REPLICATION AND TRANSCRIPTION ACTIVATION BY POLYOMAVIRUS ENHANCER MOTIFS

REPLICATION AND TRANSCRIPTION ACTIVATION BY POLYOMAVIRUS ENHANCER MOTIFS PEA1, PEA2, AND PEA3

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A Thesis

Submitted to the School of Graduate Studies

in Partial Fulfillment of the Requirements

for the Degree

Master of Science

McMaster University

August 1991

MASTER OF SCIENCE (1991) McMASTER UNIVERSITY

(Biology) Hamilton, Ontario

TITLE: Replication and Transcription Activation by Polyomavirus Enhancer Motifs PEA1, PEA2, and PEA3

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NUMBER OF PAGES: xi, 179

ABSTRACT

The polyomavirus enhancer is organized into three elements. One of these elements, Element 2, is particularly interesting because the activities of the factors which interact with it are highly regulated. There are at least three cellular proteins, PEA1, PEA2, and PEA3, which bind to adjacent sites in Element 2. These proteins are differentially active in mouse cells at different developmental stages and their activity is modulated by serum, tumor promoting agents and the products of several oncogenes. It is likely, therefore, that these cellular proteins play an important role in interpreting growth stimuli and other physiological cues in the mouse.

A plasmid was contructed which can be used to test enhancer elements for their ability to activate both transcription and DNA replication. This plasmid includes the Py origin of replication and a minimal promoter, consisting of a TATA box only, controlling expression of a reporter gene. The activity of the PEA factors was studied by cloning the binding sites for these factors into this reporter plasmid as monomers, multiple tandem copies, and in paired combinations,

and testing their ability to activate transcription and DNA replication *in vivo*. The results of these studies show that PEA1 and PEA3 can function independently and cooperatively to activate both replication and transcription. By contrast, PEA2 is unable to independently activate transcription and represses PEA1-activated transcription when the binding sites for these factors are located adjacent to one another. However, PEA2 functions cooperatively with PEA1 to activate DNA replication, and can weakly activate replication on its own.

ACKNOWLEDGEMENTS

I would like to thank my supervisor, Dr. John Hassell for financial support and advice. I am also grateful for financial support from the Ontario Graduate Scholarship program.

I remember with fondness, all those people at McGill and McMaster Universities who contributed to the many enjoyable memories I have of the last few years.

I thank Alison Cowie for reviewing my thesis and for many helpful discussions and encouraging words along the way. I also appreciate help from Carmela Civitareale for the printing of this thesis.

For all their love and support, a special thanks to my parents and to my favourite guy, Tony.

CONTRIBUTIONS BY OTHERS

Some of the plasmids containing the double-site oligonucleotides were constructed and sequenced by Paul LaChance. Synthetic oligonucleotides were synthesized by Dinsdale Gooden of the Institute for Molecular Biology and Biotechnology, McMaster University. All other experimental procedures were performed by H. Michele McWilliams.

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INTRODUCTION

A. Polyomavirus: general background

i) Introduction

Polyomavirus (Py) is a member of the papovavirus family. It was originally identified as a causative agent of parotid tumors in mice (Gross, 1953) but subsequent studies showed that Py can induce tumors in different tissues in a wide variety of species (Stewart et al., 1958). The natural host for Py is the mouse since this is the only species in which the virus can undergo a full lytic cycle to produce progeny virus. Py can infect cells from other species but either replicates poorly and yields no progeny (rats, hamsters), or fails to replicate at all (other species) (Black, 1964).

ii) Genomic Organization

Py has a circular, double stranded DNA genome which is complexed with cellular histones within the virion. The viral genome is divided into two transcription units. The early transcription unit encodes three proteins, called tumor (T) antigens, which are expressed during the initial phase of viral infection. The late genes, encoding three virion structural proteins, are expressed 12-15 hours after infection, concurrent with, or just after the start of viral

DNA replication. Transcription of early and late genes occurs in opposite directions around the genome and is controlled by a central regulatory region. This noncoding region comprises the viral origin of replication and the early and late promoters, including the Py enhancer. (see Figure 1)

