detected from the promoterless parent plasmid $p(C_2AT)_{19}$ when tested *in vitro* (Fig. 5, lane 3). Furthermore, the minimal promoter was weak by comparison to the entire Adenovirus 2 major late promoter driving transcription of the G-less cassette in $pML(C_2AT)_{19}$ (Fig. 5, compare lane 1 with lane 4), suggesting that the minimal promoter is suitable for detecting small increases in transcriptional activity.

As noted by others, (Gorski et al., 1986), transcripts shorter than full-length occasionally appeared as a result of RNA degradation or premature termination of transcription (Fig. 5, lane 4). These were not due to promiscuous initiation within the G-less cassette because the promoterless construct did not yield detectable transcripts under identical *in vitro* conditions.

To test whether the minimal promoter was responsive to transcription activators in the nuclear extract, binding sites for the activator proteins Sp1 (Dynan and Tjian, 1983) and AP-1 (Kouzarides and Ziff, 1989) were inserted immediately upstream of the TATA box. *In vitro* transcription from the minimal promoter was substantially increased by the 21 bp repeats of the SV40 promoter (which comprise six binding sites

for transcription factor Sp1; Dynan and Tjian, 1983) (Fig. 6A, compare lanes 1 and 2), and by four tandem consensus AP-1 motifs (Fig. 6A, compare lanes 1 and 3). These observations indicate both that the extract contains biologically active transcription factors, and that the minimal promoter is responsive to their action.

To test whether transcription could be activated by upstream elements in the absence of a TATA box, I constructed plasmids $p(AP1)_5371$ and $p(AP1)_5(C_2AT)_{19}$. The former bears five AP-1 binding sites upstream of the TATA box driving transcription of the G-less cassette, whereas the latter bears five AP-1 sites immediately upstream of the G-less cassette without a TATA box. When these two plasmids were tested for transcription *in vitro*, a strong signal arose from $p(AP1)_5371$ transcripts, but no transcripts were detected from the TATA-less construct, $p(AP1)_5(C_2AT)_{19}$ (Fig. 6B, compare lanes 1 and 2). Therefore, transcription of the cassette apparently requires a TATA box even when strong upstream promoter elements are present. Furthermore, no transcripts were detected from p371, $p(SP1)_6371$, or $pML(C_2AT)_{19}$ in reactions containing 0.05 ug/ml *alpha*-amanitin (Fig. 7, compare lanes 1-3 to lanes 4-6), indicating that transcripts from the minimal promoter, the minimal promoter augmented by strong upstream elements, or from the entire adenovirus 2 major late promoter, are products of functional RNA polymerase II in the

Figure 6. Effect of *cis*-acting elements on transcription *in vitro*.

A). Transcription of templates comprising the G-less cassette coupled to i) the minimal promoter on its own (lane 1), ii) the promoter with six upstream Sp1 binding sites (lane 2), or iii) the promoter with four upstream AP-1 binding sites (lane 3). Transcripts from p300, included as an internal standard, are indicated by an arrowhead. *In vitro* synthesized transcripts were analyzed as described in Materials and Methods. All lanes are of the same autoradiographic exposure times.

B) *In vitro* transcription products from a template bearing five AP-1 binding sites either upstream of the minimal promoter driving the G-less cassette (lane 1), or immediately upstream of the cassette in the absence of a TATA sequence (lane 2). Bands resulting from the internal control template, p300, are indicated. *In vitro* synthesized products were analyzed as described in Materials and Methods.

A
p371
p(SP1)6371
p(AP1)4371
p300
1
2
3
B
p(AP1)5371
p(AP1)5(C2AT)19
p300
1
2

Figure 7. Effect of alpha-amanitin on transcription in vitro.

Transcription was performed in the absence (lanes 1 to 3) or presence (lanes 4 to 5) of the toxin alpha-amanitin at 0.05 ug/ml, a concentration which is inhibits RNA polymerase II activity. Templates used in each reaction are indicated above the lanes. Bands resulting from the internal control template, p300 are indicated. In vitro synthesized products were analysed as described in Materials and Methods.

p300
1
2
3
4
5
6
p371
p(SP1)6371
pML(C2AT)19
p371
p(SP1)6371
pML(C2AT)19
+ α-amanitin

nuclear extract.

B) Transcriptional Properties of Individual Enhancer Motifs

i) AP-1 motifs

To determine whether the *in vitro* system could be used to assay for transcriptional activation conferred by individual enhancer motifs, I examined activation mediated by AP-1 motifs more carefully. The AP-1 motif has been shown to confer transcriptional activation both *in vivo* (Angel et al., 1987; Lee et al., 1987b; Lucibello et al., 1988) and *in vitro* (Lee et al., 1987a; Mermod et al., 1988; Bohmann and Tjian, 1989; Fig. 6). An oligonucleotide comprising the consensus AP-1 binding motif (Fig. 4C) was cloned as a monomer or as tandem repeats of up to five copies upstream of the TATA box of p371 to generate the p(AP1)$_n$371 series of plasmids. These plasmids were tested as templates for transcription *in vitro*.

Surprisingly, transcription of p(AP1)$_1$371, bearing a single AP-1 motif immediately upstream of the TATA box, was no more efficient than p371, bearing the minimal promoter template (Fig. 8A, compare lanes 1 and 2). By contrast, two tandem copies of the AP-1 motif increased transcription 5-fold from the TATA box, indicative of synergistic activation (Fig. 8A, lane 3). Transcription increased linearly with additional AP-1 motifs: templates bearing 3, 4, and 5 copies of the (AP1)

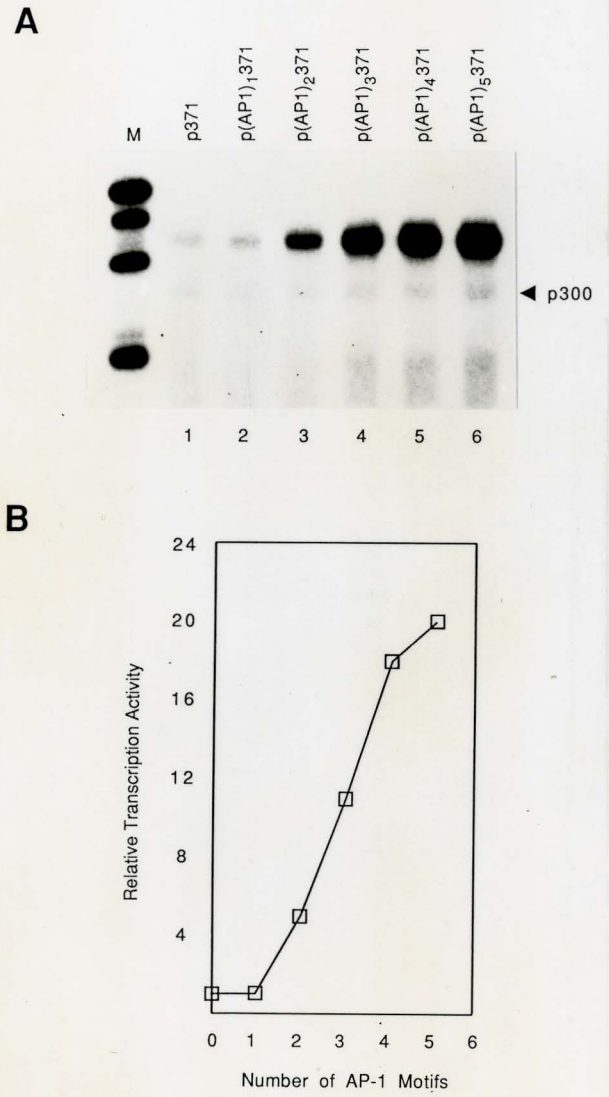
Figure 8. In vitro analysis of transcriptional activity conferred by AP-1 and PEA1 sequence motifs.

A) Transcription of the G-less cassette driven by the minimal promoter on its own (lane 1), or with 1 to 5 tandem copies of the AP-1 binding motif upstream of the TATA box (lanes 2 through 6). Bands produced by transcripts from the internal control template, p300, are indicated. Transcription reactions and analysis of in vitro products were performed as in Materials and Methods. M: RNA markers as in Figure 5.

B) Densitometric quantitation of transcripts produced in A). Transcriptional activity is given as induction ratios relative to in vitro activity of the minimal promoter template p371.

C) Transcription of the G-less cassette driven by the minimal promoter on its own (lane 1), or with 1, 3, or 4 copies of the PEA1 binding motif immediately upstream of the TATA box (lanes 2 to 4). Bands resulting from transcripts of the internal control template, p300, are indicated. Transcription reactions and analysis of in vitro products were performed as described in Materials and Methods. M: RNA markers as in Figure 5.

D) Densitometric quantitation of transcripts produced in C). Relative transcriptional activity was measured as fold-induction ratios to the amount of transcripts produced by the minimal promoter template p371.


A
M
p371
p(AP1)$_1$371
p(AP1)$_2$371
p(AP1)$_3$371
p(AP1)$_4$371
p(AP1)$_5$371
p300
1 2 3 4 5 6
B
Relative Transcription Activity
24
20
16
12
8
4
0 1 2 3 4 5 6
Number of AP-1 Motifs
C
M
p371
p(PEA1)$_1$371
p(PEA1)$_3$371
p(PEA1)$_4$371
p300
1 2 3 4
D
Relative Transcription Activity
24
20
16
12
8
4
0 1 2 3 4 5 6
Number of PEA1 Motifs

oligomer were transcribed 11-, 18-, and 20-fold better than the minimal promoter template (Fig. 8A, lanes 4-6; shown graphically in Fig. 8B).

ii) Comparison of AP-1 and PEA1 activities

The PEA1 motif has been shown to confer transcriptional activation in vivo in transfection studies both in the context of the entire Py enhancer (Martin et al., 1988) and as part of a region of the enhancer (Imler et al., 1988; Wasyslyk, B. et al., 1988; Yamaguchi et al., 1989; Murakami et al., 1990). PEA1 and AP-1 bind to the same DNA motifs (Kryszke et al., 1987; A. Cowie, unpublished results) and the activities of both factors are stimulated by serum, TPA, and the oncoprotein $p21^{Ha\text{-}ras}$ (Angel et al., 1987; Lee et al., 1987b; Ryder and Nathans, 1988; Imler et al., 1988; Yamaguchi et al., 1989). Furthermore, murine c-Jun and Jun-B proteins bind to both the AP-1 motif and the Py PEA1 site as heterodimers with Fos (Hirai et al., 1989; A. Cowie, unpublished results). Taken together, these observations suggest that PEA1 and AP-1 may be identical or closely related factors.

PEA1 is presumed to be a murine AP-1 homolog (Kryszke et al., 1987; Piette et al., 1988). Consistent with this hypothesis, I obtained results using templates bearing PEA1 binding sites in the in vitro assay analogous to those obtained for templates bearing AP-1 binding sites. No

activation was detected when a single PEA1 motif was present upstream of the TATA box (Fig. 8C, compare lanes 1 and 2), whereas templates bearing 3 and 4 PEA1 binding sites were transcribed 4- and 5-fold better than the minimal promoter template (Fig. 8C, lanes 3 and 4). Note that activation conferred by PEA1 motifs was substantially less than that induced by equivalent numbers of AP-1 binding motifs (Compare graphs in Figs. 8B and 8D). Nonetheless, both motifs were ineffective when a single copy was present on the template.

The binding motifs for PEA1 and AP-1 differ by only one nucleotide. This similarity as well as those between PEA1 and AP-1 transcriptional activities (Fig. 8) prompted me to examine whether their motifs interacted with the same factor(s) in the cell-free system. If this were true, one would expect both motifs to compete for binding by either factor, and one motif should be a better competitor for both PEA1 and AP-1 binding activities.

Nuclear extracts were incubated with monomeric (AP1) or (PEA1) oligonucleotides prior to their use in <u>in vitro</u> assays. Transcription of a template bearing two AP-1 binding sites, $p(AP1)_2371$, was reduced in extracts pre-incubated with either oligomer (Fig. 9A, compare lane 1 to lanes 2-9), although the (AP1) monomer had a greater effect (for example, compare lane 4 to lane 5; illustrated in graph of Fig. 9B). Similarly, transcription of a template bearing three PEA1

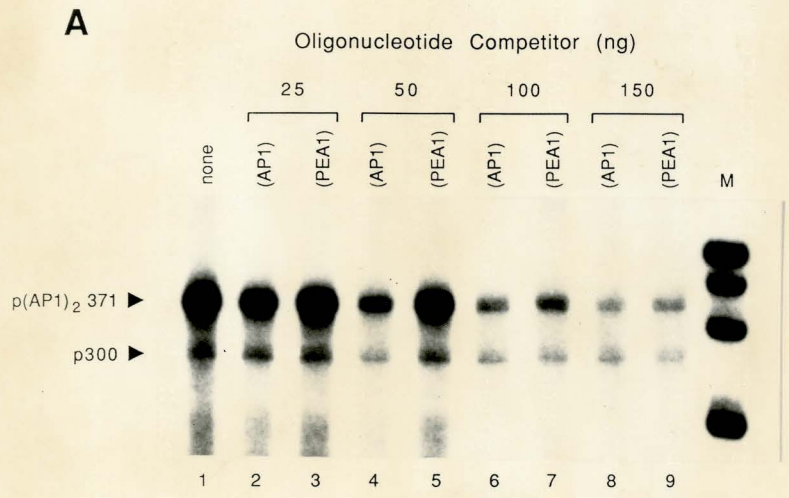
Figure 9. Comparison of transcription activation conferred by AP-1 and PEA1 motifs. Nuclear extracts were pre-incubated with monomer competitor oligonucleotides and then used for transcription as described in Materials and Methods. *In vitro* synthesized transcripts were analyzed and quantitated by densitometry as described in Materials and Methods. M: RNA markers as in Figure 5.

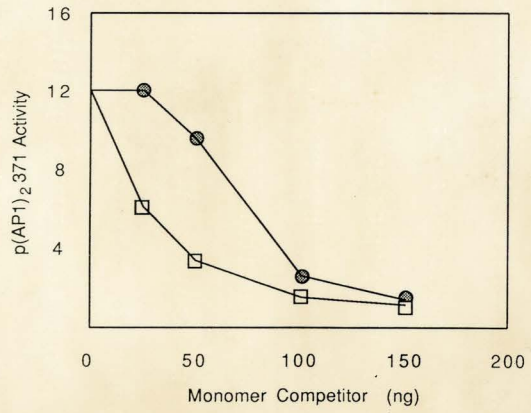
A) Transcription of a template bearing two AP-1 motifs, $p(AP1)_2371$, by nuclear extract pre-incubated with increasing concentrations of competitor (AP-1) or (PEA1) oligonucleotides as indicated above each lane. Transcripts from the internal standard, p300, are also indicated.

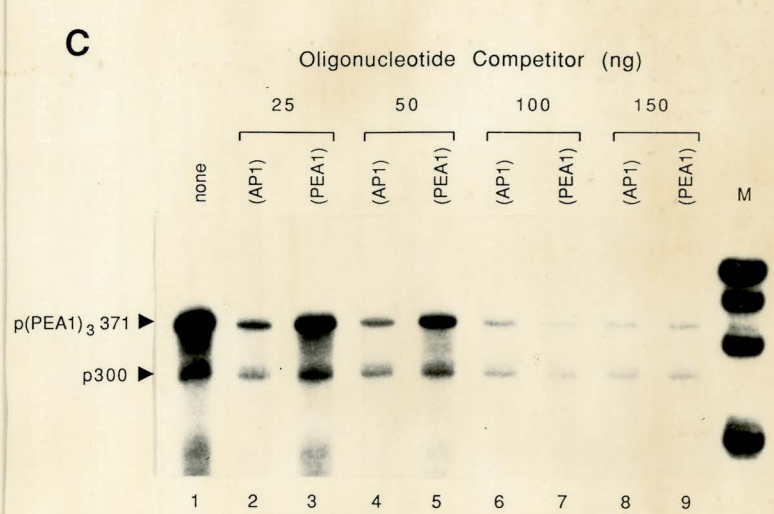
B) Densitometric quantitation of transcripts produced in A) showing inhibitory effect of both types of competitor. ●: (PEA1) monomer; □: (AP1) monomer.