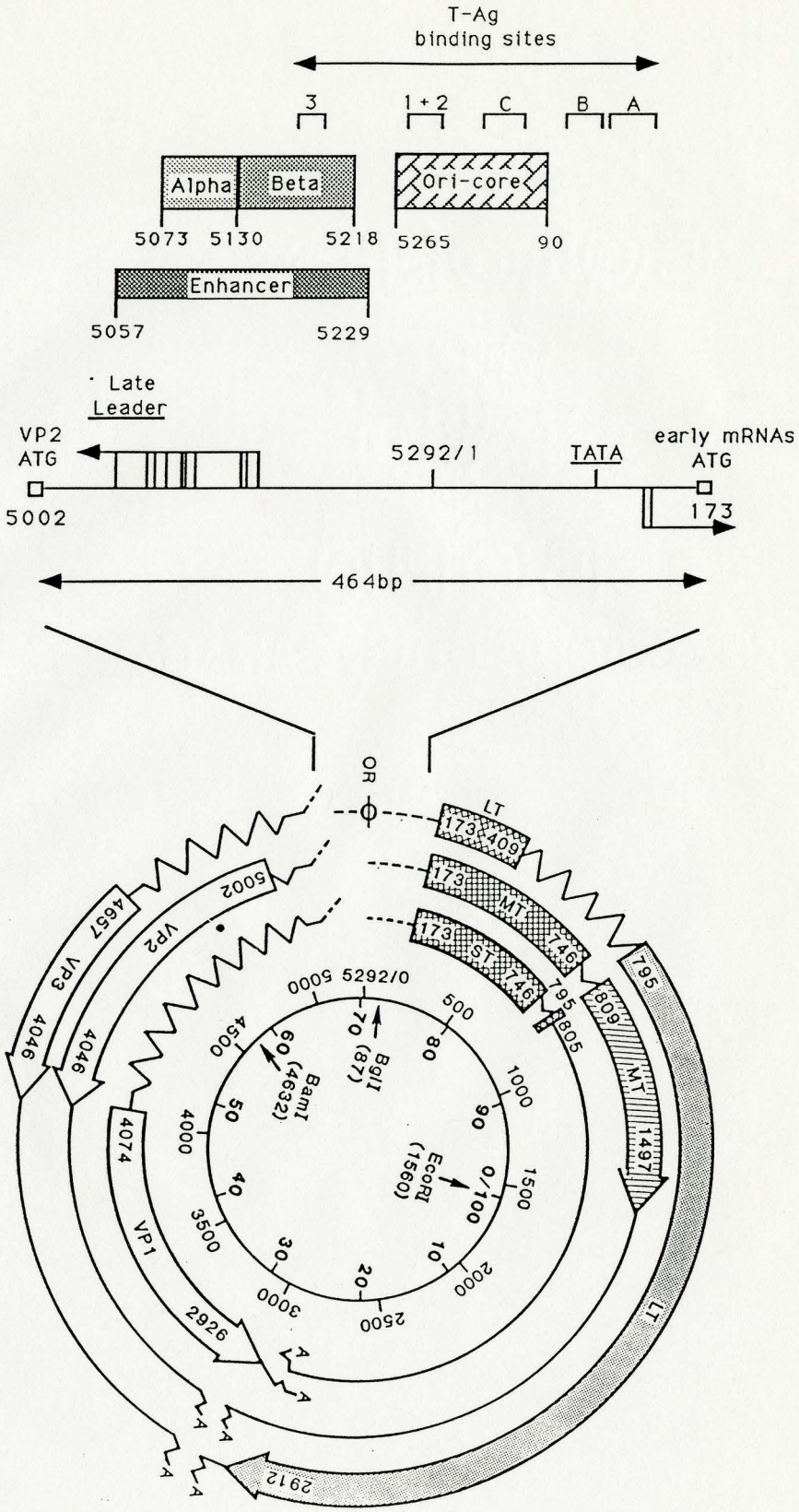
iii) Lytic Cycle

After adsorption to the host cell, the virus particle enters the cell and is transported to the nucleus where it is uncoated. The viral early transcription unit is then expressed to yield small, middle, and large T antigens. During the early stage of viral infection there are considerable changes in the host cell metabolism including increased synthesis of histones and enzymes involved in DNA replication. When sufficient levels of the viral early gene products and host cell replication factors have accumulated, viral DNA replication begins. Py large T antigen initiates this process by binding to the hexanucleotide sequence 'AGAGGC' (Pomerantz et al., 1983; Cowie and Kamen, 1984) which is present in several copies around the Py origin of replication. With the onset of DNA replication, expression of the early genes decreases and the late region is efficiently expressed, yielding the three structural proteins VP1, VP2, and VP3. These proteins are assembled into empty

Figure 1. Organization of the polyomavirus genome

A schematic diagram of the polyomavirus genome is shown at the bottom of the figure. The inner circle shows map units and nucleotide co-ordinates (Tooze 1981; Soeda et al., 1980). Regions encoding early (clockwise) and late (counter-clockwise) gene products are shown as arrows. Initiation, splice and polyadenylation sites are indicated by nucleotide numbers. OR indicates the viral origin of replication. (adapted from Tooze, 1981)

An expanded version of the non-coding region located between the early and late transcription units is shown at the top of the figure. This 464 bp region contains the Py enhancer and overlapping α and ß auxiliary replication units, as well as the origin of replication. Also shown are the early TATA box, the major early and late transcription initiation sites, and binding sites for the virally encoded early gene product, large T antigen.


T-Ag
binding sites
3
1+2
C
B
A
Alpha
Beta
Ori-core
5073
5130
5218
5265
90
Enhancer
5057
5229
Late
Leader
VP2
ATG
5292/1
TATA
early mRNAs
ATG
5002
173
464bp
OR
LT
173
409
MT
746
ST
795
805
809
MT
1497
LT
2912
A
4657
VP3
4046
VP2
5002
4046
4074
VP1
2926
5292/0
5000
500
1000
1500
2000
2500
3000
3500
4000
4500
BamI
(4632)
BglI
(87)
EcoRI
(1560)

capsids in the nucleus of the infected cell. Subsequent association with viral chromatin yields mature virion particles which are released following cell lysis. Progeny virus begin to appear by 20-25 hours post infection (reviewed in Tooze (ed), 1981).

Large T antigen has several important functions during the lytic cycle. It represses early gene expression by binding to DNA sequences around the early promoter, thereby sterically blocking initiation of transcription from this region (Cogen, 1978; Cowie and Kamen, 1984). It is also required for initiation of each new round of viral DNA replication (Franke and Ekhart, 1973). Furthermore, large T antigen seems to function as an activator of late gene expression (Kern et al., 1986; Featherstone, Ph.D. thesis, McGill University, Montreal, Quebec, 1986), although the mechanism by which it does this is unclear. In the absence of both the large T binding sites and the origin of replication, large T antigen induces a 2-4 fold increase in late promoter activity. However, in the presence of the large T binding sites, transcription from the late promoter is stimulated up to 70 fold by large T antigen (Kern et al., 1986). These results suggest that large T antigen can activate late gene expression by two independent mechanisms; one that requires DNA binding and one that doesn't.

The roles of small and middle T Ag in the lytic cycle are not well understood. Middle T antigen, by itself or together with large T, has been shown to stimulate late gene expression (Kern et al., 1986). Small T antigen may play an auxiliary role in viral DNA replication (Templeton et al., 1986).

iv) Animal Viruses as models

Animal viruses are commonly used as models to study cellular processes such as DNA replication and transcription in mammalian cells. Py and simian virus 40 (SV40), another member of the papovavirus family, are particularly attractive models because of their small size (approximately 5 kilobase pairs) and relative simplicity. Moreover, because these viruses rely extensively on cellular factors for replication and transcription of their genomes, they are thought to accurately reflect mechanisms employed by the host cell to effect these same processes. The following discussion provides a general review of current literature on eukaryotic DNA replication and transcription and then focuses on what has been learned about these processes through studies with Py in particular.

B. Eukaryotic transcription

i) Introduction

The enzymes which transcribe information from DNA to RNA are called RNA polymerases. In eukaryotes, it is RNA polymerase (pol) II, one of three RNA polymerases in the cell that makes messenger RNA (mRNA). Initiation of transcription is a complex process which requires the assembly of pol II and several other cellular factors into an active transcription complex at mRNA start sites. Regulation of pol II transcription is controlled through two classes of cis-acting DNA sequences called promoters and enhancers. Promoters are located close to the site of mRNA initiation while enhancers can be found as far as several kilobases (kb) away.

ii) Promoters and enhancers

A general strategy for identifying DNA sequences that control pol II transcription has been to delete or mutagenize regions near the transcription initiation sites of pol II transcribed genes and then analyze the effect of these alterations on mRNA levels. From studies like this, it has become apparent that the promoters and enhancers of protein-coding genes are comprised of variable arrays of transcription control elements. The particular combination and arrangement

of these cis-acting elements is thought to determine the unique pattern of expression of each pol II transcribed gene. (reviewed in Serfling and Schaffner, 1985, Maniatis et al., 1987; Mitchell and Tjian, 1989)

A common element in pol II promoters is the 'TATA' box. This A/T-rich element, with the consensus sequence TATAA/TAA/T (M. Goldberg, Ph.D. thesis, Stanford University, Stanford, Calif., 1978), is found 25 to 30 bp upstream of the transcription initiation site of many pol II transcribed genes. When mutations are introduced into this sequence, the specificity of mRNA initiation is reduced and transcripts with heterogenous 5' ends are generated (Grosschedl and Birnsteil, 1980; Grosveld et al., 1981). It seems, therefore, that one of the functions of the TATA box is to accurately position the site of RNA initiation.

Although the TATA box is found in most pol II promoters, it is not absolutely required for promoter function in vivo. Some promoters, particularly those of housekeeping genes (genes which are constitutively expressed), lack a TATA box. Moreover, the TATA box can be eliminated from some promoters which normally include this sequence element without significantly affecting the level of RNA synthesis in vivo (Benoist and Chambon, 1980; Fromm and Berg, 1982). Nevertheless, the TATA box appears to be an essential element

for transcription of some genes since deletion of this sequence can abolish RNA synthesis both *in vivo* and *in vitro* (Grosveld et al., 1981; Corden et al., 1980; Dierks et al., 1983).

In some promoters lacking a TATA box, a separate element called the 'initiator' determines the start site for transcription. This sequence overlies the start site itself and directs a low level of transcription initiation from a single internal nucleotide (Smale and Baltimore, 1989). Transcription from the initiator can be increased by the addition of a heterologous TATA box and/or other promoter proximal elements (Smale and Baltimore, 1989).

Aside from the TATA box, most pol II transcribed genes have additional sequence elements in the promoter proximal region which regulate the frequency of transcription initiation. Some of these elements, which are usually located upstream (5') of the TATA box, are common to many promoters. Examples of two elements which have been evolutionarily-conserved are the so-called 'CCAAT' box (C/TATTA/G) present upstream of all cellular globin genes (Efstratiadis et al., 1980) as well as many other unrelated genes (Graves et al., 1986), and a G/C rich sequence (GGGCGG) which can be found in multiple copies in some promoters such as the SV40 early promoter (Everett et al., 1983; Dynan and Tjian, 1983). The

contribution of these elements to promoter function varies from gene to gene. For example, mutation of the CCAAT box in the rabbit ß globin promoter reduces transcription 20-fold in vivo (Dierks et al., 1983), whereas a similar mutation in this element in the context of either the herpes simplex virus type 1 thymidine kinase (HSVtk1) promoter (McKnight et al., 1981), or the SV40 early promoter (Benoist and Chambon, 1981), has no effect on transcription. In addition to these conserved elements, some promoters contain gene-specific elements which regulate transcription in response to particular stimuli such as heat shock or physiological agents such as hormones and growth factors.

Upstream promoter elements (UPE) can function in either orientation to modulate transcription initiation but, in general, are unable to function when moved further than 150 bp from the TATA box (McKnight et al., 1982; Takahashi et al., 1986). The function of some UPE may also require that they be positioned on the same face of the DNA helix as the TATA box. For example, it has been demonstrated that the ability of the G/C rich elements in the SV40 promoter to activate transcription is significantly reduced when they are placed on the opposite side of the DNA helix relative to the TATA box (Takahashi et al., 1986).

Together, the promoter proximal elements direct transcription in a low but specific manner. Transcription can be greatly stimulated by additional cis-linked regulatory sequences located further away from the transcription initiation site. These promoter distal elements are called enhancers.

Enhancers were first identified in the genomes of the papovaviruses, SV40 and Py, where they were shown to stimulate transcription from the viral early promoters up to 1000 fold in vivo (Moreau et al., 1981; Banerji et al., 1981; deVilliers and Schaffner, 1981). Enhancers have subsequently found associated with many other viral and cellular genes (reviewed in Gluzman, 1985). Sequence analysis of many enhancers has identified several conserved elements but no unique sequence common to all enhancers has been found (Laimins et al., 1982).

Unlike promoter-proximal elements, enhancers can activate transcription in a distance-independent manner. This phenomenon was first observed when it was shown that the SV40 enhancer could activate transcription from a cis-linked promoter even when placed as far as 3 kb away from the transcription initiation site (Banerji et al, 1983). Although the enhancers of many cellular genes are found just upstream of their promoter, there are other cases in which the enhancer for a gene has been located as far as 10 kb away (Pinkert et

al., 1987), or in a position downstream to the promoter. The immunoglobulin (Ig) heavy chain enhancer, for example, is present within an intron downstream of the transcription initiation site for this gene (Gilles et al., 1983).

Enhancers may function in a wide variety of cell types, as is the case for the SV40 and Py enhancers (Laimins et al., 1982; deVilliers et al., 1982), or, like the Ig enhancer which is only active in lymphoid cell lines (Gilles et al, 1983), their activity may be restricted to certain cell types. An additional property of some enhancers is the ability to modulate transcription in response to environmental stimuli such as growth factors, phorbol esters and heavy metal ions. Both positive and negative regulatory effects are mediated through enhancers (for review see Maniatis, et al, 1987).

Some enhancers contain direct tandem repeats of sequences. The SV40 enhancer, for example, has two copies of a 72 bp element. The repeated sequences are functionally redundant since deletion of one of the two copies does not alter transcription efficiency (Banerji et al., 1981). Furthermore, mutations within the 72 bp repeat which impair enhancer activity can be compensated by duplications in nonmutated regions of the enhancer (Herr and Gluzman, 1985; Herr and Clarke, 1986). Other enhancers lack direct repeats

but still involve elements scattered over 100 to 300 bp that appear to be functionally redundant. For example, the Py enhancer can be divided into multiple elements that have little sequence homology, but can still functionally compensate for each other (Veldman et al., 1985; Mueller et al., 1984, 1988). These observations have led to the realization that enhancers are not continuous units but rather they are comprised of many smaller elements which function cooperatively to activate transcription.

Detailed analysis of the SV40 enhancer has provided further insight into enhancer structure. Experiments performed in several labs suggest that at least two distinct levels of organization exist within enhancers. Three 15-20 bp long sequences, A, B, and C, were identified as important transcription elements in the SV40 enhancer on the basis of viral revertant studies (Herr and Gluzman, 1985; Herr and Clarke, 1986). Subsequent experiments demonstrated that elements A, B, and C have little or no intrinsic activation properties of their own when present in single copies in front of a promoter, but duplication of a single element or combinations of two different elements efficiently enhance transcription. Cooperation between elements can be observed even when they are separated by up to 100 bp (Ondek et al., 1987, 1988). Analysis of yet smaller sequence motifs from

within these elements revealed that A, B, and C are themselves composed of subunits. These 8-10 bp subunits, called 'enhansons' (Ondek et al., 1988), differ from elements because they are very sensitive to changes in spacing. Five or ten bp insertions between enhansons dramatically affects the ability of the element to activate transcription. However, subunits from different elements can be mixed and matched to create functional heterologous or homologous pairs, indicating some flexibility in their arrangement (Ondek et al., 1988; Fromental et al., 1988). The combination of two enhansons to create enhancer elements is thought to represent a second level of organization within enhancer structure. (see Figure 2B)

Together, these studies show that enhancer activity is generated by the combined action of multiple individual elements which cooperate to activate transcription. It seems that a critical number of elements are required to constitute an active enhancer. This requirement can be met by combinations of many different elements or by multimerization of only a few elements.

iii) Sequence-specific transcription factors

The regulatory sequences which have been identified by genetic analyses in pol II promoters and enhancers

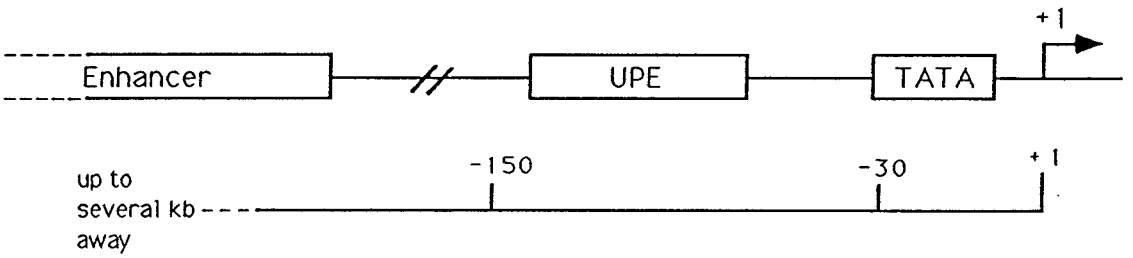
Figure 2. Regulatory regions of eukaryotic pol II transcribed genes

A) Schematic diagram showing the organization of the regulatory region of a typical pol II transcribed gene. Negative numbers give the distance from the site of transcription initiation.