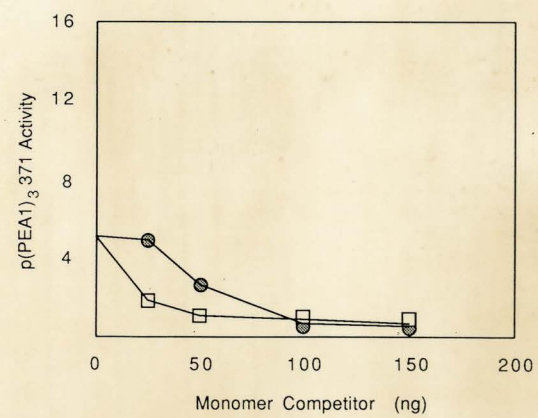
C) Transcription of a template bearing three PEA1 motifs, $p(PEA1)_3371$, by nuclear extract pre-incubated with increasing concentrations of competitor (AP-1) or (PEA1) oligonucleotides as indicated above each lane. Transcripts from the internal standard, p300, are also indicated.

D) Densitometric quantitation of transcripts produced in C) showing inhibitory effect of both types of competitor. ●: (PEA1) monomer; □: (AP1) monomer.


A
Oligonucleotide Competitor (ng)
25
50
100
150
none
(AP1)
(PEA1)
M
p(AP1)2 371
p300
1 2 3 4 5 6 7 8 9


B
16
12
8
4
p(AP1)2 371 Activity
0 50 100 150 200
Monomer Competitor (ng)


C
Oligonucleotide Competitor (ng)
25
50
100
150
none
(AP1)
(PEA1)
M
p(PEA1)3 371
p300
1 2 3 4 5 6 7 8 9

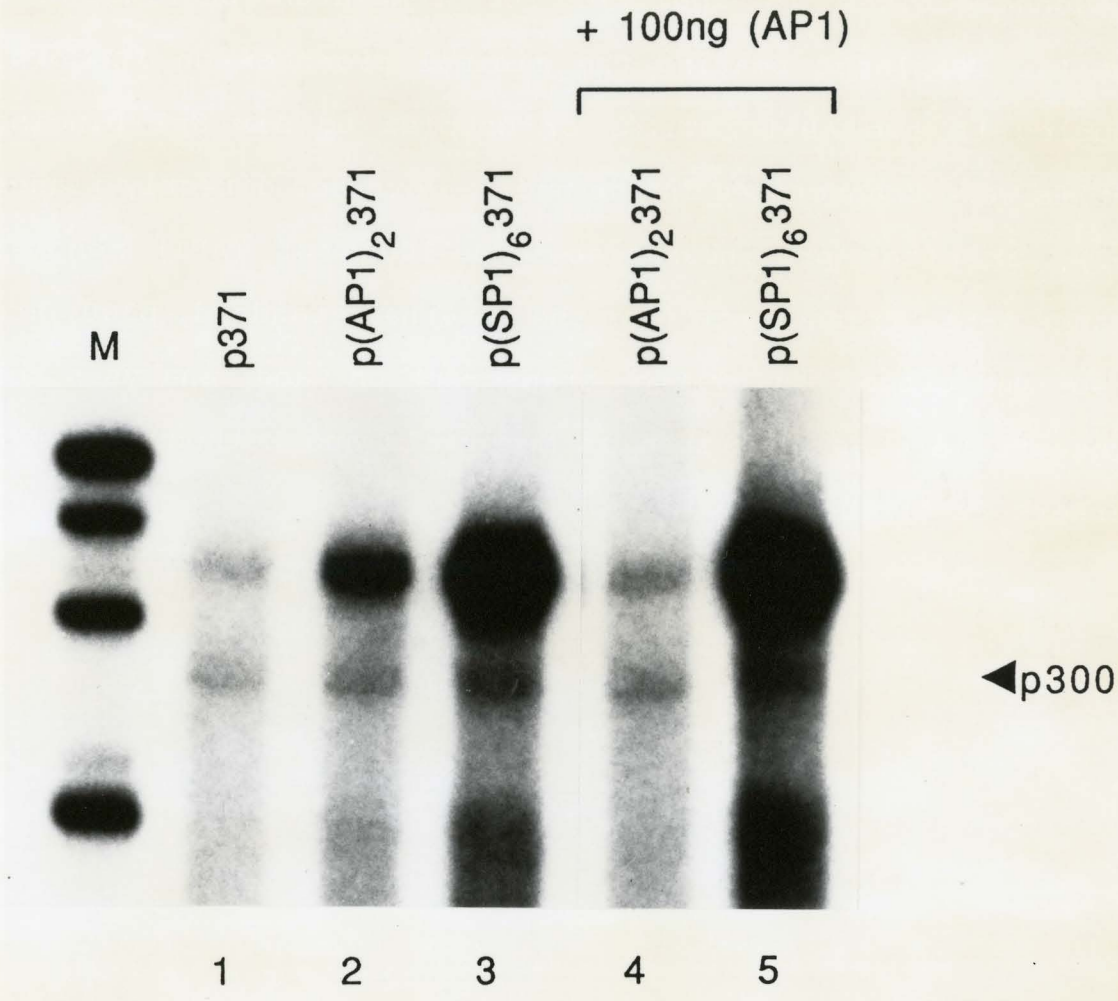

D
16
12
8
4
p(PEA1)3 371 Activity
0 50 100 150 200
Monomer Competitor (ng)

motifs, p(PEA1)$_3$371, was reduced by pre-incubation with either oligonucleotide (Fig. 9C, compare lane 1 to lanes 2-9), and again the (AP1) monomer was more effective in reducing transcription (for example, compare lanes 2 and 3; see graph in Fig. 9D). For both templates, a 50% reduction in transcription activity was achieved with 3-fold less (AP1) competitor than with (PEA1) competitor (compare curves in Figs. 9B and 9D). These results suggest that the PEA1 motif is a low-affinity binding site for the same factors that interact with the AP-1 motif, and that the reduced activity associated with (PEA1) multimers relative to (AP1) multimers may be due to the lower affinity of AP-1 for the PEA1 motif.

Importantly, these results also show that although a dimer of the AP-1 motif is required to activate transcription, a monomer bearing a single AP-1 or PEA1 site is capable of inhibiting AP-1 activity, presumably by competing with the template for AP-1 binding. Therefore, the factor which requires two AP-1 motifs to activate transcription (Fig. 8A, lane 3) is capable of binding to a single AP-1 motif.

As a control, I measured the ability of the (AP1) monomer to affect transcription of p(SP1)$_6$371, a template bearing six binding sites for the general transcription factor Sp1 (Dynan and Tjian, 1983) juxtaposed to the TATA box. Pre-incubation of extracts with (AP1) monomer reduced AP-1 transcription activity (Fig. 10, compare lanes 1 and 2 to lane

Figure 10. Inhibition of transcription by (AP1) monomer oligonucleotides is specific for templates bearing AP-1 motifs. Templates bearing the minimal promoter on its own, coupled to two AP-1 motifs, or coupled to six Sp1 binding motifs were transcribed in the nuclear extract directly (lanes 1-3), or after pre-incubation with 100ng of (AP1) monomer (lanes 4-5). Transcripts from the internal standard, p300, are also indicated. *In vitro* transcription and analysis of products were as described in Materials and Methods. M: RNA markers as in Figure 5.


+ 100ng (AP1)
M
p371
p(AP1)2371
p(SP1)6371
p(AP1)2371
p(SP1)6371
◀p300
1
2
3
4
5

4), whereas it did not affect transcription of $p(SP1)_6371$ (Fig. 10, compare lane 3 to lane 5). These results demonstrate that inhibition of transcription by the (AP1) monomer was specific for templates bearing AP-1/PEA1 binding sites.

iii) PEA2 motifs

I next attempted to define the transcriptional properties of PEA2, a factor that binds adjacent to the AP-1/PEA1 motif in element 2 of the Py enhancer (Fig. 4A). PEA2 protects the sequence 5'-GACCGCA-3' from digestion by DNase I (Piette and Yaniv, 1987). The site has been reported to be required <u>in vivo</u> for element 2 to activate both transcription (Mueller et al., 1988) and DNA replication (Muller et al., 1988) in transient assays using murine 3T3 cells. By contrast, others have reported that PEA2 functions as a repressor of PEA1 in thymidine kinase-deficient mouse L cells and in F9 murine EC cells (Wasylyk et al., 1988), and to have no activity in replication assays in C127 cells (Murakami et al., 1990). To investigate PEA2 activity in the FM3A extract, I used the (PEA2) monomer (Fig. 4C). The monomer comprises a sequence shown to complex with a factor distinct from PEA1 (and PEA3) in band shift assays using murine 3T3 cell nuclear extracts (Piette and Yaniv, 1987). The (PEA2) oligomer was cloned immediately upstream of the minimal promoter in p371 as a monomer or as multiple tandem copies, and the resulting

plasmids were tested for transcription *in vitro*.

In constrast to effects seen in 3T3 cells (Mueller et al., 1988), no activation was observed when up to five PEA2 binding sites were cloned upstream of the minimal promoter using the FM3A extract (Fig. 11, compare lane 1 to lanes 2-5). PEA2, therefore, does not appear to function as an independent transcription activator. Note, however, that the templates bearing PEA2 motifs were transcribed at the same level as the minimal promoter template, suggesting that any PEA2 binding activity in FM3A cells does not repress basal promoter action, unlike PEA2 activity in L cells (Wasylyk et al., 1988).

iv) PEA3 motifs

The PEA3 motif, 5'-AGGAAG-3', has been shown to be recognized by a factor distinct from PEA1 and PEA2, and has been reported to confer activation of both transcription (Martin et al., 1988; Wasylyk et al., 1989; Yamaguchi et al., 1989) and DNA replication (Muller et al., 1988; Murakami et al., 1990) in transient assays. The PEA3 motif is recognized by the Ets family of transcription factors (Gunther et al., 1989; Wasylyk et al., 1990; Xin et al., manuscript in preparation). The (PEA3) oligomer comprising the known PEA3 binding site (Fig. 4C) was constructed to investigate the transcriptional activity of PEA3 *in vitro*.

Figure 11. *In vitro* transcription of templates bearing multiple PEA2 binding motifs. Templates bearing the TATA box on its own (lane 1) or linked to 1 to 5 tandem copies of the (PEA2) oligomer (lanes 2-5) were transcribed in FM3A nuclear extracts. Transcription and analysis of products were done as described in Materials and Methods. Transcripts produced from the internal control template, p300, are indicated. M: RNA markers as in Figure 5.

M
p371
p(PEA2)$_1$371
p(PEA2)$_2$371
p(PEA2)$_4$371
p(PEA2)$_5$371
◀p300
1
2
3
4
5

No activation was observed when a single PEA3 motif was placed upstream of the minimal promoter (Fig. 12A, compare lanes 1 and 2). By contrast, templates bearing 2, 3, and 5 copies of the (PEA3) oligonucleotide were transcribed 2-, 7-, and 9-fold better than templates bearing one PEA3 binding site upstream of the TATA box (Fig. 12A, compare lane 2 to lanes 3-5). In the particular experiment shown in Fig. 12A, the signal from the internal standard p300 in lane 3 appears anomalously low. However, $p(PEA3)_2371$ was consistently transcribed 2- to 4-fold better than the minimal promoter template p371 in similar experiments (for example, see Fig. 13B, lane 3) . PEA3, therefore, appears to be similar to AP-1/PEA1 in that it requires a minimum of two binding sites to activate transcription from the minimal promoter (see graph in Fig. 12B).

Competition experiments were performed to verify that the activity associated with multiple PEA3 motifs was due to a factor that could bind to a single PEA3 site. As expected, pre-incubation of FM3A extract with (PEA3) monomer decreased transcription of $p(PEA3)_2371$ (Fig. 13A, compare lane 1 to lane 5). For controls, FM3A extracts were also pre-incubated with (PEA2) or (AP-1) monomers prior to their use in transcription assays. Pre-incubation of extract with (PEA2) monomer had no effect on transcription of $p(PEA3)_2371$ (Fig. 13A, compare lane 1 to lanes 2 and 4). Conversely, pre-incubation with the (AP1)

Figure 12. In vitro transcription of templates bearing multiple PEA3 binding motifs.

A) Templates bearing the TATA box on its own (lane 1) or linked to 1 to 5 tandem copies of the (PEA3) oligomer (lanes 2-5) were transcribed in FM3A nuclear extracts. Transcription and analysis of products were done as described in Materials and Methods. Transcripts produced from the internal control template, p300, are indicated. M: RNA markers as in Figure 5. **B)** Densitometric quantitation of transcripts produced in **A)**. Transcriptional activity is given as induction ratios relative to the minimal promoter template, p371.