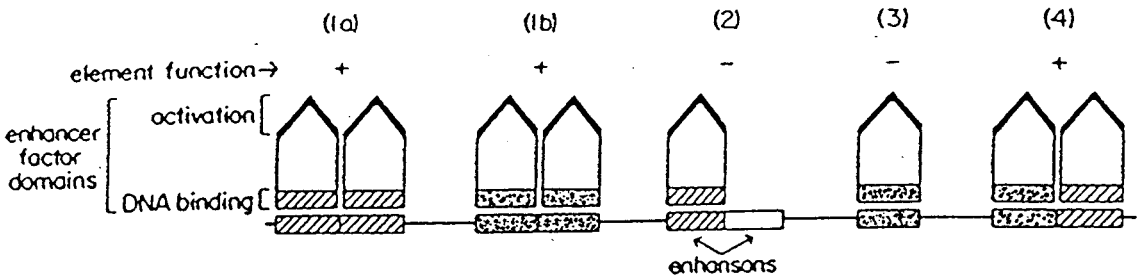
B) Schematic diagram illustrating the 'enhanson' model of enhancer structure (Ondek et al., 1988). The short 8-10 bp enhansons are depicted as shaded boxes. Shown above, are the sequence-specific transcription factors which bind to the enhansons. Individual enhansons have no activity (-) but combinations of two homologous or heterologous enhansons closely adjuxtaposed create a functional enhancer element (+).

C) Illustration showing how <u>trans</u>-acting factors bound at an enhancer located far from the transcription initiation site are thought to activate transcription through interactions with proximally bound factors. In this model, the DNA between the enhancer and promoter loops out to allow proteins bound at these regions to interact. This diagram also illustrates how the close adjuxtaposition of two proteins bound at adjacent enhansons might allow for the formation of a functional activation domain which could interact with a target in the general transcription initiation complex to promote transcription.

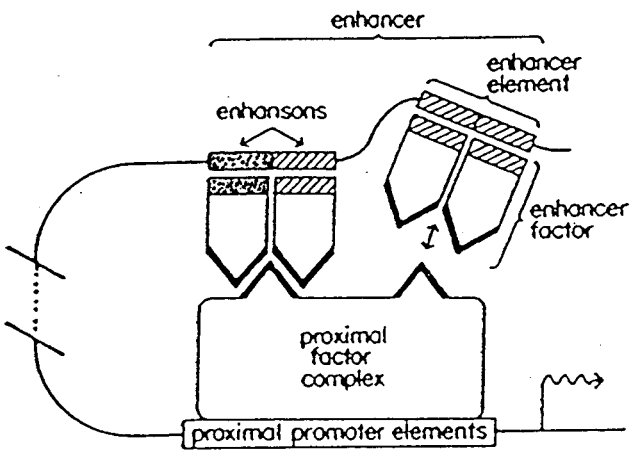
A


+1
Enhancer
UPE
TATA
-150
-30
+1
up to
several kb
away

B


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

C


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

constitute binding sites for cellular proteins. This was demonstrated initially by *in vivo* competition experiments which showed that transcription from a reporter gene driven by the SV40 enhancer was inhibited by cotransfection of a second plasmid carrying the SV40 enhancer alone (Schöler and Gruss, 1984; Wildeman et al., 1984). This result suggested that elements within the SV40 enhancer interact with cellular factors that are present in limiting amounts in the cell. Subsequently, DNase I and dimethyl sulphate protection analysis of the SV40 enhancer showed that cell nuclear extracts contain DNA binding proteins which interact specifically with the sequences identified by functional analysis to be important for transcriptional regulation (Wildeman et al., 1986; Davidson et al., 1986). The correlation between factor binding and transcription activation was further confirmed when it was shown that the introduction of point mutations into the SV40 enhancer which interfere with protein binding *in vitro*, also reduce transcription *in vivo* (Wildeman et al., 1986; Zenke et al., 1986).

Analysis of many promoters and enhancers has revealed that there are a multitude of different discrete nucleotide sequences which constitute binding sites for different cellular factors (Wingender, 1988). Some transcription

factors such as SP1 (Dynan and Tjian, 1983) and Oct1 (Sive and Roeder, 1986; Sturm et al., 1987) are present in many different cell types. These factors modulate the transcription of a wide variety of viral and cellular genes. The expression of other factors, such as Oct2 and MyoD, is restricted to certain cell types. For example, Oct2 is only found in lymphoid cell lines (Staudt et al., 1986) and MyoD expression is restricted to muscle cells (Buskin et al., 1989). These factors play key roles in the transcription of cell-specific genes. There exists another large group of transcription factors whose activities are inducible. Because induction can occur in the absence of protein synthesis, activation is thought to involve the modification of pre-existing cellular factors. Several different types of mechanisms involved in the post-translational regulation of sequence-specific transcription factors have been observed (reviewed in Maniatis et al., 1987) and a few are discussed below.

Treatment of cells with phorbol esters such as 12-O-tetradecanoyl-phorbol 13-acetate (TPA) to stimulate protein kinase C (PKC) activity dramatically increases the activity of many enhancers, suggesting that the activity of some transcription factors may be regulated by phosphorylation (Imbra and Karin, 1986; Wasylyk et al., 1987). More direct

evidence for the role of phosphorylation in modulating transcription factor activity comes from recent experiments showing that the activation of PKC results in the rapid dephosphorylation of a protein called c-jun which is a major component of the transcription complex AP1. Dephosphorylation of c-jun results in increased DNA binding activity of AP1 and increased transcription of genes containing AP1 binding sites (Boyle et al., 1991). Phosphorylation also controls the activity of the yeast heat shock transcription factor (HSF), however in this instance, phosphorylation increases the activation properties of HSF that is already bound to heat shock promoter elements (Sorger and Pelham, 1988). Since many transcription factors are phosphoproteins, it is likely that phosphorylation is important in controlling the activity of some of these proteins.

Protein-protein interactions are also important in the regulation of some transcription factors. For example, the lymphoid-specific factor NF-kB is regulated by association with another protein called IkB. When complexed to IkB, NF-kB is sequestered in the cytoplasm where it is unable to activate transcription. Treatment of cells with TPA, cytokines, and other growth factors causes the complex to dissociate, allowing transport of NF-kB to the nucleus where it can bind to its recognition sequence and activate

transcription (Baeuerle and Baltimore, 1988). Other factors which are negatively regulated by protein-protein interactions include AP2 and the yeast transcription factor GAL4.

There are also a number of transcription factors that are positively regulated by association with another protein. The herpes virus immediate early protein Vmw65 is a potent activator of transcription but since it lacks a DNA binding domain, Vmw65 must form a complex with the cellular DNA binding protein Oct1 in order to activate transcription (Goding and O'Hare, 1989). Steroid hormone receptors are able to bind to DNA and activate transcription only after they have been induced to undergo conformational changes by hormone binding (reviewed in Beato, 1985).

The discovery that some transcription factors are comprised of multiple protein subunits has revealed additional levels of complexity in the regulation of eukaryotic transcription. The AP1 transcription factor, for example, is a complex of several polypeptides encoded by multiple genes including c-jun, junB, junD, fos, and fra1 (for review see Curran and Franza, Jr, 1988). Various combinations of homo- and hetero-dimers involving these proteins can bind to AP1 sites. Since it has recently been demonstrated that at least two members of the AP1 family, cjun and junB, have different activation properties (Chiu et al., 1989), it is likely that

competitive binding of the various forms of AP1 to the AP1 recognition sequence plays a role in regulating the activity of this factor.