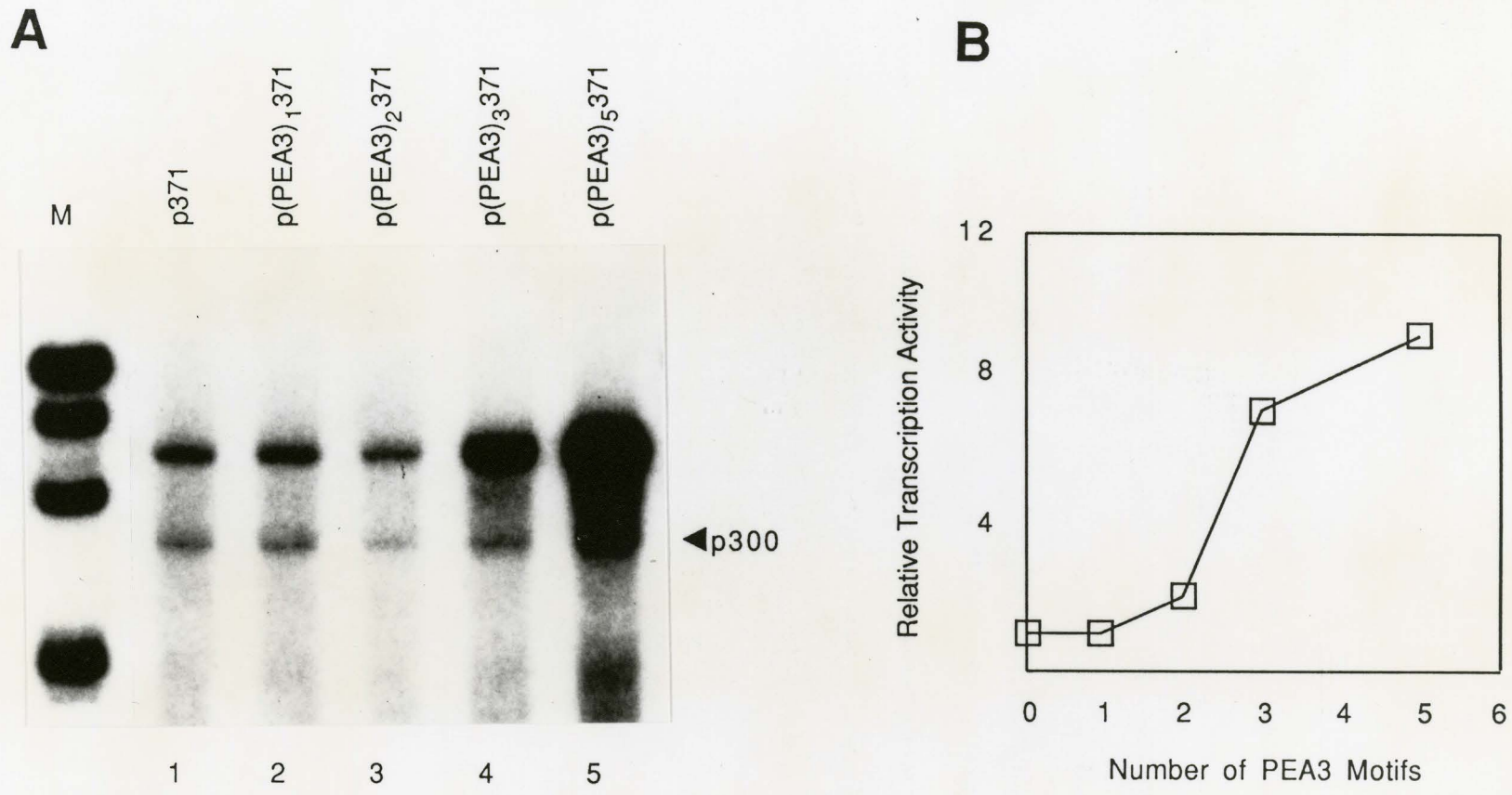
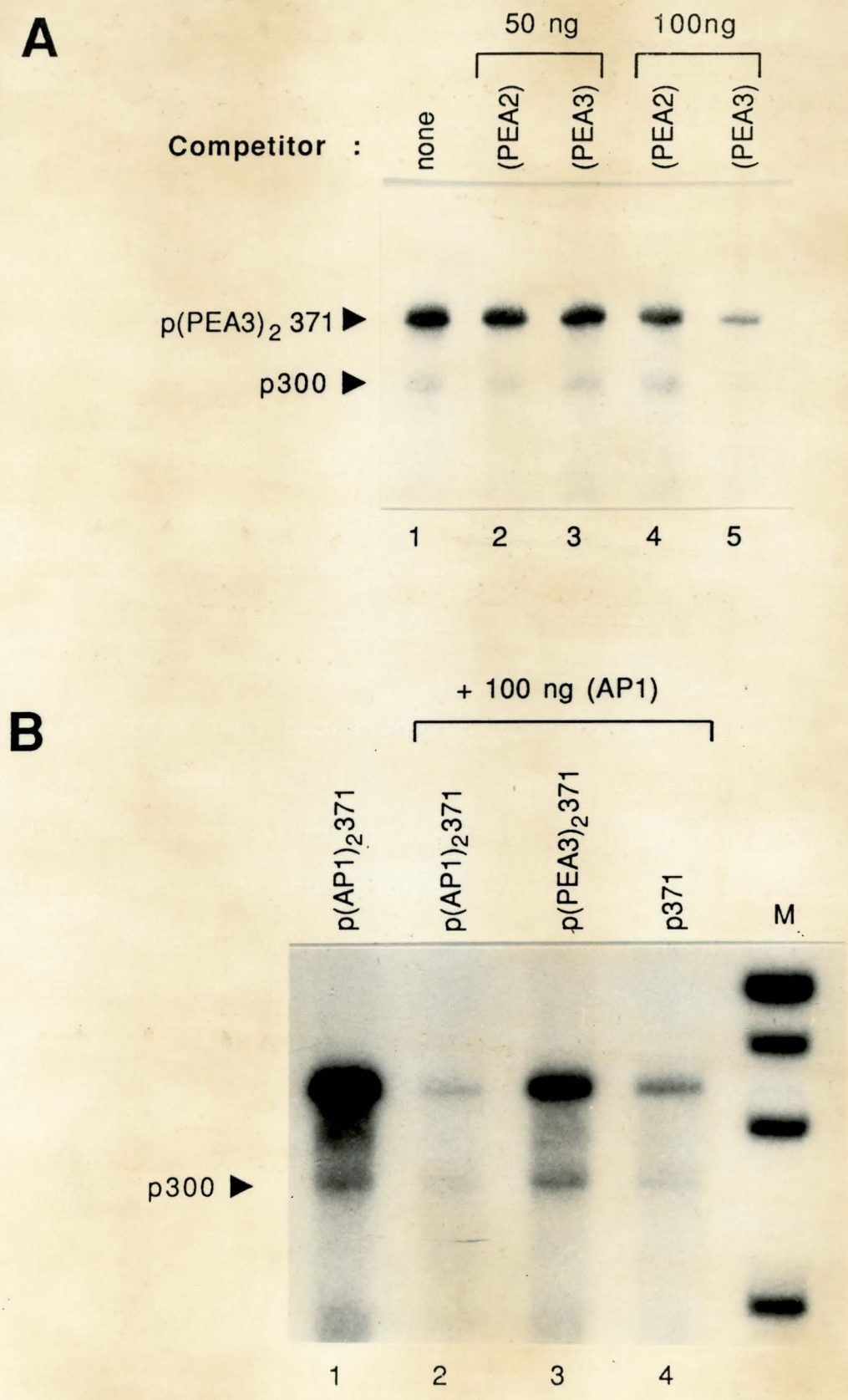

A
M
p371
p(PEA3)$_1$371
p(PEA3)$_2$371
p(PEA3)$_3$371
p(PEA3)$_5$371
◀p300
1
2
3
4
5
B
Relative Transcription Activity
12
8
4
0
1
2
3
4
5
6
Number of PEA3 Motifs

Figure 13. Transcription activation conferred by PEA3 motifs is inhibited by (PEA3) monomers.

A) In vitro transcription of a template bearing two PEA3 binding motifs upstream of the TATA box, $p(PEA3)_2371$, in extracts pre-incubated with either (PEA2) or (PEA3) oligonucleotide competitor. Competitors and their concentrations are indicated above each lane. Transcripts from the internal standard, p300, are also indicated. Transcription reactions and product analysis were performed as described in Materials and Methods.

B) In vitro transcription by untreated FM3A extract (lane 1) or after pre-incubation with (AP1) monomer (lanes 2-4). Templates used in each reaction are indicated above each lane; transcription and analysis of products were as described in Materials and Methods. Transcripts from the internal control template, p300, are also indicated. M: RNA markers as in Figure 5.


A
50 ng
100ng
Competitor :
none
(PEA2)
(PEA3)
(PEA2)
(PEA3)
p(PEA3)2 371
p300
1
2
3
4
5
B
+ 100 ng (AP1)
p(AP1)2371
p(AP1)2371
p(PEA3)2371
p371
M
p300
1
2
3
4

monomer abolished AP-1 activity (Fig. 13B, compare lanes 1 and 2), but did not affect PEA3 activity, because p(PEA3)$_2$371 was still transcribed 4x better than the minimal promoter template in these conditions (Fig. 13B, compare lanes 3 and 4).

C) AP-1 and PEA3 Binding Activities

Although AP-1/PEA1 and PEA3 can bind to a single copy of their motifs (Piette and Yaniv, 1987; Wasylyk C. et al., 1989; this study), a minimum of two adjacent motifs was required for either protein to stimulate transcription <u>in vitro</u> (Figs. 8D and 12B). This requirement may reflect co-operative binding of these factors to two adjacent sites. There have been a number of reports in which co-operative binding between the same or different transcription factors was demonstrated by titration curves using band shift assays (Davidson et al., 1988; Hardy and Shenk, 1989; LeBowitz et al., 1989; Janson and Pettersson, 1990). Therefore, I used the same method to investigate whether AP-1 or PEA3 bind co-operatively to adjacent copies of their motifs.

i) AP-1 Binding Activity

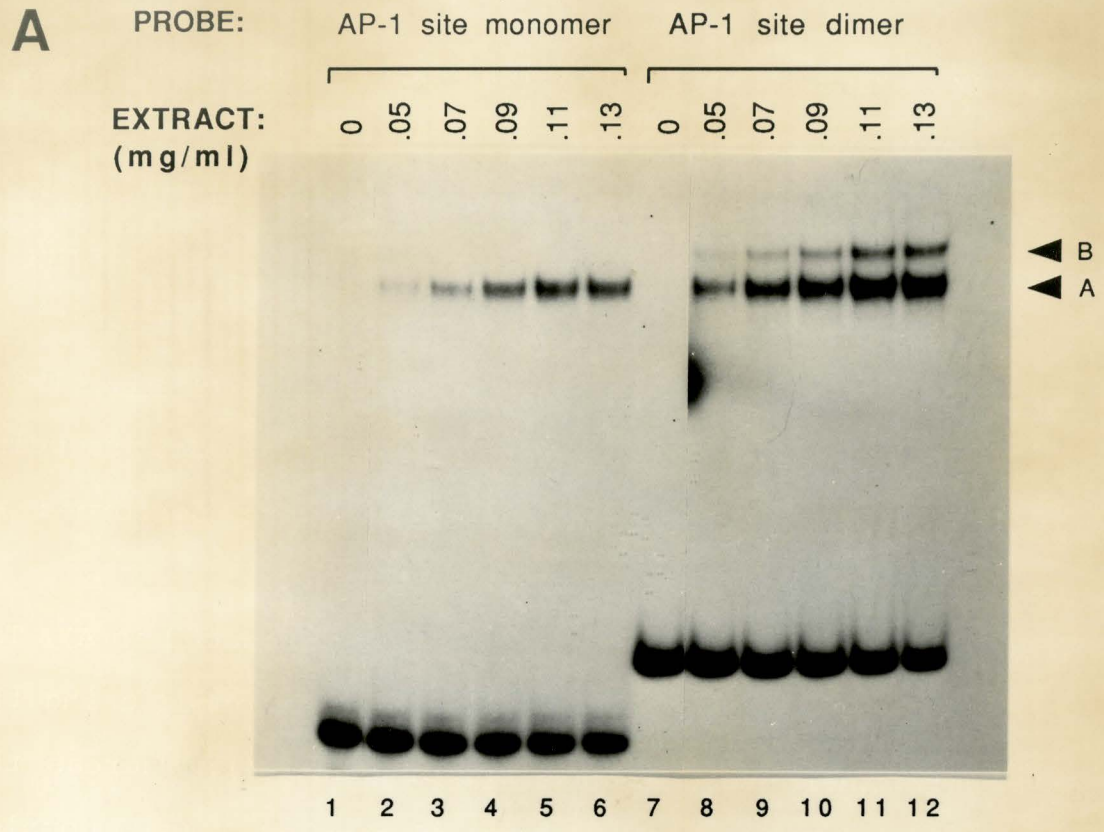
Band shift assays were used to measure the ability of AP-1 to bind to a single DNA motif compared to its ability to bind to

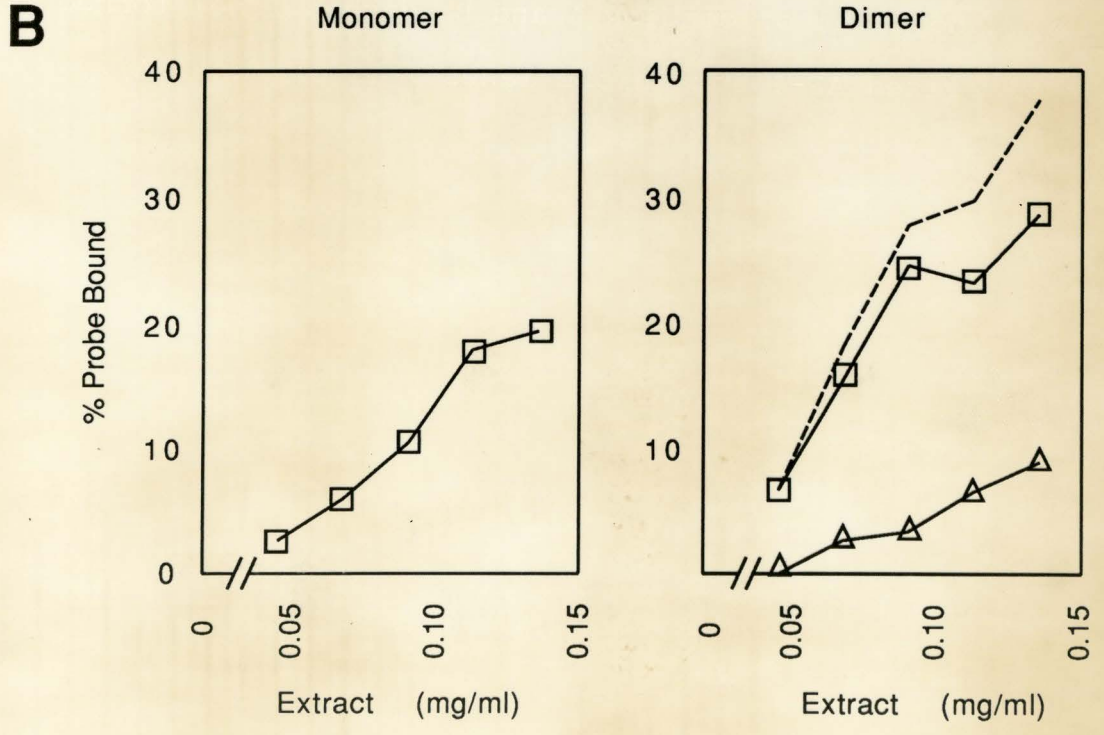
two adjoining motifs. A single complex, A, formed with a probe bearing one AP-1 motif (Fig. 14A, lanes 1-6). The abundance of this complex increased linearly with increasing concentrations of extract. By contrast, two complexes were detected in binding reactions containing the probe bearing two adjoining AP-1 motifs: complex A which co-migrated with the complex formed with the one site probe; and complex B which migrated more slowly than complex A (Fig. 14A, lanes 7-12). I interpret complex A to result from one AP-1 heterodimer binding to the probe bearing two AP-1 motifs, and complex B to be composed of two AP-1 heterodimers bound to this probe. These results suggest that AP-1 does not bind co-operatively to the two site probe because complexes A and B both appear at the same extract concentration, and the abundance of both increase linearly with increasing extract concentration (depicted in graphs of Fig. 14, panel B). Furthermore, these binding reactions were done with extract concentrations (0.05-0.15 mg/ml) far below those used in the transcription reactions (2 mg/ml) suggesting that all sites are already occupied in protein saturating conditions during transcription in vitro. These observations suggest that synergistic transcriptional activation by AP-1/PEA1 does not result from co-operative factor binding.

Figure 14. Band shift titration experiment using probes comprising AP-1 motifs.

A) Band shift reactions were done using a probe bearing a single AP-1 site (lanes 1-6), or a probe comprising a dimerized AP-1 motif (lanes 7-12). Increasing concentrations of FM3A extract (prepared according to Shapiro et al., 1988) were used as indicated above each lane (in mg/ml), producing complexes A and B as shown. Reactions were performed as described in Materials and Methods.

B) Densitometric quantitation of complexes produced in A). The panel on the left shows amount of complex A formed using the single site AP-1 probe (lanes 1-6 of panel A); whereas the right-side panel shows the amount of complex A (□), complex B (△), and total of complex A and B (dashed line) formed with the dimerized AP-1 site probe lanes 7-12 of panel A).


A
PROBE:
AP-1 site monomer
AP-1 site dimer
EXTRACT:
(mg/ml)
0
.05
.07
.09
.11
.13
0
.05
.07
.09
.11
.13
B
A
1 2 3 4 5 6 7 8 9 10 11 12


B
Monomer
Dimer
% Probe Bound
40
30
20
10
0
0
0.05
0.10
0.15
Extract (mg/ml)

ii) PEA3 Binding Activity

Band shift assays using probes bearing either a single PEA3 motif or two adjoining PEA3 motifs yielded results analogous to those for AP-1. One major complex was formed with the probe bearing a single PEA3 motif, and the amount of this complex increased linearly with increasing extract concentrations (Fig. 15A, lanes 1-6). The same complex, indicated as complex A, and a minor complex of lesser mobility, designated complex B, formed with the probe bearing two adjoining PEA3 motifs (Fig. 15A, lanes 7-12). Assuming that complex A resulted from one PEA3 factor bound to the probe, and complex B from two PEA3 factors bound to the probe, these results indicate that PEA3 does not bind co-operatively to the two site probe because the abundance of both complexes increased linearly with increasing extract concentrations (see graphs in Fig. 15B). Synergistic activation of transcription by PEA3 is therefore unlikely to be due to co-operative binding to DNA.

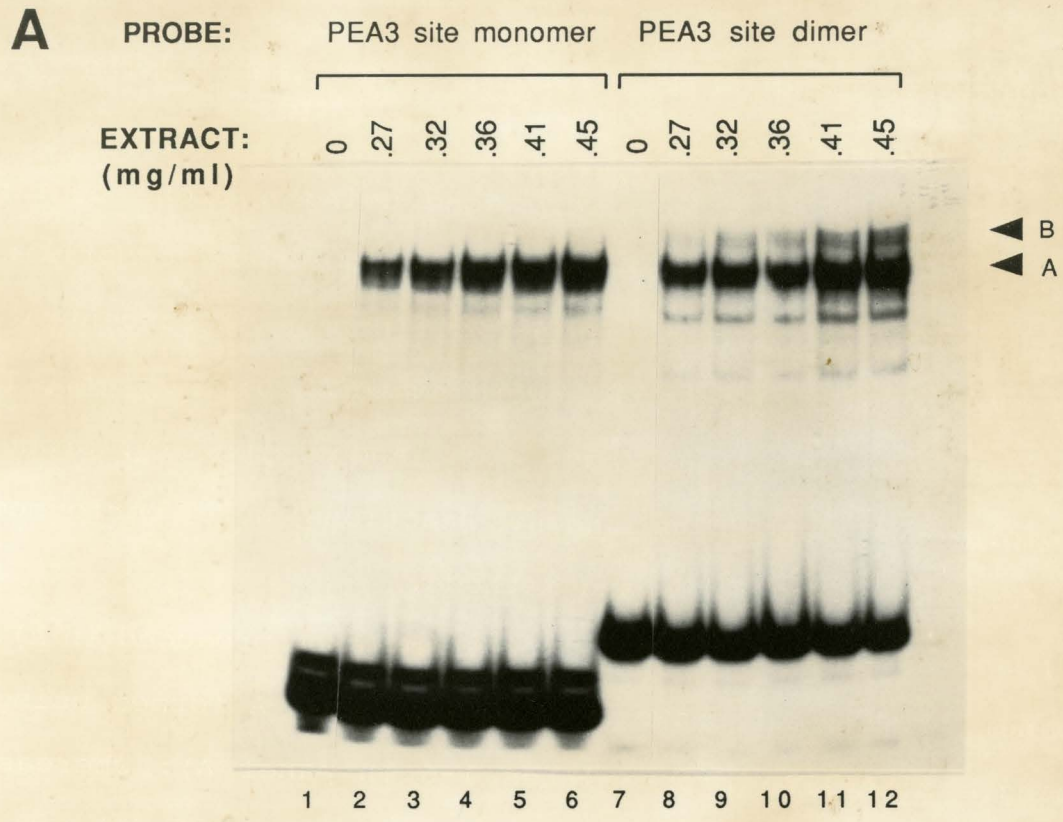
D) Transcriptional Activities of Paired, Heterologous Polyomavirus Enhancer Motifs

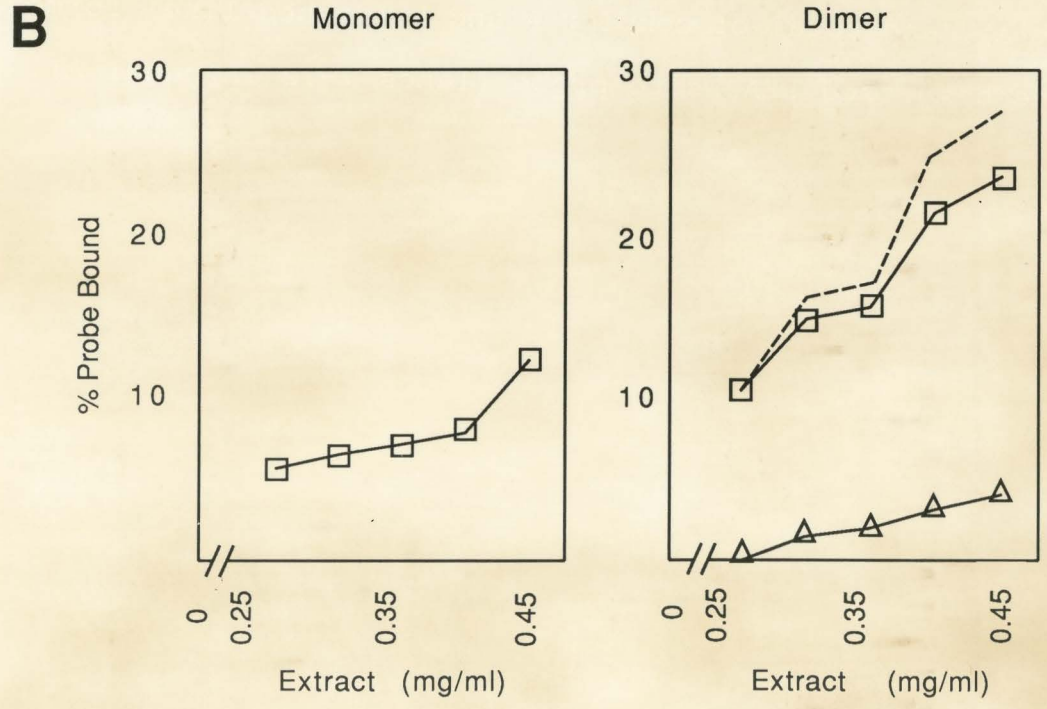
Transcription <u>in vitro</u> was not stimulated on templates bearing a single copy of either the PEA1, PEA2, or PEA3 motifs, even though they occur as single sites in the Py enhancer. It

Figure 15. Band shift titration experiment using probes comprising PEA3 motifs.

A) Band shift reactions were done using a probe bearing a single PEA3 site (lanes 1-6), or a probe comprising a dimerized PEA3 motif (lanes 7-12). Increasing concentrations of FM3A extract (prepared according to Shapiro et al., 1988) were used as indicated above each lane (in mg/ml), producing complexes A and B as shown. Reactions were performed as described in Materials and Methods.

B) Densitometric quantitation of complexes produced in A). The panel on the left shows amount of complex A formed using the single site PEA3 probe (lanes 1-6 in panel A); whereas the right-side panel shows the amount of complex A (□), complex B (Δ), and total of complex A and B (dashed line) formed with the dimerized PEA3 site probe (lanes 7-12 in panel A).


A
PROBE:
PEA3 site monomer
PEA3 site dimer
EXTRACT: (mg/ml)
0 .27 .32 .36 .41 .45 0 .27 .32 .36 .41 .45
B
A
1 2 3 4 5 6 7 8 9 10 11 12


B
Monomer
Dimer
% Probe Bound
30
20
10
0
0.25
0.35
0.45
Extract (mg/ml)

seemed plausible that the factors which bind to them acted in conjunction with each other to stimulate transcription. The alpha-core, comprising sites for all three factors, has previously been shown to function as an independent enhancer when multimerized, though not as a monomer, in transient assays (Veldman et al., 1985). Therefore, I tested whether paired combinations of Py enhancer motifs would confer transcriptional activity in the cell-free system.

i) Paired PEA1 and PEA2 Motifs

PEA2 was unable to activate transcription in vitro on its own (Fig. 11), but might do so in conjunction with PEA1, an independent transcription activator. I used a series of oligomers to determine whether PEA2 could activate transcription in vitro in conjunction with PEA1, and in the absence of PEA3. The oligonucleotide (PEA1/PEA2) comprises binding sites for PEA1 and PEA2 as they are found in the wild-type Py enhancer, whereas (PEA1/mPEA2) and (mPEA1/PEA2) are mutated versions whose mutations block binding of PEA2 and PEA1, respectively (Fig. 4C; Piette and Yaniv, 1987).

Templates bearing one or four copies of (PEA1/PEA2) upstream of the minimal promoter were transcribed at a level equal to that of the minimal promoter template (Fig. 16, compare lanes 2 and 3 to lane 1). PEA2, therefore, does not appear to activate transcription in vitro either by itself or

Figure 16. In vitro transcription of templates bearing paired PEA1 and PEA2 binding motifs. Transcription reactions were done according to Materials and Methods using templates bearing i) the TATA box on its own (lane 1); ii) coupled to one or four copies of an oligomer comprising paired PEA1 and PEA2 motifs (lanes 2-3); or iii) coupled to multiple tandem copies of mutant versions of the oligonucleotide in which binding by either PEA1 or PEA2 is blocked by point mutations (lanes 4-5). Transcripts from the internal standard, p300, are also indicated. Analysis of transcripts was done as in Materials and Methods.

p300
1
2
3
4
5
p371
p(PEA1/PEA2)$_1$371
p(PEA1/PEA2)$_4$371
p(PEA1/mPEA2)$_4$371
p(mPEA1/PEA2)$_7$371

in conjunction with PEA1.

Templates bearing four copies of (PEA1/mPEA2) were also transcribed at basal levels (Fig. 16, lane 4). This result is in contrast to the activity conferred by the wild-type PEA1 binding sites in the $p(PEA1)_n371$ templates. The inactivity of the (PEA1/mPEA2) multimers suggests that the basal level of transcription for $p(PEA1/PEA2)_4371$ was not due to repression of PEA1 activation by PEA2. Templates bearing 7 copies of the (mPEA1/PEA2) oligomer are also transcribed at basal levels (Fig. 16, lane 5), which, in agreement with results obtained using the $p(PEA2)_n371$ templates (Fig. 11), indicates that PEA2 is not an independent activator of transcription.

ii) Paired PEA3 and PEA1 motifs

To examine whether PEA1 and PEA3 activated transcription in conjunction with each other (and in the absence of PEA2), I used the oligonucleotide (PEA3/PEA1) (Fig. 4C) which contains binding sites for PEA3 and PEA1 as they are found in the wild-type Py enhancer. (PEA3/PEA1) is similar to both the PB oligonucleotide of Wasylyk et al., (1988), and the M2A oligonucleotide of Yamaguchi et al., (1989). Both PB and M2A were shown to activate transcription when present as multimers in transient assays.

A template bearing a single copy of (PEA3/PEA1)

upstream of the minimal promoter was transcribed at the same level as the minimal promoter template (Fig. 17, compare lanes 1 and 2). However, templates bearing two or three tandem repeats of this oligomer were transcribed 4- and 5-fold better, respectively, than templates bearing one or no copies (Fig. 17, lanes 2-3). This <u>in vitro</u> data agrees with the <u>in vivo</u> data of Wasylyk et al. (1988), and Yamaguchi et al. (1989).

To determine whether the effect of the (PEA3/PEA1) multimers was dependent on intact binding sites for both PEA3 and PEA1, I tested mutant versions (PEA3/mPEA1) and (mPEA3/PEA1), in which double point mutations abolish binding of PEA1 or PEA3, respectively (Fig. 4C; Wasylyk et al., 1989). Templates bearing multiple copies of either of these two mutant oligomers were transcribed at levels equal to that of the template bearing the TATA box on its own (Fig. 17, lanes 5 and 6). Therefore, it appears that both the PEA1 and PEA3 sites must be occupied for these factors to promote efficient transcription of these templates. This suggests that some co-operation between them may occur despite the apparent basal activity of the p(PEA3/PEA1)$_1$371 template (Fig. 17, lane 2).

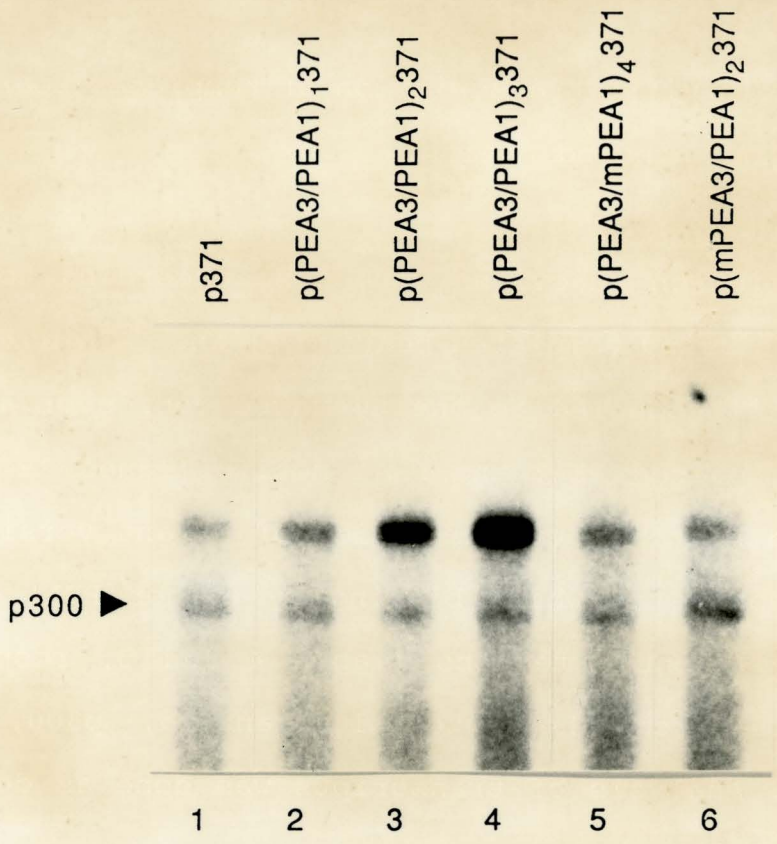
Note that the wild-type binding motifs for either PEA1 or PEA3 in the mutant oligonucleotides are identical to the corresponding motifs in the (PEA1) and (PEA3) oligomers. However, multimers of the latter activate transcription,

Figure 17. In vitro transcription of templates bearing paired PEA3 and PEA1 binding motifs.

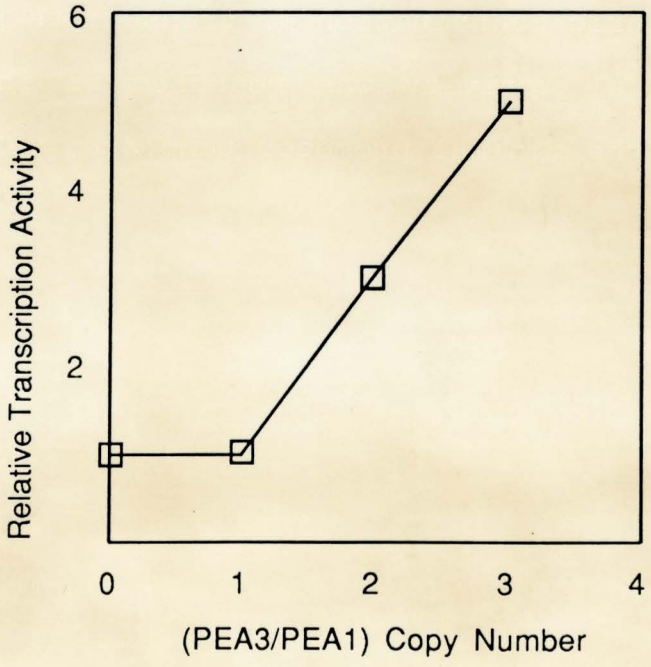
A) Transcription reactions were done according to Materials and Methods using templates bearing i) the TATA box on its own (lane 1); ii) coupled to one to three copies of an oligomer comprising paired PEA3 and PEA1 motifs (lanes 2-4); or iii) coupled to multiple tandem copies of mutant versions of the oligonucleotide in which binding by either PEA1 or PEA3 is blocked by point mutations (lanes 4 and 5, respectively). Transcripts from the internal standard, p300, are also indicated. Analysis of transcripts was done as described in Materials and Methods.

B) Densitometric quantitation of transcripts produced in A). Transcriptional activity was measured as induction ratios relative to the minimal promoter template, p371.

A


p371
p(PEA3/PEA1)$_1$371
p(PEA3/PEA1)$_2$371
p(PEA3/PEA1)$_3$371
p(PEA3/mPEA1)$_4$371
p(mPEA3/PEA1)$_2$371
p300
1
2
3
4
5
6

B


Relative Transcription Activity
6
4
2
0
1
2
3
4
(PEA3/PEA1) Copy Number

whereas multimers of the former do not; transcription is activated when multimers of PEA1 and/or PEA3 motifs are juxtaposed, but not when they are separated by a mutated binding motif. The mutated motifs result in an additional 5 bp, or one half helical turn in the DNA, between successive wild-type motifs, i.e. with respect to spacing in transcriptionally active multimers of these motifs. These observations suggest that, as is the case of enhansons in the SV40 enhancer, spacing constraints are critical for function of the PEA1 and PEA3 motifs in the Py enhancer.