There are several other examples in which multiple factors have been found to bind to the same sequence. In HeLa cells, for example, differential splicing of mRNA for the CTF/NF1 gene produces several proteins which can bind to the CCAAT element found in many promoters and enhancers. Each of these CCAAT binding proteins has a different version of a carboxy-terminal activation domain which could conceivably be regulated to activate transcription in different contexts (Santoro et al., 1988). Hormone receptors also share similarities in their DNA binding specificities. The glucocorticoid and progesterone receptors both bind to the same sequences in the MMTV LTR enhancer with subtle differences that can influence the relative strength of binding to different sites (Chalepakis et al., 1988). Thus it appears that competition between different transcription factors for the same DNA sequence may be a relatively common phenomenon. Furthermore, the studies described above suggest that this may be an important means by which different transcriptional regulatory signals can be mediated through a single sequence element in different cell types or in response to different environmental stimuli.

From these examples, it is apparent that many of the sequence-specific factors which control pol II transcription are themselves highly regulated. Regulation occurs through a variety of mechanisms, including cell-specific gene expression, post-translational modifications, and/or protein-protein interactions. The discovery that the products of some nuclear oncogenes such as v-jun and v-fos are modified forms of cellular transcription factors (Setoyama et al., 1986; Bohmann et al., 1987; Angel et al., 1988; Chiu et al., 1988; Struhl, 1988; Vogt and Bos, 1990) suggests that inappropriate expression of these factors can have profound consequences on normal cellular functions.

iv) Functional domains of sequence-specific transcription factors

Structural analysis of many sequence-specific transcription factors has shown that they generally consist of separable functional domains, including a DNA binding domain as well as one or more transcription activation domains (reviewed in Mitchell and Tjian, 1989).

Several DNA binding structures have been identified in transcription factors. In general, these DNA binding domains are relatively small, consisting of 60 to 100 amino acids. Common motifs include the helix-turn-helix (homeo)

domain, zinc fingers, leucine zippers, and a helix-loop-helix homology domain (reviewed in Johnson and McKnight, 1989).

The helix-turn-helix motif was initially described as a DNA binding motif in the bacteriophage proteins CRO and λ repressor (Pabo and Sauer, 1984). Crystallography studies revealed that these proteins share a common DNA binding domain composed of two alpha helices separated by a beta turn. In eukaryotes, a related motif called the 'homeodomain' was identified as a highly conserved region in proteins involved in the regulation of Drosophila embyrogenesis (McGinnis et al., 1984). Evidence that the homeodomain (HD) is directly involved in sequence-specific DNA binding came from experiments showing that a fusion protein containing only the HD of the drosophila engrailed protein linked to ß-galactosidase can bind to the engrailed recognition site *in vitro* (Desplan et al., 1985). Other eukaryotic factors which possess this domain are the octamer binding proteins, Oct1 and Oct2 and the pituitary-specific factor Pit1 (Staud et al., 1986; Sturm et al., 1987; Ingraham et al., 1988).

A second type of DNA binding structure, the zinc finger, is present in a variety of transcriptional regulatory proteins including the pol III factor TFIIIA, the yeast transcriptional activator GAL4, the mammalian factor SP1, and

steroid hormone receptors (Miller et al., 1985; Kadagona et al., 1987; Beato, 1989). The zinc finger motif consists of two pairs of histidine or cysteine amino acids separated by a nonconserved segment of about 12 amino acids. The DNA binding structure is formed through the interaction of a single zinc atom with the 4 conserved histidine or cysteine residues such that the intervening amino acids loop out to form a finger-like projection (reviewed in Johnson and McKnight, 1989). DNA specificity is believed to be determined by the nonconserved stretch of amino acids at the finger tip which is presumed to make specific contacts with the DNA recognition sequence. This is supported by experiments which demonstrate that mutations introduced specifically into the finger region of the glucocorticoid receptor destroy the ability of the protein to bind DNA (Hollenberg et al., 1987).

A third structural motif, the leucine zipper, has been shown to mediate both protein dimerization and DNA binding. This motif is characterized by multiple leucine residues spaced precisely 7 amino acids apart. It has been proposed that this domain forms an alphahelical structure with the leucine residues aligned along one face of the helix and that protein dimerization occurs through the interdigitation of two such structures present on homologous or heterologous protein subunits (Landshulz et al., 1988). Dimerization via the

leucine zipper is necessary but not sufficient for binding of the protein complex to DNA. An additional, positively charged amino acid region adjacent to the leucine zipper is also required for DNA binding. The leucine zipper is believed to bring the basic domains of two subunits together into a structure which can bind DNA in a sequence-specific manner. Transcription factors which possess this leucine zipper/ basic domain include C/EBP (Landschulz et al., 1988), GCN4 (Hope and Struhl, 1987), jun, and fos (Rasone et al., 1989).

A different DNA binding/dimerization motif, known as the helix-loop-helix motif (Murre et al., 1989), has recently been identified in proteins implicated in the developmental control of gene expression. Members of this group include the proteins MyoD, myf-5, and myogenenin which play critical roles in muscle cell differentiation. These proteins form homo- and hetero-dimers through interactions involving the helix-loop-helix structure and, as is the case for leucine zipper proteins, an adjacent basic domain is required for protein-DNA interactions (Davis et al., 1990).

The transcription activation domains of sequence-specific binding proteins are not as well defined as the DNA binding structures. Transcription factors can have more than one activation domain and often these regions show no obvious structural similarities. Activation domains studied so far

are characterized by a high proportion of a particular amino acid. For example, the activation regions of the yeast transcription factors GAL4 and GCN4 (Ma and Ptashne, 1987a, 1987b; Hope et al., 1988), as well as the Herpes virus trans-activator Vmw65 (Triezenberg et al, 1988), have a significant percentage of glutamic acid and aspartic acid residues. These negatively charged amino acids are believed to be arranged along one face of an amphipathic alpha helix (for review see Ptashne, 1988). The mammalian factor SP1 has multiple activation domains, two of which are composed of approximately 25% glutamine (gln) residues (Courey and Tjian, 1988; Courey et al., 1989). Other transcription factors such as Oct1, Oct2, and AP2 also have gln-rich regions, some of which have been implicated in the activation function of these proteins (Sturm et al., 1988: Clerc et al., 1988; Williams et al., 1988). A third type of activation domain found in CTF/NF1, c-jun and Oct2 is characterized by a relatively high proportion (20-30%) of proline residues (Struhl, 1988; Mermod et al., 1989; Bohmann and Tjian, 1989).

v) Regulation of pol II transcription

Sequence-specific transcription factors are believed to regulate gene expression by influencing some aspect of the rate of assembly or stability of active transcription

complexes at mRNA start sites. Biochemical studies have identified four activities, TFIIA, -B, -D, and -E/F, required in addition to RNA pol II for basal level transcription (Matsui et al., 1980; Samuels et al., 1982). The first step in the assembly of a functional initiation complex correlates with the binding of TFIID to the TATA sequence (Nakajima et al., 1988, Sawadogo and Roeder, 1985a). The TATA-TFIID complex is stabilized by interaction with TFIIA and subsequent binding of TFIIB, TFIIE/F, and pol II leads to the formation of a complete transcription initiation complex (Buratowski et al., 1989). Sequence-specific transcription factors may influence any one or more of the steps involved in transcription initiation complex assembly.

TFIID is considered a likely target for sequence-specific transcription factors because of the critical role it plays in initiating the assembly of the general transcription complex. Moreover, it has been observed that binding of the cellular transcription factors USF (Sawadogo and Roeder, 1985b), GAL4 (Horikoshi et al., 1988a), and ATF (Horikoshi et al., 1988b) to their respective sites upstream of mRNA start sites causes qualitative changes in the interaction of TFIID with the downstream TATA sequence and, at least for ATF, this interaction has been shown to promote complex assembly with the rest of the general transcription

factors (Horikoshi et al., 1988b; Hai et al., 1988).