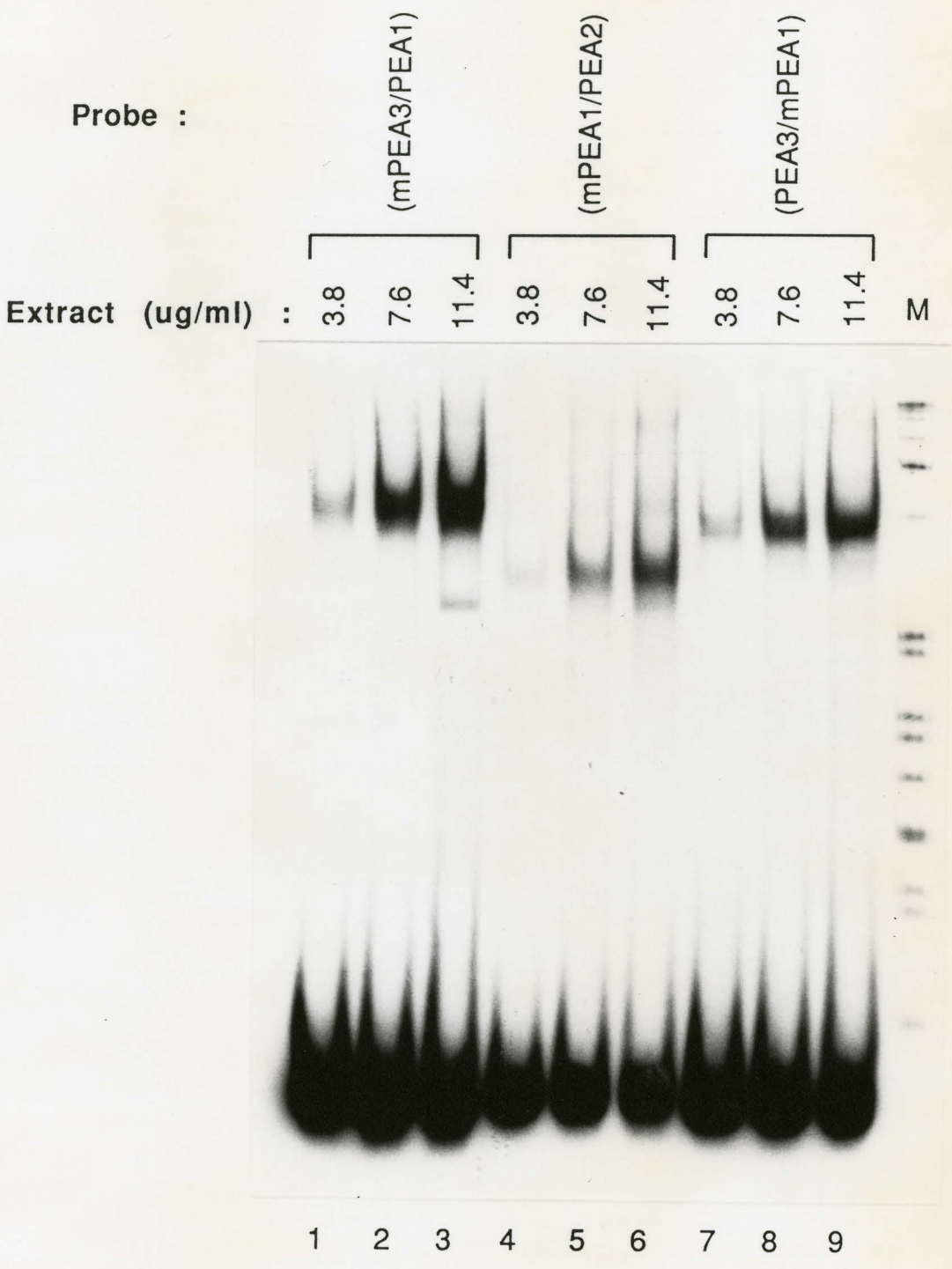
In the natural viral sequence, the binding sites for PEA3 and PEA1 (as determined by DNase I protection studies) are adjoined with no intervening nucleotides. It is notable that when the transcriptionally inactive monomer (PEA3/PEA1) is cloned as an active multimer, the re-iterated sequence comprises seven base pairs between the 3' end of the first PEA1 site and the 5' border of the next PEA3 site downstream. It may be that this increased space between successive PEA1 and PEA3 sites now enables the corresponding factors to promote efficient transcription. Supporting this interpretation of events is the fact that 10 bp and 9 bp of intervening sequence exist between the transcriptionally active (PEA1) and (PEA3) multimers, respectively. If this is the case, the conditions required for PEA1 and PEA3 activities *in vivo* and *in vitro* do not correspond.

E) Binding Activities of PEA1, PEA2, and PEA3 in FM3A Extracts

PEA1, PEA2 and PEA3 were each identified as binding activities in nuclear extracts of murine 3T6 cells by band shift assays (PEA1 and PEA2: Piette and Yaniv, 1987; PEA3: Martin et al., 1988; Satake et al., 1988). Each factor can bind to its motif independent of the other two (Piette and Yaniv, 1987; Wasylyk et al., 1989) although the binding of PEA2 may be enhanced or stabilized by PEA1 (Martin et al., 1988). I wished to verify that the FM3A nuclear extract used for the transcription assays contained equivalent binding activities for PEA1, PEA2, and PEA3 as had previously been shown for 3T6 cells. Moreover, I wanted to ensure that the lack of transcriptional activation observed with templates bearing the oligomers (mPEA1/PEA2) (Fig. 16, lanes 4-5), (PEA3/mPEA1), and (mPEA3/PEA1) (Fig. 17, lanes 5-6), was not due to the inability of the appropriate factor to bind to its wild-type site. Therefore, band shift assays were performed using DNA fragments comprising a single copy of these oligonucleotides as radiolabeled probes.

A unique band of reduced mobility was detected for each probe using an FM3A extract (Fig. 18, lanes 1, 4, and 7). The amount of complex formed with (mPEA3/PEA1) (Fig. 18, lanes 1-3), with (mPEA1/PEA2) (Fig. 18, lanes 4-6), and with (PEA3/mPEA1) (Fig. 18, lanes 7-9), increased linearly with

Figure 18. Band shift assay for DNA-binding factors in an FM3A nuclear extract. Probes were prepared as outlined in Materials and Methods comprising binding motifs for PEA1 (lanes 1-3), PEA2 (lanes 4-6), or PEA3 (lanes 7-9). FM3A extracts were prepared according to Dignam, et al., (1983), and used at the protein concentrations indicated above each lane. Band shift reactions were performed as described in Materials and Methods. M: molecular weight markers.


Probe :
(mPEA3/PEA1)
(mPEA1/PEA2)
(PEA3/mPEA1)
Extract (ug/ml) :
3.8
7.6
11.4
3.8
7.6
11.4
3.8
7.6
11.4
M
1 2 3 4 5 6 7 8 9

increasing concentrations of extract. Therefore, PEA1, PEA2, and PEA3 binding activities are present in FM3A extracts, and each binds independently to the single copy of its motif in the oligonucleotides used in this study.

DISCUSSION

A) Functional *In Vitro* Assay for Enhancer Motifs

Enhancers are major determinants of gene expression, both during development and in adult organisms. Enhancers consist of functionally redundant, modular DNA elements that operate by interacting with *trans*-acting protein factors. The unique array of *cis*-acting elements in a particular enhancer are thought to determine its functional characteristics such as tissue or developmental specificity. To define the properties of these *cis*-acting elements (presumably reflecting those of the factors that recognize them), and to investigate their structural arrangement in a functional enhancer, a cell-free system was developed to assay for transcriptional activity conferred by individual enhancer sequence motifs.

The minimal promoter of p371 directs transcription at a low basal level, and is responsive to activation by a number of upstream sequence elements including Sp1, AP-1, PEA1, and PEA3 binding motifs. *In vitro* transcription assays can be used to complement results from *in vivo* transient assays, and are necessary for biochemical analysis of transcriptional phenomena. The vector p371 was not designed for, and offers no particular advantages to, use *in vivo*. However, other vectors have been generated that allow the same gene constructs to be tested for transcription activity *in vivo* and *in vitro* (Grosschedl and Birnstiel, 1982; Sergeant et al.,

1984; Westin et al., 1987).

The cell-free system employing the vector p371 should be a useful tool in future studies. In addition to identifying and characterizing other enhancer sequence motifs that confer activation (or repression) of transcription, the system can be used to facilitate structure/function studies of cloned or purified transcription factors using nuclear extracts specifically depleted of that factor. A similar approach was used, for example, for the biochemical analysis of transcriptional activation by Jun (Bohmann and Tjian, 1989). Since the TATA box is both necessary and sufficient for transcription of the G-less cassette, the system can also be used to investigate interactions of transcription factors with TFIID. Similar studies have been done using a truncated adenovirus promoter driving the G-less cassette (Sawadogo and Roeder, 1985b). In this study, the cell-free system was used to define the properties of three DNA motifs found within the polyomavirus enhancer.

B) Roles of PEA1, PEA2, and PEA3

Element 2 of the polyomavirus enhancer contains partially overlapping binding sites between nt 5108 and 5130 for at least three factors, PEA1, PEA2, and PEA3 (Piette and Yaniv,

1987; Martin et al., 1988; Wasylyk et al., 1989). To date, no factors have been identified that interact with sequences upstream of nt 5108 within element 2; those sequences may operate by interacting with an unidentified factor(s), or by excluding nucleosomes from the enhancer region to increase accessibility for transcription factors.

The contributions to transcriptional enhancement of PEA1, PEA2, and PEA3 are not understood. I attempted to define their activities in vitro, through their sequence recognition motifs. The results of these experiments show that the PEA1 and PEA3 motifs, but not the PEA2 motif, confer activation of transcription. The oligonucleotides used in this report (Figure 4C) have also been cloned into reporter plasmids bearing the CAT gene, and results from CAT assays after transfection of FM3A cells with these constructs are qualitatively identical to my in vitro data (M. McWilliams, unpublished results). Therefore, both in vitro and in vivo observations indicate that PEA1 and PEA3 are transcription activator proteins, but that PEA2 is not.

PEA1 is thought to be a murine homolog of the transcription activator AP-1 (Kryzske et al., 1987; Piette et al., 1988). The AP-1 family of proteins is known to recognize a range of degenerate motifs (Halazonetis et al., 1988). Furthermore, there are reports that AP-1 also recognizes the binding sites of AP-4 (Mermod et al., 1988), and of the

Octamer Binding Proteins (Takimoto et al., 1989). By contrast, it has also been reported that the AP-1 consensus binding site in the Gibbon Ape Leukemia Virus LTR is recognized by a complex of two proteins in the Gibbon T cell line MLA144, and that neither member of this binding activity is related to Jun or Fos proteins (Quinn et al., 1989). Therefore, it would not have been unprecedented for the PEA1 binding activity in FM3A cells to be unrelated to AP-1 heterodimers.

In support of the notion that PEA1 is an AP-1 homolog, the PEA1 motif behaved as a low affinity AP-1 binding site in FM3A extracts. The transcription properties conferred by the PEA1 motifs were qualitatively indistinguishable from those conferred by AP-1 motifs, arguing that PEA1 in murine FM3A cells is related or identical to AP-1 activity. A more definite answer as to whether the two factors are identical will require either molecular cloning of PEA1 or its purification from cell homogenates.

AP-1/PEA1 activated transcription synergistically; multiple AP-1/PEA1 motifs were required for transcriptional activation in the cell-free system even though a single motif was sufficient for factor binding. Furthermore, data from band shift assays suggests that synergistic activation was not due to co-operative binding, and indicate instead that all factor binding sites on the template were saturated under the conditions used for transcription <u>in vitro</u>, rendering any

effects of co-operative binding irrelevant. Synergistic activation of transcription under such saturating conditions has also been demonstrated for other transcription factors (Carey et al., 1990; Lin et al., 1990).

Both the AP-1 and Py PEA1 motifs are recognized by murine c-Jun and Jun-B as heterodimers with Fos protein (Hirai et al., 1990; A. Cowie, unpublished results; reviewed in Kouarides and Ziff, 1989). In agreement with the *in vitro* results of this study, Chiu et al., (1989) have shown that *trans*-activation by Jun-B *in vivo* may require synergistic interactions between multiple homodimers bound to adjacent sites on the DNA, whereas c-Jun is an efficient activator of a single site. It is not known which species of Jun protein activated transcription in the *in vitro* assay, although FM3A cells must express both c-*jun* and *jun*-B because cDNA clones of both have been isolated from a *lambda* phage expression library made from FM3A cytoplasmic RNA (A. Cowie, unpublished results). However, the activities of homodimeric Jun proteins tested by Chiu et al. are difficult to correlate with the activity observed *in vitro* because the latter may be due to heterodimers of Jun and Fos proteins.

PEA3, like AP-1/PEA1, also activated transcription in the cell-free system synergistically. PEA3 was capable of binding to a single copy of its motif *in vitro*, but a minimum of two adjoining PEA3 motifs was required to promote efficient

transcription. Also analogous to observations with AP-1, PEA3 protein did not bind co-operatively to multiple PEA3 sites in band shift assays, indicating that synergistic activation is not due to co-operative factor binding. The recent cloning of PEA3 (Xin et al., manuscript in preparation) should facilitate experiments to determine the nature of the mechanism by which PEA3 stimulates transcription.

Synergistic activation of transcription may be a common property shared by transcription activators. It has been argued that synergistic activation by factors from widely divergent species indicates the existence of a common target protein (Carey et al., 1990; Lin et al., 1990). Transcription factors may interact with any of the protein components of the pre-initiation complex, or perhaps another elusive unidentified cellular component of an active transcription complex which does not interact with DNA. Furthermore, it has been proposed that synergy among weak transcription activators is an essential feature of the control of gene expression. Ptashne (1988) has argued that the presence of strong activation surfaces in the cell would unavoidably result in a deleterious general inhibition of transcription by a phenomenon described as "squelching." In this scenario, strong activator domains would indiscriminately interact with the limited supply of target proteins, causing the latter to be unavailable for the formation of active transcription

complexes, and thereby decrease or squelch cellular transcription in general. Synergistic activation by multiple transcription factors, whose activator domains are too weak individually to interact with their targets, could be a way to avoid squelching.

Multiple adjacent binding sites are required for the function of either AP-1 or PEA3 *in vitro*. By contrast, a survey of promoters carrying AP-1 or PEA3 binding sites revealed that these sites rarely occur as adjoining multimers in nature. Notable exceptions are the enhancers of different wild-type isolates or mutant polyomaviruses with expanded host cell ranges that contain duplications of short segments of the *alpha*-core region (Ruley and Fried, 1983; Melin et al., 1985). The PEA3 and AP1/PEA1 motifs frequently occur, however, juxtaposed to each other. They are juxtaposed in the wild-type Py enhancer, and in the promoters for c-*fos*, collagenase, stromelysin, and interleukin-2 (Wasylyk et al., 1989; 1990; Gutman and Wasylyk, 1990). It seems possible that, although co-operative interactions are relatively unimportant for factor binding (this study; Wasylyk et al., 1989), the association of the motifs for PEA3 and AP-1 may be significant for their function beyond mere binding. In addition, multimers of an oligonucleotide comprising juxtaposed PEA3 and PEA1 sites activated transcription in the cell-free system. These observations suggest that PEA3 and AP-1/PEA1 interact with

each other to stimulate transcription synergistically.

Supporting the hypothesis that PEA1 and PEA3 interact synergistically, it has been demonstrated that the c-Ets-1 protein, which recognizes the PEA3 motif, co-operates with Fos/Jun heterodimers to activate transcription from the Py enhancer alpha-core in transient assays (Wasylyk et al., 1990). Furthermore, both the PEA3 and the PEA1 motifs were required for this activation, in agreement with the results of the present in vitro study. Recently, it has been shown that PEA3 acts synergistically with AP-1 to generate high levels of transcription from the collagenase gene promoter induced by either TPA or non-nuclear oncoproteins (Gutman and Wasylyk, 1990).

The role of PEA2 remains unclear. Multimers of the PEA2 sequence motif conferred neither transcriptional activation nor repression of basal promoter activity in the cell-free system. In vivo data from transient assays supports the in vitro results of this report: multimers of either the (PEA2) or the (PEA1/PEA2) oligomer did not activate transcription of a reporter gene in FM3A cells (M. McWilliams, unpublished). Multimers of the PEA2 motif were unable to confer activation of DNA replication in transient assays, whereas both the PEA1 and PEA3 motifs could activate replication, after transfection of C127 or COP5 cells (Murakami et al., 1990).

Others have reported the PEA2 motif to contribute an activity that represses transcription (Wasylyk et al., 1988). Recently, however, a fourth DNA-binding factor distinct from PEA2 has been identified that binds to a sequence motif which completely encompasses the PEA2 motif in element 2 (Furukawa et al., 1990). In that report, the repressor activity in F9 EC cells associated with the PEA2 motif by Wasylyk et al., (1990) has been attributed instead to this new factor, PEBP4. PEBP4 is thought to compete with both PEA1 and PEA2 for binding to the same region of Py. The relative concentrations of each factor could thereby determine the activity of the alpha-core (Furukawa et al., 1990).

Although the results of the present study suggest that PEA2 is neither an independent activator nor repressor of transcription, there are other plausible explanations for the inactivity of (PEA2) multimers in the in vitro assay. Previous work on the behavior of this motif in vivo employed constructs in which it was a part of a larger region of the enhancer (Veldman et al., 1985; Mueller, et al., 1988; Muller et al., 1988). Perhaps, as suggested by Martin, et al., (1988) and the results of Wasylyk et al., (1988), the PEA2 motif is functional in transcription only in conjunction with both PEA1 and PEA3. Perhaps PEA2 has different transcriptional activities in different cell lines.

C) Element 2 Motifs as Enhansons

Ondek et al., (1988) have proposed an "enhanson" model for the functional organization of enhancers. This model proposes that enhancer motifs either correspond to or are composed of discrete functional units called enhansons. In addition, enhansons function only when stringent requirements are fulfilled concerning the spacing between successive enhanson motifs. Other investigators have divided the enhansons of the SV40 enhancer into three classes which have distinct requirements for activity: Class A enhansons generate a functional enhancer when multimerized or when associated with a second Class A enhanson; Class B enhansons display no enhancer activity when multimerized on their own, but can when they are associated with a class A enhanson; Class C enhansons possess intrinsic enhancer activity, since there are no stringent spacing requirements involved when this type of enhanson is multimerized to generate an enhancer (Fromental et al., 1988; Kanno et al., 1989).

Element 2 of the Py enhancer comprises at least three motifs that are candidates for enhanson status: the binding sites for PEA1, PEA2, and PEA3. If the PEA2 motif is in fact an enhanson, it remains a possibility that the (PEA2) and (mPEA1/PEA2) multimers were inactive in the in vitro assay because the PEA2 binding sites are spaced at intervals

unsuitable for activity in the constructs used.

Yamaguchi et al., (1989) have already speculated that PEA1 and PEA3 motifs act as enhansons. It was noted in the present study that spacing between adjacent PEA1 or PEA3 motifs may be critical for activation by their corresponding factors. Multimers of (PEA1), (PEA3), and (PEA3/PEA1) conferred activation, whereas multimers of (PEA3/mPEA1), (mPEA3/PEA1), and (PEA1/mPEA2), in which the identical wild-type motifs are separated by an additional 3 to 5 base pairs due to the presence of the mutated factor binding sites, are inactive. Interestingly, it has been suggested that the spacing between PEA3 and AP-1 (PEA1) motifs, both in the Py enhancer and in the collagenase promoter, modulates the level transcriptional activation induced by oncogene expression (Gutman and Wasylyk, 1990).

The (PEA3/PEA1) oligomer, comprising binding sites for both PEA3 and PEA1, was active as a multimer, but not as a monomer, even though it was shown that dimers of either the PEA3 site or the PEA1 site are active. A monomer oligonucleotide equivalent to the (PEA3/PEA1) sequence has been shown to activate transcription <u>in vivo</u> (Wasylyk et al., 1990). The reason for this discrepancy with the <u>in vitro</u> results presented here are not known, although the (PEA3/PEA1) monomer may have very low activity which is not detectable by the <u>in vitro</u> assay with any degree of certainty.

Further experiments using templates bearing multimers of PEA1, PEA2, or PEA3 motifs spaced at different intervals are required to clarify the role of spacing in the cell-free assay. However, the results of this study demonstrate that PEA1 and PEA3 motifs, although inactive as single copies, augment transcription as closely juxtaposed homologous or heterologous multimers (summarized in Table 1). Therefore, the PEA1 and PEA3 motifs may be the minimal structural sub-units of Polyomavirus enhancer element 2, and fulfill the definition of enhansons. The PEA1 and PEA3 motifs can therefore be categorized tentatively as Class A enhansons of the Py enhancer.

Our knowledge of the mechanisms by which enhancers stimulate transcription is, at best, incomplete. In particular, the means by which enhancers operate from up to ten kilobases away from the transcription start site is not understood.

Several models have been proposed for the mechanism of enhancer action. In the looping model, the intervening DNA between the enhancer sequences and the RNA start site form a loop such that proteins bound to enhancer motifs can make physical contact with proteins bound to promoter proximal elements. This model would allow enhancer binding factors to interact, perhaps synergistically, not only with each other but with a common target found at or near the transcription

Table 1. In vitro transcriptional activity of enhancer motifs. Representative results are shown of transcriptional activation by the enhancer motifs tested in the cell-free system. Synthetic oligonucleotides comprising factor binding motifs (listed in the left-most column) were cloned immediately upstream of the TATA box in the minimal promoter template, p371. These templates were tested for transcriptional activity in vitro as described in Materials and Methods. **a:** Activity is given as induction ratios relative to the activity of the minimal promoter template; **b:** activity of the TATA box driving transcription on its own.

Table 1. *In vitro* transcriptional activity of enhancer motifs

Oligonucleotide Insert	Copy Number	Activity[a]
none[b]	0	1
(AP1)	1	1
	2	5
	3	11
	4	18
	5	20
(PEA1)	1	1
	3	4
	4	5
(PEA2)	1	1
	2	1
	4	1
	5	1
(PEA3)	1	1
	2	2
	3	7
	5	9
(PEA3/PEA1)	1	1
	2	3
	3	5
(PEA3/mPEA1)	4	1
(mPEA3/PEA1)	1	1
	2	1
(PEA1/PEA2)	1	1
	4	1
(PEA1/mPEA2)	2	1
	4	1
(mPEA1/PEA2)	3	1
	7	1

initiation site. Looping is probably the most viable model of enhancer action in the light of recent experimental findings (Irani et al., 1983; Mukherjee et al., 1988; Muller et al., 1989). In the sliding model, enhancers and/or proteins bound to enhancer sequences recruit a target factor which then slides along the DNA to the start site to promote efficient transcription. Other models propose that enhancers somehow induce a conformational change in the chromatin fiber which promotes RNA pol II activity, or act to direct the surrounding regions of chromatin to a nuclear compartment enriched in transcription factors (recent reviews of enhancer mechanisms can be found in Schleif, 1988; Jeang and Khoury, 1988; Marriott and Brady, 1989).

The experiments of this study did not investigate orientation independent activation of transcription, or enhancer action at a distance. It is known, however, that the TATA box alone is not sufficient for the full response to a remote enhancer *in vivo*, which requires the presence of one or two factor binding motifs immediately upstream of the TATA box (Dierks et al., 1983; Green et al., 1983). It would be a straightforward exercise to generate templates for testing the ability of enhancer motifs to function in the cell-free system under such circumstances; *cis*-acting elements, such as PEA1/AP-1 and PEA3 binding sites, can be cloned into p371 in opposite orientation to the minimal promoter, at various

distances, or downstream of the G-less cassette, and tested for transcriptional activity *in vitro*. This remains as an interesting avenue for future studies.

REFERENCES

Acheson, N.H. 1981. Efficiency of processing of viral RNA during the early and late phases of productive infection by polyomavirus. J. Virol. 37: 628-635

Akmal, M., A. El-Ghor, and R.D. Burk. 1989. DNase I hypersensitive site maps to the HBV enhancer. Virology 172: 478-488

Allison, L.A., J.K. Wong, V.D. Fitzpatrick, M. Moyle, and C.J. Ingles. 1988. The C-terminal domain of the largest subunit of RNA polymerase II of S. cerevisiae, D. melanogaster, and mammals: a conserved structure with an essential function. Mol. Cell. Biol. 8: 321-329

Allison, L.A. and C.J. Ingles. 1989. Mutations in RNA polymerase II enhance or suppress mutations in GAL4. Proc. Natl. Acad. Sci. USA 86: 2794-2798

Angel, P., M. Imagawa, R. Chiu, B. Stein, R.J. Imbra, H.J. Rahmsdorf, C. Jonat, P. Herrlich, and M. Karin. 1987. Phorbol ester-inducible genes contain a common cis element recognized by a TPA-modulated trans-acting factor. Cell 49: 729-739

Amati, P. 1985. Polyoma regulatory region: a potential probe for mouse cell differentiation. Cell 43: 561-562

Banerji, J., S. Rusconi and W. Schaffner. 1981. Expression of a beta-globin gene is enhanced by remote SV40 DNA sequences. Cell 27: 299-308

Banerji, J., L. Olson, and W. Schaffner. 1983. A lymphocyte-specific cellular enhancer is located downstream of the joining region in immunoglobulin heavy chain genes. Cell 33: 729-740

Barbacid, M. 1986. Mutagens, oncogenes and cancer. Trends Genet. 2: 188-192

Bartolomei, M.S., N.F. Halden, C.R. Cullen, and J.L. Corden. 1988. Genetic analysis of the repetitive carboxyl-terminal domain of the largest subunit of mouse RNA polymerase II. Mol. Cell. Biol. 8: 330-339

Basilico, C., Y. Matsuyo, and H. Green. 1970. The interaction of polyomavirus with mouse-hamster somatic hybrid cells. Virology 41: 295-299

Bennett, E.R., M. Naujokas, and J.A. Hassell. 1989. Requirements for species-specific papovavirus DNA replication. J. Virol. 63: 5371-5385

Berk, A.J., and M.C. Schmidt. 1990. How do transcription factors work? Genes Dev. 4: 151-155

Bhat, N.K., R.J. Fisher, S. Fujiwara, R. Ascione, and T.S. Papas. 1987. Temporal and tissue-specific expression of mouse ets genes. Proc. Natl. Acad. Sci. USA 84: 3161-3165

Boccara, M., and F. Kelly. 1978. Expression of polyoma virus in heterokaryons between embryonal carcinoma cells and differentiated cells. Virology 90: 147-150

Bohmann, D., W. Keller, T. Dale, H.R. Scholer, G. Tebb, and I.W. Mallaj. 1987. A transcription factor which binds to the enhancer of SV40, immunoglobulin heavy chain, and U2 snRNA genes. Nature 325: 268-272

Bohmann, D., and R. Tjian. 1989. Biochemical analysis of transcriptional activation by Jun: differential activity of c- and v-jun. Cell 59: 709-717

Bohnlein, E., and P. Gruss. 1986. Interaction of distinct nuclear proteins with sequences controlling the expression of polyomavirus early genes. Mol. Cell. Biol. 6: 1401-1411

Borelli, E., R. Hen, and P. Chambon. 1984. Adenovirus-2 E1a products repress enhancer-induced stimulatin of transcription. Nature 312: 608-612

Boulukos, K.E., P. Pognonec, A. Begue, F. Galibert, J.C. Gesquiere, D. Stehelin, and J. Ghysdael. 1988. Identification in chickens of an evolutionarily conserved cellular ets-2 gene (c-ets-2) encoding nuclear proteins related to the products of the c-ets proto-oncogene. EMBO J. 7: 697-705

Bourgaux, P. 1964. The fate of polyomavirus in hamster, mouse, and human cells. Virology 23: 46-54

Brandl, C.J., and K. Struhl. 1989. Yeast GCN4 transcriptional activator protein interacts with RNA polymerase II in vitro. Proc. Natl. Acad. Sci. USA. 86: 2652-2656

Buratowski, S., S. Hahn, L. Guarente, and P.A. Sharp. 1989. Five intermediate complexes in transcription initiation by RNA polymerase II. Cell 56: 549-561

Campbell, B.A., and L. P. Villarreal. 1986. Lymphoid and other tissue-specific phenotypes of polyomavirus enhancer recombinants: positive and negative combinational effects on enhancer specificity and activity. Mol. Cell. Biol. 6: 1401-1411

Campbell, B.A., and L.P. Villarreal. 1988. Functional analysis of the individual enhancer core sequences of polyomavirus: cell-specific uncoupling of DNA replication from transcription. Mol. Cell Biol. 8: 1993-2004

Cao, Z., E.A. Barron, A.J. Carrillo, and Z.D. Sharp. 1987. Reconstitution of cell-type-specific transcription of the rat prolactin gene in vitro. Mol. Cell Biol. 7: 3402-3408

Carey, M., Y.-S. Lin, M.R. Green, and M. Ptashne. 1990. A mechanism for synergistic activation of a mammalian gene by GAL4 derivatives. Nature 345: 361-364

Caruso, M., C. Iacobini, C. Passananti, A. Felsani, and P. Amati. 1990. EMBO J. 9: 947-955

Chiu, R., P. Angel, and M. Karin. 1989. Jun-B differs in its biological properties from, and is a negative regulator of, c-Jun. Cell 59: 979-986

Chung, S., V. Folson, and X. Wooley. 1983. DNase-I hypersensitive sites in the chromatin of immunoglobulin K light chain genes. Proc. Nat. Acad. Sci. USA 80: 2427-2431

Courey, A.J., and R. Tjian. 1988. Analysis of Sp1 in vivo reveals multiple transcriptional domains, including a novel glutamine-rich activation motif. Cell 55: 887-898

Courey, A.J., D.A. Holzman, S.P. Jackson, and R. Tjian. 1989. Synergistic activation by the glutamine-rich domains of human transcription factor Sp1. Cell 59: 827-836

Cremisi, C., and C. Babinet. 1986. Negative regulation of early polyomavirus expression in mouse embryonal carcinoma cells. J. Virol. 59: 761-763

Davidson, I., C. Fromental, P. Augereau, A. Wildeman, M. Zenke, and P. Chambon. 1986. Cell-type specific protein binding to the enhancer of simian virus 40 in nuclear extracts. Nature 323: 544-548

Davidson, I., J.H. Xiao, R. Rosales, A. Staub, and P. Chambon. 1988. The HeLa cell protein TEF-I binds specifically and cooperatively to two SV40 enhancer motifs of unrelated sequence. Cell 54: 931-942

Davison, B.L., J.M. Egly, E.R. Mulvihill, and P. Chambon. 1983. Formation of stable pre-initation complexes between eukaryotic class B transcription factors and promoter sequences. Nature 301: 680-686

De Pamphilis, M.L. 1988. Transcriptional elements as components of eukaryotic origins of DNA replication. Cell 52; 635-638

De Simone, V., G. La Mantia, L. Lania, and P. Amati. 1985. Polyomavirus mutation that confers a cell specific cis-advantage for viral DNA replication. Mol. Cell Biol. 5: 2142-2146

deVilliers, J., and W. Schaffner. 1981. A small segment of polyomavirus DNA enhances the expression of a cloned beta-globin gene over a distance of 1400 base pairs. Nucleic Acids Res. 9: 6251-6264

deVilliers, J., W Schaffner, C. Tyndall, S. Lupton, and R. Kamen. 1984. Polyomavirus DNA replication requires an enhancer. Nature 312: 242-246

Dignam, J.D., R.M. Lebowitz, and R. G. Roeder. 1983. Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. Nucleic Acids Res. 11: 1475-1489

Dougherty, J.P., P. Augereau, and P. chambon. 1986. The mouse immunoglobulin heavy-chain gene enhancer contains sequences that inhibit transcritpion in vitro in HeLa cell extracts. Mol. Cell. Biol. 6: 4117-4121

Dynan, W.S., and R. Tjian. 1983. The promoter-specific transcription factor Sp1 binds to upstream sequences in the SV40 early promoter. Cell 35: 79-87

Elgin, S.C. 1988. The formation and function of DNase I hypersensitive sites in the process of gene activation. J. Biol. Chem. 263: 19,259-19,262

Ephrussi, A., G.M. Church, S. Tonegawa, and W. Gilbert. 1985. B-lineage-specific interactions of an immunoglobulin enhancer with cellular factors in vivo. Science 227: 134-140

Evans, R.M.. and S.M. Hollenberg. 1988. Zinc fingers: gilt by association. Cell 52: 1-3