The genes encoding TFIID activity have been isolated from yeast, drosophilia, and humans. Nucleotide sequence comparisons between these genes indicate that a large region at the carboxy terminus of TFIID is highly conserved (greater than 80% sequence identity), whereas the N-terminal regions are completely divergent. Truncated forms of recombinant human TFIID, expressing only the conserved carboxy terminus were shown to be sufficient for both TATA box binding and for mediating basal transcription in TFIID-depleted nuclear extract (Peterson et al., 1990). However, the full-length recombinant protein is required in order to observe transcriptional activation by the sequence-specific transcription factor SP1, suggesting that the N-terminus may be required for mediating trans-activation by upstream factors.

The interaction of sequence-specific transcription factors with TFIID may regulate transcription by increasing the ability of the general transcription initiation complex to compete with nucleosomes for binding in the vicinity of the mRNA start site. It has been demonstrated for at least two transactivators, the pseudorabies immediate early protein (Workman et al., 1988) and GAL4 (Workman et al., 1991), that these proteins facilitate TFIID binding to the promoter and

that this step prevents subsequent repression of promoters by nucleosome assembly in vitro. However, physical exclusion of nucleosomes from the promoter is not sufficient for transcription activation, since derivatives containing only the GAL4 DNA binding domain are unable to alleviate promoter repression during chromatin assembly even though they remain stably bound to the promoter. By contrast, GAL4 derivatives containing activation domains significantly reduce repression of transcription during nucleosome assembly, thereby leading to a net activation of transcription over basal levels. Thus, the ability of a protein to bind stably in chromatin is separable from its ability to activate transcription. Workman et al., (1991) propose that activation domains of sequence-specific transcription factors facilitate some rate-limiting step of preinitiation complex assembly, possibly by increasing the binding of TFIID, pol II, or one of the other general factors to the promoter and that this step is sensitive to repression by nucleosome assembly.

Activation domains may contact their target in the general initiation complex directly or this interaction may be mediated by other proteins. There are several lines of evidence which suggest that transcription activation occurs through intermediary proteins, termed "coactivators". First, overexpression of some transcriptional activator proteins can

inhibit transcription from promoters containing binding sites for the factor. This phenomenon, which is called "squelching" (Gill and Ptashne, 1988), is believed to occur because excess quantities of the activator, which remain free in solution, may interact with limiting quantities of an essential target protein, thereby sequestering it away from the promoter. This target is not one of the general initiation factors since squelching affects activated but not basal transcription (Berger et al., 1990). Furthermore, recent experiments demonstrate that purified recombinant forms of TFIID which are functional for basal transcription are unable to mediate activation by sequence-specific transcription factors. By constrast, partially purified forms of TFIID from nuclear extracts can function in both basal and activated transcription (Pugh and Tjian, 1990; Hoey et al., 1990). These findings suggest that partially purified preparations of TFIID contain other factors required for activated transcription. There may be different coactivators to mediate the interactions of different types of activation domains with the basal transcription machinery. This prediction is based on experiments by Pugh and Tjian (1990), which show that the activity required for transcriptional activation by the gln-rich domain of SP1 is not capable of mediating activation by the pro-rich domain of CTF/NF1.

It is not clear how sequence-specific transcription factors promote transcription when they are bound to enhancer elements located far from the transcription initiation site. One model proposes that distally bound factors are brought close to the transcription initiation site through protein-protein interactions with factors bound to UPE (Ptashne, 1986; Courey et al., 1989; see Figure 2C). This model is supported by experiments which demonstrate that the distance-independent property of enhancers requires the presence of UPE in the promoter. For example, deletion of the SP1 binding sites in the SV40 early promoter dramatically reduces the ability of the SV40 enhancer to activate transcription from a distance (Moreau et al., 1981; Everett et al., 1983; Takahashi et al., 1986). Activity can be restored if the enhancer sequences are moved to a position immediately adjacent to the TATA box (Treisman and Maniatis, 1985).

In summary, transcription by RNA pol II is regulated by transcription factors which bind to specific sequence elements in the promoters and enhancers of pol II transcribed genes. The binding of transcription factors to their recognition sites is thought to facilitate the formation of initiation complexes at mRNA start sites, although the precise mechanisms involved in this process are still unclear. It is apparent, however, that both protein-protein and protein-DNA

interactions are important for regulation of transcription by RNA pol II.

C. Eukaryotic DNA replication

i) Introduction

Relatively little is known about the molecular mechanisms involved in the control of cellular DNA replication, largely because of the enormous complexity of eukaryotic genomes. Studies of animal viruses (reviewed in DePamphilis, 1988) and lower eukaryotes such as yeast (Linskens et al., 1988) have shown that eukaryotic replication origins correspond to specific DNA sequences of a few hundred nucleotides or less. However, recent evidence suggests that mammalian replication origins may be much larger entities. Vaughn et al., (1990) show that a putative cellular replication origin located downstream of the dihydrofolate reductase gene (DHFR) in chinese hamster cells consists of a broad initiation zone extending over 28 kb. Another difference between viral and cellular replication is that, unlike cellular DNA replication, the viral genome undergoes multiple rounds of DNA replication during a single cell cycle. Despite these differences, the mechanisms involved in the

replication of DNA viruses likely reflect the cellular process to a large extent. For example, during the lytic cycle, Py and SV40 replicate in the nucleus of the host cell as chromosomes whose histone composition and nucleosome structure are essentially the same as cellular chromatin. Moreover, with the exception of a single virally-encoded protein, large T antigen, replication of these viruses is entirely dependent upon host cell proteins.

ii) SV40 and Py replication

The replication origins of Py and SV40 are composed of two functionally distinct domains (Tyndall et al., 1981; Bergsma et al., 1982, Muller et al., 1983, deVilliers et al., 1984). The ori-core domain is absolutely required for DNA replication and specifies the site where replication initiates. Auxiliary domains, which are dispensable under some conditions, determine the efficiency and cell specificity of replication.

The Py and SV40 ori-core sequences are approximately 65 bp in length and contain multiple binding sites for the virally-encoded early protein, large T antigen. Conserved elements within the ori-cores include a 15 to 17 bp A/T rich stretch at one end, a central 13 to 15 bp G/C rich palindrome which includes two repeats of the large T antigen binding site

on each strand, and an imperfect repeat at the early border of ori-core. Mutations in any of these three elements impair ori-core function (Triezenberg and Folk, 1984).

Although the ori-core domain is sufficient for SV40 DNA replication, and to a lesser extent Py replication, auxiliary domains comprised of sequences outside the core region significantly increase the efficiency of initiation (Tyndall et al., 1981; Bergsma et al., 1982; Muller et al.,1983; deVilliers et al., 1984; Muller et al., 1988). Some of these sequences represent additional binding sites for large T antigen. In the case of Py, the strongest affinity binding site for large T antigen lies outside ori-core (Pomerantz et al, 1983; Cowie and Kamen, 1984). However, sequences which map within the viral enhancers and promoters are even more important for replication activation. For example, the presence of the SV40 enhancer or 21 bp promoter elements next to the SV40 ori-core increases the efficiency of SV40 DNA replication <u>in vivo</u> 10-100 fold (Bergsma et al., 1982). Py DNA replication is stimulated even more dramatically by the presence of auxiliary domains (Tyndall et al., 1981; Muller et al., 1983; deVilliers et al., 1984). Either one of two fuctionally redundant domains, α or ß, which correspond to elements within the Py enhancer, activate Py replication (Muller et al., 1983, 1988; see Figures 1 and 3).