Fire, A., M. Samuels, and P.A. Sharp. 1984. Interactions between RNA polymerase II, factors, and template leading to accurate transcription. J. Biol. Chem. 259: 2509-2516

Fried, M., and D.M. Crothers. 1981. Equilibria and kinetics of the lac repressor-operator interactions by polyacrylamide gel electrophoresis. Nucl. Acids Res. 9:6505-6525

Fromental, B., M. Kanno, H. Nomiyama, and P. Chambon. 1988. Cooperativity and hierarchical levels of functional organization in the SV40 enhancer. Cell 54: 943-953

Fromm, M. and P. Berg. 1982. SV40 early and late region promoter function are enhanced by the 72 base pair repeat inserted at distant locations and inverted orientations. Mol. Cell Biol. 3: 991-999

Furukawa, K., Y. Yamaguchi, E. Ogawa, K. Shigesada, M. Satake, and Y. Ito. 1990. A ubiquitous repressor interacting with an F9 cell-specific silencer and its functional suppression by diofferentiated cell-specific positive factors. Cell Growth and Diff. 1: 135-147

Garcia-Blanco, M.A., R.G. Clerc, and P.A. Sharp. 1989. The DNA-binding homeodomain of the Oct-2 protein. Genes Dev. 3: 739-745

Garner, M.M. and A. Revzin. 1981. A gel electrophoresis method for quantifying the binding of proteins to specific DNA regions: application to components of the E. coli lactose operon regulatory system. Nucl. Acids Res. 9: 3047-3060

Gentz, R., F.J. Rauscher III, C. Abate, and T. Curran. 1989. Parallel association of Fos and Jun leucine zippers juxtaposes DNA binding domains. Science 243: 1695-1699

Gill, G., and M. Ptashne. 1988. Negative effect of the transcriptional activator GAL4. Nature 334: 721-724

Gillies, S.D., S.L. Morrison, V.T. Oi, and S. Tonegawa. 1983. A tissue-specific transcripiton enhancer element is located in the major intron of a rearranged immunoglobulin heavy chain gene. Cell 33: 717-728

Giniger, E. and M. Ptashne. 1987. Transcription in yeast activated by a putative amphipathic alpha helix linked to a DNA binding unit. Nature 330: 670-672

Goding, C.R. and P. O'Hare. 1989. Herpes Simplex Virus Vmw65-Octamer Binding Protein interaction: a paradigm for combinatorial control of transcription. Virology 173: 363-367

Gorski, K., M. Carneiro, and U. Schibler. 1986. Tissue-specific in vitro transcription from the mouse albumin promoter. Cell 47: 767-776

Gross, L. 1953. A filterable agent, recovered from Ak leukemic extracts, causing salivary gland carcinomas in C3H mice. Proc. Soc. Exp. Biol. Med. 83: 414

Gutman, A., and B. Wasylyk. 1990. The collagenase gene promoter contains a TPA and oncogene-responsive unit encompassing the PEA3 and AP-1 binding sites. EMBO j. 9: 2241-2246

Halazonetis, T.D., K. Georgopoulos, M.E. Greenberg, and P. Leder. 1988. C-Jun dimerizes with itself and with c-Fos, forming complexes of different DNA binding affinities. Cell 55: 917-924

Hardy, S. and T. Shenk. 1989. E2F from adenovirus-infected cells binds cooperatively to DNA containing two properly oriented and spaced recognition sites. Mol. Cell. Biol. 9: 4495-4506

Hartshorne, T.A., H. Blumberg, and E.T. Young. 1986. Sequence homology of the yeast regulatory protein ADR1 with Xenopus transcription factor TFIIIA. Nature 320: 283-287

Hassell, J.A., W.J. Muller, and C.R. Mueller. 1986. The dual role of the polyomavirus enhancer in transcription and DNA replication. Cancer Cells 4: 561-569

Hearing, P. and T. Schenk. 1983. The adenovirus type 5 E1a transcriptional control region contains a duplicated enhancer element. Cell 33: 695-703

Heberlein, U., and R. Tjian. 1988. Temporal pattern of alcohol dehydrogenase gene transcription reproduced by Drosophila stage-specific embryonic extracts. Nature 331: 410-

415

Helmann, J.D., and M.J. Chamberlain. 1988. Sturcture and function of bacterial sigma factors. Annu. Rev. Biochem. 57: 839-872

Hen, R., E. Borelli, and P. Chambon. 1985. Repression of the immunoglobulin heavy chain enhancer by the adenovirus-2 E1a products. Science 230: 1391-1394

Herbomel, P., B. Bourachot, and M. Yaniv. 1984. Two distinct enhancers with different cell specificities co-exist in the regulatory region of polyoma. Cell 39: 653-662

Herr, W. and Y. Gluzman. 1985. Duplications of a mutated SV40 enhancer restore its activity. Nature 313: 711-714

Herr, W. and J. Clarke. 1986. The SV40 enhancer is composed of multiple functional elements that can compensate for one another. Cell 45: 461-470

Hirai, S.-I., R.-P. Ryseck, F. Mechta, R. Bravo, and M. Yaniv. 1989. Characterization of jun-D: a new memberr of the jun proto-oncogene family. EMBO j. 8: 1433-1439

Hollenberg, S.M. and R.M. Evans. 1988. Multiple and cooperative trans-activation domains of the human glucocorticoid receptor. Cell 55: 899-906

Homa, F.L., J.C. Glorioso, and M. Levine. 1988. A specific 15-bp TATA box promoter element is required for expression of a herpes simplex virus type 1 late gene. Genes Dev. 2: 40-53

Hope, I.A. and K. Struhl. 1986. Functional dissection of a eukaryotic transcriptional activator protein, GCN4, of yeast. Cell 46: 885-894

Hope, I.A., and K. Struhl. 1987. GCN4, a eukaryotic transcriptional activator protein, binds as a dimer to a target DNA. EMBO J. 6: 2781-2784

Horikoshi, M., T. Hai, Y.-S. Lin, M.R. Green, and R.G. Roeder. 1988. Transcription factor ATF interacts with the TATA factor to facilitate establishment of a preinitiation complex. Cell 54: 1033-1042

Horikoshi, M., C.K. Wang, H. Fujii, J.A. Cromlish, P.A. Weil, and R.G. Roeder. 1989. Cloning and structure of a yeast gene encoding a general transcription initiation factor TFIID that binds to the TATA box. Nature 341: 299-303

Imler, J.L., C. Schatz, C. Wasylyk, B. Chatton, and B. Wasylyk. 1988. A Harvey-ras responsive transcription element is also responsive to a tumor-promoter and to serum. Nature 332: 275-278

Jakobovits, E.B., S. Bratosin, and Y. Aloni. Formation of a nucleosome-free region in SV40 minichromosommes dependent upon a restricted segment of DNA. 1982. Virology 120: 340-348

Janson, L. and U. Pettersson. 1990. Cooperative interactions between transcription factors Sp1 and OTF-1. Proc. Natl. Acad. Sci. USA 87: 4732-4736

Johnson, P.F., W. Landshculz, B.J. Graves, and S. McKnight. 1987. Identification of a rat liver nuclear protein that binds to the enhancer core element of three animal viruses. Genes and Dev. 1: 133-146

Johnson, P.F., and S.L. Mcknight. 1989. Eukaryotic transcriptional regulatory proteins. Annu. Rev. Biochem. 58: 799-839

Jones, N.C., P.W.J. Rigby, and E.B. Ziff. 1988. Trans-acting protein factors and the regulation of eukaryotic transcription: lessons from studies on DNA tumor viruses. Genes Dev. 2: 267-281

Jongstra, J., T.L. Reudelhuber, P. Oudet, C. Benoist, C.-B. Chae, J.M. Jeltsch, D.J. Mathis, and P. Chambon. 1984. Induction of altered chromatin structures by SV40 enhancer and promoter elements.

Kadanoga, J.T., K.R. Carner, F.R. Maziarz, and R. Tjian. 1987. Isolation of a cDNA encoding transcirption factor SP1 and functional analysis of the DNA binding domain. Cell 51: 1079-1090

Kakidani, H., and M. Ptashne. 1988. GAL4 activates gene expression in mammalian cells. Cell 52: 161-167

Kanno, M., C. Fromental, A. Staub, F. Ruffenach, I. Davidson, and P. Chambon. 1989. The SV40 TC-II (kappa-B) and the related H-2KsuprscrB enhansons exhibit different cell type specific and inducible proto-enhancer activities, but the SV40 core sequence and the AP-2 binding site have no enhanson properties. EMBO J. 8: 4205-4212

Katinka, M., M. Yaniv, M. Vasseur, and D. Blangy. 1980. Expression of polyoma early functions in mouse embryonal carcinoma cells depends on sequence rearrangements in the beginning of the late region. Cell **20**: 393-399

Kingston, R.E., A.S. Baldwin, and P.A. Sharp. 1985. Transcription control by oncogenes. Cell **41**: 3-5

Kovesdi, I. M. Satake, K. Furukawa, R. Reichel, Y. Ito, and J.R. Nevins. A factor discriminating between the wild-type and mutant polyomavirus enhancer. 1987. Nature 328: 87-89

Kouzarides, T., and E. Ziff. 1989. Behind the Fos and Jun leucine zipper. Cancer Cells **1**: 71-76

Krainer, A.R., T. Maniatis, B. Ruskin, and M.R. Green. 1984. Normal and mutant human beta-globin pre-mRNAs are faithfully and efficiently spliced in vitro. Cell 36: 993-1005

Kryszke, M.-H., J. Piette, and M. Yaniv. 1987. Induction of a factor that binds to the polyomavirus A enhancer on differentiation of embryonal carcinoma cells. Nature 328: 254-256

Landschulz, W.H., P.F. Johnson, and S.L. McKnight. 1989. The DNA binding domain of the rat liver nuclear protein C/EBP is bipartite. Science **243**: 1681-1688

Laimins, L.A., G. Khoury, C. Gorman, B. Howard, and P. Gruss. 1982. Host-specific activation of transcription by tandem repeats from simian virus 40 and Moloney murine sarcoma virus. Proc. Nat. Acad. Sci. USA **79**: 6453-6457

Laimins, L.A., P. Gruss, R. Possatti, and G. Khoury. 1984. Characterization of enhancer elements in the long terminal repeat of Moloney murine sarcoma virus. J. Virol. 49: 183-189

LeBowitz, J.H., R.G. Clerc, M. Brenowitz, and P.A. Sharp. 1989. The Oct-2 protein binds cooperatively to adjacent octamer sites. Genes Dev. 3: 1625-1638

Lech, K. K. Anderson, and R. Brent. 1988. DNA-bound Fos proteins activate transcription in yeast. Cell 52: 179-184

Lee, K.A.W., A. Bindereif, and M.R. Green. 1988. A small-scale procedure for preparation of nuclear extracts that support efficient transcription and pre-mRNA splicing. Gene Anal. Techn. 5: 22-31

Lee, W., A. Haslinger, M. Karin, and R. Tjian. 1987a. Activation of transcription by two factors that bind promoter and enhancer sequences of the human metallothionein gene and SV40. Nature 325: 368-372

Lee, W. P. Mitchell, and R. Tjian. 1987b. Purified transcription factor AP-1 interacts with TPA-inducible enhancer elements. Cell 49: 741-752

Lillie, J.W. and M.R. Green. 1989b. Activator's target in sight. Nature 341: 279-280

Lin, Y.-S., M.F. Carey, M. Ptashne, and M.R. Green. 1988. GAL4 derivatives function alone and synergistically with mammalian activators in vitro. Cell 54: 659-664

Lin, Y.-S., M. Carey, M. Ptashne, and M.R. Green. 1990. How different eukaryotic transcriptional activators can cooperate promiscuously. Nature 345: 359-361

Lusky, M., and M.R. Botchan. 1986. Transient replication of bovine papilloma virus type I plasmids: cis and trans requirements. Proc. Natl. Acad. Sci. USA 83: 3609-3613

Ma, J., and M. Ptashne. 1987. Deletion analysis of GAL4 defines two transcriptional activating segments. Cell 48: 847-853

Macchi, M., J.-M. Bornert, I. Davidson, M. Kanno, R. Rosales, M. Vigneron, J.-H. Xiao, C. Fromental and P. Chambon. 1989. EMBO J. 8: 4215-4227

Maione, R., A. Felsani, L. Pozzi, M. Caruso, and P. Amati. 1989. Polyomavirus genome and polyomavirus enhancer-driven gene expression during myogenesis. J. Virol. 63: 4890-4897

Maniatis, T., E.F. Fritsch, and J. Sambrook. 1982. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

Maniatis, T., S. Goodbourn, and J.A. Fischer. 1987. Regulation of inducible and tissue-specific gene expression. Science 236: 1237-1245

Manley, J.L., A. Fire, A. Cano, P.A. Sharp, and M.L. Gefter. 1980. DNA-dependent transcription of adenovirus genes in a soluble whole-cell extract. Proc. Natl. Acad. Sci. USA 77: 3855-3859

Manley, J.L., A. Fire, M. Samuels, and P.A. Sharp. 1983. In vitro transcription: whole-cell extract. Methods in Enzymol. 101: 568-585

Marriott, S.J., and J.N. Brady. 1989. Enhancer function in viral and cellular gene regulation. Biochimica et Biophysica Acta 989: 97-110

Martin, M.E. J. Piette, M. Yaniv, W.-J. Tang, and W.R. Folk. 1988. Activation of the polyomavirus enhancer by a murine activator protein 1 (AP-1) homolog and two contiguous proteins. Proc. Natl. Acad. Sci. USA, 85: 5839-5843

Mattern, C.F., K.K. Takemoto, and W.A. Daniel. 1966. Replication of polyoma virus in mouse embryo cells: electron microscopic observations. Virology 30: 242-256

Maxam, A.M., and W. Gilbert. 1980. Sequenceing end-labeled DNA with base-specific chemical cleavages. Methods Enzymol. 65: 499-560

Melin, F., H. Pinon, C. Reiss, C. Kress, N. Montreau, and D. Blangy. 1985. Common feature of polyomavirus mutants selected on PCC4 embryonal carcinoma cells. EMBO J. 4: 1799-1803

Melton, D.A., P.A. Krieg, M.R. Rebagliati, T. Maniatis, K. Zinn, and M.R. Green. 1984. Efficient in vitro synthesis of biologically active RNA and RNA hybridization probes from plasmids containing a bacteriophage SP6 promoter. Nucl. Acids Res. 12: 7035-7056

Mermod, N., T.J. Williams, and R. Tjian. 1988. Enhancer binding factors AP-4 and AP-1 act in concert to activate SV40 late transcription in vitro. Nature 332: 557-561

Mermod, N., E.A. O'Neill, T.J. Kelly, and R. Tjian. 1989. The proline-rich transcriptional activator of CTF/NF-1 is distinct from the replication and DNA binding domain. Cell 58: 741-753

Meyer, M.E., H. Gronermeyer, B. Turcotte, M.T. Bocquel, T. Tusset, and P. Chambon. 1989. Steroid hormone receptors compete for factors that mediate their enhancer function.