These results, and others, suggest that transcription factors binding to enhancer and promoter elements may play a role in regulating DNA replication. This will be discussed in more detail later.

The binding of large T antigen to sites within ori-core is thought to initiate DNA replication. Large T antigen possesses intrinsic helicase activity and this activity is believed to catalyze the unwinding of the two DNA strands in the origin, thus establishing a template for DNA replication. The unwinding reaction requires the presence of the A/T rich segment of the origin which may contribute to melting of the DNA strands in this region. In addition to sequence-specific elements, the T antigen-mediated unwinding reaction requires accessory proteins contributed by the host cell. One such factor is a single-stranded DNA binding protein which prevents reassociation of the single strands exposed during the unwinding reaction (reviewed in Challberg and Kelly, 1989).

Initiation of DNA synthesis results in the establishment of two replication forks which move in opposite directions around the viral genome. At each fork, one of the newly synthesized strands grows continuously (the leading strand) while the other strand (the lagging strand) grows by the joining together of small segments of DNA that have been initiated from RNA primers.

Mammalian cells contain four DNA polymerases (pol) called α, ß, γ, and δ (Fry and Loeb, 1986; So and Downey, 1988). It is believed that both the α and δ polymerase activities are required for SV40 and Py replication. Although it is not exactly clear how the two polymerases share the responsibility of replicating the viral genomes, one model proposes that DNA pol α serves as the lagging strand polymerase, whereas DNA pol δ catalyzes elongation of the leading strand (Downey et al., 1988). This model is consistent with the biochemical properties of the two enzymes. For example, DNA pol α has a primase activity which is capable of synthesizing the short RNA primers required for DNA elongation along the lagging strand. Furthermore, studies have shown that DNA pol α is not a highly processive enzyme since it only polymerizes 100 nucleotides per binding event. While this property would be detrimental to the leading strand polymerase, the lagging strand polymerase only needs to be able to polymerize short DNA segments to join up the RNA primers. By contrast, DNA pol δ is a highly processive enzyme. In the presence of a 37 kilodalton (kd) cellular protein called PCNA (proliferating cell nuclear antigen) DNA pol δ can catalyze the polymerization of at least 1000 nucleotides per binding event. This property would be beneficial for a leading strand polymerase. (reviewed in

Challberg and Kelly, 1989) Large T antigen has been shown to bind to DNA polymerase α-primase and this interaction may be important for recruiting polymerase to its site of action at ori-core (Smale and Tjian, 1986).

Additional cellular proteins are required for replication of the SV40 and Py genomes. These include topoisomerases (Yang et al., 1987) which unwind the parental strands as the replication forks advance. Topoisomerases are also required to separate the newly synthesized daughter duplexes at the completion of DNA replication. A helicase activity also participates in unwinding the parental DNA ahead of the replication forks. It is not clear whether this function is provided by the intrinsic helicase activity of large T antigen or by cellular helicases.

iii) Role of transcription factors in replication activation

The activation of DNA replication by enhancers and other transcription elements is not limited to SV40 and Py. The auxiliary domains of the adenovirus, bovine papillomavirus, and epstein barr virus replication units also comprise enhancer elements (reviewed in DePamphilis, 1988). There are several lines of evidence suggesting that the same sequence elements which activate transcription also activate replication. Tang et al., (1987) generated mutations within

the Py enhancer by chemical mutagenesis and showed that transcription and replication were both impaired by these mutations in a qualitatively similar manner. Moreover, revertants which possess single nucleotide changes in the mutated enhancer are competent in both replication and transcription. Transcription elements from heterologous sources can activate SV40 and Py replication (deVilliers et al., 1984; Bennett et al., 1989) and furthermore, the cell specificity of DNA replication correlates with the cell-specific activity of the element for transcription. For example, substitution of the Py enhancer with the lymphoid-specific mouse immunoglobulin gene enhancer restricts Py replication to mouse lymphocytes (deVilliers et al., 1984). Finally, it has been shown that reiteration of a sequence element which corresponds to the binding site for a single transcription factor can activate replication from ori-core (Bennett-Cook and Hassell, 1991; Coulber et al., personal communication).

Although it is clear that the same sequence elements, and by inference the same DNA binding proteins, regulate both replication and transcription, several observations suggest that there are some differences involved in the activation of these two processes. First, the number of transcription factor binding sites required to activate replication is fewer

than is needed for activation of transcription (Veldman et al., 1985; Muller et al., 1988). This implies that there is a quantitative difference in the levels of activation required for replication and transcription. Second, enhancer elements can only activate replication when they are positioned immediately adjacent to the late border of ori-core (Muller et al., 1983, 1988; Innis and Scott, 1984). This contrasts with the ability of enhancer elements to activate transcription in a position-independent manner.

The mechanism by which transcription factors activate DNA replication is not clear. Recent experiments by Bennett-Cook and Hassell, (1991) show that both the activation and DNA binding domains of transcription factors are required for activation of DNA replication. This may imply that some of the components involved in transcription, such as RNA polymerase or one of the other general initiation factors, may participate in replication as well. However, since it has been shown that SV40 DNA replication is completely resistant to α-amanitin, a specific inhibitor of RNA pol II, it is clear that the activation of replication is not dependent upon a transcriptionally-active form of RNA pol II (Decker et al., 1987).

D. Polyomavirus enhancer

i) Introduction

The Py enhancer comprises a 172 bp fragment located between nucleotides (nt) 5057 and 5229 (or between -385 and -213 relative to the major start sites for the early mRNAs; numbering system as described by Soeda et al, 1980)(Figure 1). These sequences regulate transcription from the early promoter and replication of the viral genome (Jat et al., 1982; Mueller et al., 1984; deVilliers et al., 1984). Within the enhancer are several short sequences which share homology with other enhancers such as the SV40 enhancer, the adenovirus E1A enhancer, the c-fos enhancer, and the mouse immunoglobulin heavy chain enhancer. Genetic analyses of the Py enhancer reveal that no one sequence, including any of the conserved elements, are absolutely required for enhancer activity. Like other enhancers studied to date, the Py enhancer is composed of a mosaic of different elements which together determine the overall activity of the enhancer (Mueller et al., 1984, 1988; Veldman et al., 1985; Tang et al., 1987).

The Py enhancer has several interesting properties. It has different activities in mouse cells at different developmental stages. For example, the enhancer is inactive in undifferentiated mouse embryonal carcinoma (EC) cells but

is active after these cells have been induced to differentiate in culture (Herbomel et al., 1984). Variant strains of Py that can grow in undifferentiated EC cells have mutations within the enhancer (Melin et al., 1985; Fujimura, 1986; Caruso et al., 1990).

A second interesting feature of the Py enhancer is that its activity is regulated by the products of several oncogenes. For example, Py enhancer activity is stimulated by the product of the Ha-ras oncogene (Imler et al., 1988; Satake et al., 1988; Yamaguchi et al., 1989), whereas the adenovirus E1A proteins repress its activity (Borelli et al., 1984). In addition, at least three cellular proto-oncogenes, c-ets, c-fos, and c-jun (or related members from these gene families), are known to bind to various sequences within the Py enhancer (Wasylyk et al., 1990; Alison Cowie, unpublished results).