Mitchell, P.J., C. Wang, and R. Tjian. 1987. Positive and negative regulation of transcription in vitro: enhancer-binding protein AP-2 is inhibited by SV40 T Antigen. Cell 50: 847-861

Mitchell, P.J., and R. Tjian. 1989. Transcriptional regulation in mammalian cells by sequence-specific DNA binding proteins. Science 245: 371-378

Moreau, P., R. Hen, B. Wasylyk, R. Everett, M.P. Gau, and P. Chambon. 1981. The SV40 72 base pair repeat has a striking effect on gene expression both in SV40 and other chimeric recombinants. Nucl. Acids Res. 9: 6047-6068

Moscovici, C., J. Samarut, L. Guzzolo, and M.G. Moscovici. 1981. Myeloid and erythroid neoplastic responses to avian defective leukemia viruses in chicken and in quail. Virol. 113: 765-768

Mueller, C.R., A.-M. Mes-Masson, M. Bouvier, and J.A. Hassell. 1984. Location of sequences in polyomavirus DNA that are required for early gene expression in vivo and in vitro. Mol. Cell Biol. 4: 2594-2609

Mueller, C.R., W.J. Muller, and J.A. Hassell. 1988. The polyomavirus enhancer comprises multiple functional elements. J. Virol. 62: 1667-1678

Müller, R. 1986. Proto-oncogenes and differentiation. Trends Biochem. Sci. 11: 129-132

Muller, W.J., C.R. Mueller, A.-M. Mes, and J.A. Hassell. 1983. Polyomavirus origin for DNA replication comprises multiple genetic elements. J. Virol. 47: 586-599

Muller, W. J., D. Dufort, and J.A. Hassell. 1988. Multiple subelements within the polyomavirus enhancer function synergistically to activate DNA replication. Mol. Cell Biol. 8: 5000-5015

Murakami, Y., M. Asano, M. Satake, and Y. Ito. 1990. A tumor promoting phorbol ester, TPA, enhances polyomavirus DNA replication by activating the function of the viral enhancer. Oncogene 5: 5-13

Nakabeppu, Y., K. Ryder, and D. Nathans. 1988. DNA binding activities of three murine Jun proteins: stimulation by Fos. Cell 55: 907-915

Nelson, C., V.R. Albert, H.P. Elsholtz, L.I. Lu, and M.G. Rosenfeld. Activation of cell-specific expression of rat growth hormone and prolactin genes by a common transcription factor. Science 239: 1400-1405

Ondek B., A. Shepard, and W. Herr. 1987. Discrete elements within the SV40 enhancer region display different cell-specific enhancer activities. EMBO J. 6: 1017-1025

Ondek, B., L. Gloss, and W. Herr. 1988. The SV40 enhancer contains two distinct levels of organization. Nature 333: 40-45

Ostapchuk, P., J.F.X. Diffley, J.T. Bruder, B. Stillman, A.J. Levine, and P. Hearing. 1986. Interaction of a nuclear factor with the polyomavirus enhancer region. Proc. Natl. Acad. Sci. USA 83: 8550-8554

Pabo, C.O., and R.T. Sauer. 1984. Protein-DNA recognition. Annu. Rev. Biochem. 53: 293-321

Padgett, R.A., S.M. Mount, J.A. Steitz, and P.A. Sharp. 1983. Splicing of messenger RNA precursors is inhibited by antisera to small nuclear riboprotein. Cell 35: 101-107

Peden, K.W.C., J.M. Pipas, S. P. White, and D. Nathans. 1980. Isolation of mutants of animal virus in bacteria. Science 209: 1392-1396

Pfeifer, K., K.S. Kim, S. Kogan, and L. Guarente. 1989. Functional dissection and sequence of yeast HAP1 activator. Cell 56: 291-301

Piette, J., M.-H. Kryszke, and M. Yaniv. 1985. Specific interaction of cellular factors with the B enhancer of polyoma virus. EMBO J. 4: 2675-2685

Piette, J., and M. Yaniv. 1985. Specific interaction of cellular factors with the beta-enhancer of polyomavirus. EMBO J. 4: 2675-2685

Piette, J., and M. Yaniv. 1986. Molecular analysis of the interaction between an enhancer binding factor and its DNA target. Nucl. Acids Res. 14: 9595-9611

Piette, J., and M. Yaniv. 1987. Two different factors bind to the alpha domain of the polyomavirus enhancer, one of which also interacts with the SV40 and c-fos enhancers. EMBO J. 6: 1331-1337

Piette, J., S.-I. Hirai, and M. Yaniv. 1988. Constitutive synthesis of activator protein 1 transcription factor after viral transformation of mouse fibroblasts. Proc. Natl. Acad. Sci. USA 85: 3401-3405

Pognonec, P., K.E. Boulukos, and J. Ghysdael. 1988. The c-ets-1 protein is chromatin associated and binds to DNA in vitro. Oncogene 4: 691-697

Ptashne, M. 1988. How eukaryotic transcriptional activators work. Nature 335: 683-689

Quinn, J.P., A.R. Farina, K. Gardner, H. Krutzsch, and D. Levens. 1989. Multiple components are required for sequence recognition of the AP1 site in the gibbon ape leukemia virus enhancer. Mol. Cell. Biol. 9: 4713-4721

Radke, K. H. Beug, S. Kornfeld, and T. Graf. 1982. Transformation of both erythroid and meyloid cells by E26, an avian leukemia virus that contains the myb gene. Cell 31: 643-653

Rao, V.N., M. Zweig, T.S. Papas, and E.S. Reddy. 1987. Expression in E. coli of erg: a novel gene in humans related to the ets oncogene. Oncogene Res. 2: 95-101

Rao, V.N., K. Huebner, M. Isobe, A. ar-Rushdi, C. M. Croce, and E.S. Reddy. elk, tissue-specific ets-related genes on chromosomes X and 14 near translocation breakpoints. Science 244: 66-70

Reddy, E.S., V.N. Rao, and T.S. Papas. The erg gene: a human gene related to the ets oncogene. Proc. Natl. Acad. Sci. USA 84: 6131-6135

Rochford, R., Campbell, B.A., and L.P. Villarreal. 1990. Genetic analysis of the enhancer requirements for polyomavirus DNA replication in mice. J. Virol. 64: 476-485

Ruley, H.E., and M. Fried. 1983. Sequence repeats in a polyomavirus DNA region important for gene expression. J. Virol. 47: 233-237

Ryder, K. and D. Nathans. 1988. Induction of protooncogene c-jun by serum growth factors. Proc. Natl. Acad. Sci. USA 85: 8464-8467

Ryder, K., A. Lanahan, E. Perez-Albuerne, and D. Nathans. 1989. Jun-D: A third member of the Jun gene family. Proc. Natl. Acad. Sci. USA 86: 1500-1503

Saragosti, S., G. Moyne, and M. Yaniv. 1980. Absence of nucleosomes in a fraction of SV40 chromatin between the origin of replication and the region coding for the late leader RNA. Cell 20: 65-73

Sassone-Corsi, P., J.P. Dougherty, B. Wasylyk, and P. Chambon. 1984. Stimulation of in vitro transcription from heterologous promoters by the SV40 enhancer. Proc. Natl. Acad. Sci. USA 81: 308-312

Sassone-Corsi, P., A. Wildeman and P. Chambon. 1985. A trans-acting factor is responsible for the simian virus 40 enhancer activity in vitro. Nature 313: 458-463

Sassone-Corsi, P., C. Fromental, and P. Chambon. 1987. A trans-acting factor represses the activity of the polyoma virus enhancer in undifferentiated embryonal carcinoma cells. Oncogene Res. 1: 113-119

Satake, M. T. Ibaraki, and Y. Ito. 1988. Modulation of polyomavirus enhancer binding proteins by Ha-ras oncogene. Oncogene 3: 69-78

Sawadogo, M., and R.G. Roeder. 1985a. Factors involved in specific transcrption by human RNA polymerase II; analysis by a rapid and quantiative in vitro assay. Proc. Natl. Acad. Sci. USA 82: 4394-4398

Sawadogo, M., and R.G. Roeder. 1985b. Interaction of a gene-specific transcription factor with the adenovirus major late promoter upstream of the TATA box region. Cell 43: 165-175

Schirm, S., J. Jiricny, and W. Schaffner. 1987. The SV40 enhancer can be dissected into multiple segments, each with a different cell type specificity. Genes Dev. 1: 65-74

Schöler, H.R., and P. Gruss. 1984. Specific interaction between enhancer-containing molecules and cellular components. Cell **36**: 403-411

Schöler, H.R., and P. Gruss. 1985. Cell type specific transcriptional enhancement in vitro requires the presence of trans-acting factors. EMBO J. **4**: 3005-3013

Schuh, R., W. Aicher, U. Gaul, S. Cote, A. Preiss, D. Maier, and E. Seifert. 1986. A conserved family on nuclear proteins containing structural elements of the finger protein encoded by Kruppel, a Drosophila segmentation gene. Cell **47**: 1025-1032

Schutte, J., J. Viallet, M. Nau, S. Segal, J. Fedorko, and J. Minna. 1989. jun-B inhibits and c-fos stimulates the transforming and trans-activating activities of c-jun. Cell **59**: 987-997

Sen, R., and D. Baltimore. 1986. Multiple nuclear factors interact with the immunoglobulin enhancer sequences. Cell **46**: 705-716

Serfling, E., M. Jasin and W. Schaffner. 1985. Enhancers and eukaryotic gene transcription. Trends in Genetics **1**: 224-230

Sergeant, A., D. Bohmann, H. Zentgraf, H. Weiher, and W. Keller. 1984. A transcription enhancer acts in vitro over distances of hundreds of base-pairs on both circular and linear templates but not on chromatin-reconstituted DNA

Shapiro, D.J., P.A. Sharp, W.W. Wahli, and M.J. Keller. 1988. A high-efficiency HeLa cell nuclear transcription extract. DNA 7: 47-55

Soeda, E., J. Arrand, N. Smolar, J. Walsh, and B. Griffin. 1980. Coding potential and regulatory signals of the polyomavirus genome. Nature 283: 445-453

Sopta, M., Z.F. Burton, and J. Greenblatt. 1989. Structure and associated DNA-helicase activity of a general transcription initiation factor that binds to RNA polymerase II. Nature 341: 410-414

Stern, S., M. Tanaka, and W. Herr. 1989. The Oct-1 homeodomain directs formation of a multiprotein-DNA complex with the HSV transactivator VP16. Nature 341: 624-630

Stewart, S.E., B.E. Eddy, and N. Borgese. 1958. Neoplasms in mice inoculated with a tumor agent carried in tussue culture. J. Natl. Cancer Inst. 20: 1223

Strauss, F., and A. Varshavsky. 1984. A protein binds to a satellite DNA repeat at three specific sites that would be brought into mutual proximity by DNA folding in the nucleosome. Cell 37: 889-901

Struhl, K. 1989. Molecular mechanisms of transcriptional regulation in yeast. Annu. Rev. Biochem. 58: 1051-1077

Sturm, R.A., and W. Herr. 1988. The POU domain is a bipartite DNA-binding structure. Nature 336: 601-604

Sturm, R.A., G. Das, and W. Herr. 1988. The ubiquitous octamer-binding protein Oct-1 contains a POU domain with a homeo box subdomain. Genes Dev. 2: 1582-1599

Swartzendruber, D.E., T.D. Friedrich, and J.M. Lehman. 1977. Resistance of teratocarcinoma stem cells to infection with simian virus 40. J. Cell. Physiol. 93: 25-30

Takimoto, M., J.P. Quinn, A.R. Farina, L.M. Staudt, and D. Levens. 1989. Fos/Jun and Octamer Binding Protein interact with a common site in a negative element of the human c-myc gene. J. Biol. Chem. 264: 8992-8999

Tanaka,K., K. Chowdhury, K.S.S. Chang, M. Israel, and Y. Ito. 1982. Isolation and characterisation of polyomavirus mutants which grow in murine embryonal carcinoma and trophoblast cells. EMBO J. 1: 1521-1527

Tang, W.-J., S.L. Berger, S.J. Treizenberg, and W.R. Folk. 1987. Nucleotides in the polyomavirus enhancer that control viral transcription and DNA replication. Mol. Cell Biol. 7: 1681-1690

Tooze, J. 1981. DNA tumor viruses, part 2/revised, 2nd ed. Cold Spring Harbour Laboratory, Cold Spring Harbor, New York.

Tora, L., J. White, C. Brou, D. Tasset, N. Webster, E. Scheer, and P. Chambon. 1989. The human estrogen receptor has two independent nonacidic transcritpional activation functions. Cell 59: 477-487

Treisenberg, S.J., R.C. Kingsbury, and S.L. McKnight. 1988. Functional dissection of VP16, the trans-activator of Herpes Simplex virus immediate early gene expression. Genes Dev. 2: 718-729

Treisman, R., M.R. Green, and T. Maniatis. 1983. cis and trans activation of globin gene transcription in transient assays. Proc. Natl. Acad. Sci. USA 80: 7428-7432

Tseng, R.W., and F.K. Fujimura. 1988. Multiple domains in the polyomavirus B enhancer are required for productive infection in F9 embryonal carcinoma cells. J. Virol. 62: 2890-2895

Tyndall, C., G. LaMantia, C.M. Thacker, J. Favalaro, and R. Kamen. 1981. A region of the polyomavirus genome between the replication origin and the late protein coding sequences is required in cis for both early gene expression and viral DNA replication. Nucl. Acids Res. 9: 6231-6250

Veldman, G.M. S. Lupton, and R. Kamen. 1985. Polyomavirus enhancer contains multiple redundant sequence elements that activate both DNA replication and gene expression., Mol. Cell Biol. 5: 649-658

Velcich, A., F.G. Kern, C. Basilico, and E.B. Ziff. 1986. Adenovirus E1a proteins repress expression from polyomavirus early and late promoters. Mol. Cell. Biol. 6: 4019-4025

Vogt, P.K. and R. Tjian. 1988. Jun: a transcriptional regulator turned oncogenic. Oncogene 3: 3-7

Wasylyk, B., C. Wasylyk, P. Augereau, and P. Chambon. 1983. The SV40 72 bp repeat preferentially potentiates transcription starting from proximal natural or substitute promoter elements. Cell 32: 503-514

Wasylyk, B. J.L. Imler, B. Chatton, C. Schatz, and C. Wasylyk. 1988. Negative and positive factors determine the activity of the polyomavirus enhancer alpha domain in undifferentiated and differentiated cell types. Proc. Natl. Acad. Sci. USA 85: 7952-7956

Wasylyk, B., C. Wasylyk, P. Flores, A. Begue, D. Leprince, and D. Stehelin. 1990. The c-ets proto-oncogenes encode transcription factors that cooperate with c-Fos and c-Jun for transcriptional activation. Nature 346: 191-193

Wasylyk, C., J.L. Imler, J. Perez-Mutl, and B. Wasylyk. 1987. The c-Ha-ras oncogene and a tumor promoter activate the polyomavirus enhancer. Cell 48: 525-534

Wasylyk, C., J.L. Imler, and B. Wasylyk. 1988. Transforming but not immortalizing oncogenes activate the transcription factor PEA1. EMBO J. 7: 2475-2483

Wasylyk, C., P. Flores, A. Gutman, and B. Wasylyk. 1989. PEA3 is a nuclear target for transcription activation by non-nuclear oncogenes. EMBO J. 8: 3371-3378

Weiher, H., M. Konig, and P. Gruss. 1983. Multiple point mutations affecting the simian virus 40 enhancer. Science 219: 626-631

Weiher, H., and M.R. Botchan. 1984. An enhancer sequence from bovine papilloma virus DNA consists of two essential regions. Nucl. Acids Res. **12**: 2901-2916

Weil, P.A., D.S. Luse, J. Segall, and R.G. Roeder. 1979. Selective and accurate initiation of transcription at the AD2 major late promoter in a soluble system dependent on purified RNA polymerase II and DNA. Cell **18**: 469-484

Wildeman, A.G., P. Sassone-Corsi, T. Trundstrom, M. Zenke, and P. Chambon. 1984. Stimulation of in vitro transcription from the SV40 early promoter by the enhancer involves a specific trans-acting factor. EMBO J. **3**: 3129-3133

Xin, J.-H., A. Cowie, P. Lachance, and J.A. Hassell. Molecular cloning and characterization of PEA3, a new member of the ets oncogene family. Manuscript in preparation.

Yamaguchi, Y. M. Satake, and Y. Ito. 1989. Two overlapping sequence motifs within the polyomavirus enhancer are independently the targets of stimulation by both the tumor promoter 12-O-tetradecnoylphorbol-13-acetate and the Ha-ras oncogene. J. Virol. **63**: 1040-1048

Yamamoto, K.K., G.A. Gonzalez, P. Menzel, J. Rivier, and M.R. Montminy. 1990. Characterization of a bipartite activator domain in transcription factor CREB. Cell **60**: 611-617

Yong, C.B., A. Cowie, and J.A. Hassell. Synergistic activation of transcription by PEA1 and PEA3, two factors that bind to the polyomavirus enhancer. Manuscript in preparation.

Zenke, M., T. Grundstrom, H. Matthes, M. Wintzerith, C. Schatz, A. Wildeman, and P. Chambon. 1986. Multiple sequence motifs are involved in SV40 enhancer function. EMBO J. 5: 387-397