The Py enhancer can be divided into three functional elements (Mueller et al., 1988; see Figure 3). Individually, elements 1, 2, and 3 function poorly or not all, to activate transcription in mouse 3T3 cells, but pairs of elements act synergistically to enhance transcription to almost the same level as the wild type (wt) enhancer (Mueller et al., 1988). Linker mutagenesis and deletion analysis suggest that elements 1, 2, and 3 are composed of smaller subelements (Mueller et

al., 1988; Veldman et al., 1985).

In addition to activating Py early gene expression, sequences within the enhancer also activate viral DNA replication (deVilliers et al., 1984; Muller at al., 1983, 1988). Replication of Py DNA requires two functionally distinct cis-acting components. The origin core, located between nt 5265 and 90, includes binding sites for Py large T antigen and specifies the site of replication initiation. The second component consists of one of two functionally redundant sequences in the Py enhancer. These auxiliary replication sequences, called alpha (α) and beta (ß), correspond to elements 2 and 3 of the enhancer (Hassell et al., 1986). The cellular factors which bind to these sequences appear to influence the efficency and cell specificity of DNA replication.

Studies from different labs have shown that multiple cellular factors from various cell types bind to sequences within the Py enhancer. At least three factors bind to the region between nt 5108-5130 in enhancer element 2. Py enhancer A binding protein 1 (PEA1), which is thought to be a murine homolog to the human transcription factor AP1, and PEA2, a putative negative regulatory factor in EC cells, recognize adjacent sites from nt 5114-5130 (Piette and Yaniv, 1987; Martin et al., 1988; Wasylyk et al., 1988). A third

factor, named PEA3 (Martin et al., 1988; Yamaguchi et al., 1989), binds over the adenovirus homology region from nt 5108-5113, bordering the PEA1 site. Other proteins such as EF-C (Ostapchuk et al., 1986; Fujimura, 1986), C/EBP (Piette and Yaniv, 1986), and PEB1 recognize enhancer sequences between nt 5155 and 5195 in enhancer element 3. (see Figure 3)

ii) The alpha-core region of Element 2

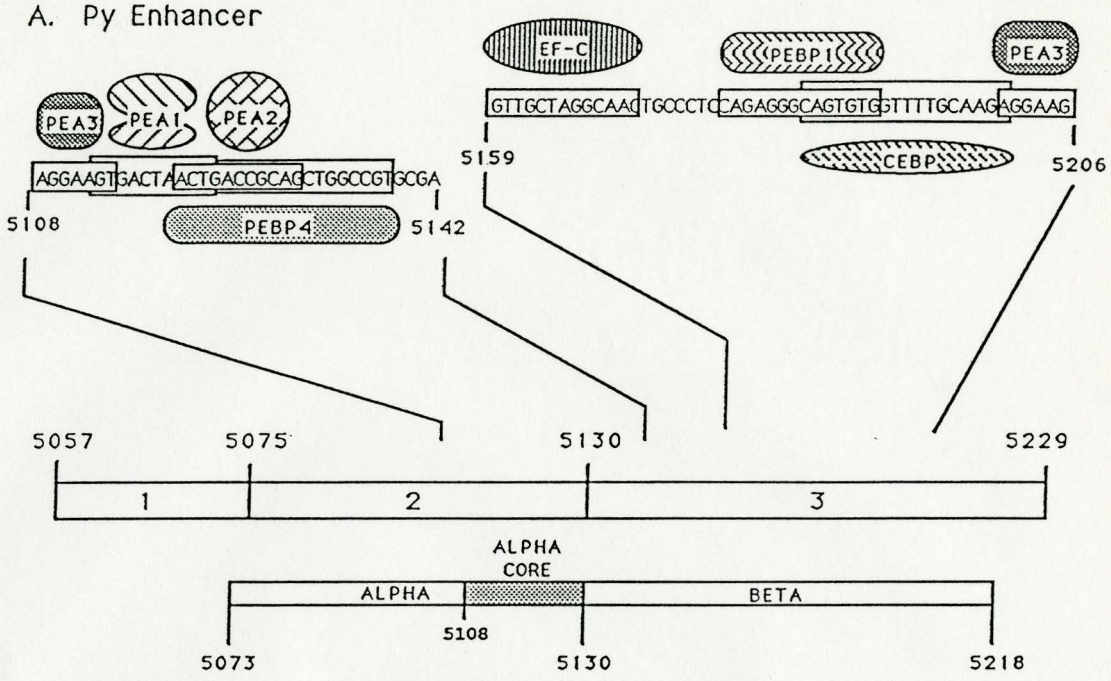
The region in Element 2 of the Py enhancer which binds the three PEA factors (nt 5108-5130) is particularly interesting. This 23 bp sequence, which corresponds to the alpha-core (α-core) element defined by Muller et al (1988), displays many of the properties of the entire enhancer, suggesting that it plays an important role in determining the overall activity of the enhancer. Like the wt enhancer, this sequence element does not function in undifferentiated mouse EC cells, but is active after these cells differentiate (Wasylyk et al., 1988). Also, regulation of Py enhancer activity by serum, the tumor promoting agent TPA, and oncogenes such as Ha-ras and v-jun is mediated through this region.

Figure 3. Cellular proteins interacting with sequences within the polyomavirus enhancer

A) Diagram illustrating the binding of cellular proteins to sequences within the Py enhancer. Enhancer elements 1, 2, and 3, as well as the overlapping α and ß auxiliary replication elements are shown as boxes with nucleotide numbers denoting the boundaries of these regions. Alpha-core is shown as a shaded box within the α auxiliary replication domain. Cellular proteins are indicated by the various stippled and striped symbols, and the sequences to which they bind within the Py enhancer are shown.

B) Sequences of the double-stranded oligonucleotides that were cloned into JSCAT-ORI. These oligonucleotides contain sequences which correspond to motifs found in the Py enhancer alpha-core element. Cellular factors which interact with these sequences are shown schematically above the oligonucleotides and the sequences protected from DNase I digestion by the bound proteins are underlined. Lower case letters indicate linker sequences; point mutations that block factor binding are boxed.

A. Py Enhancer



B. Oligonucleotides

i) Single sites

(AP1)
gatcGTGACTCAGCGCG
CACTGAGTCGCGCctag

(PEA1)
gatcGTGACTAACTGCG
CACTGATTGACGCctag

(PEA2)
gatcAACTGACCGCAG
TTGACTGGCGTCctag

(PEA3)
gatcCAGGAAGTGAC
GTCCTTCACTGctag

(PEBP4)
gatcAACTGACCGCAGCTGGCCGT
TTGACTGGCGTCGACCGGCActag

(mPEA3)
gatctCATCAAGTGAC
aGTAGTTCACTGctag

ii) Double sites

(PEA3+PEA1)
gatcTAGGAAGTGACTAACG
ATCCTTCACTGATTGCctag

(PEA3+mPEA1)
gatcTAGGAAGTAACTAACG
ATCCTTCATTGATTGCctag

(mPEA3+PEA1)
gatcTAAAAAGTGACTAACG
ATTTTTCACTGATTGCctag

(PEA1+PEA2)
gatcTGACTAACTGACCGCAGG
ACTGATTGACTGGCGTCCctag

(PEA1+mPEA2)
gatcTGACTAACTGACCACAGG
ACTGATTGACTGGTCCctag

(mPEA1+PEA2)
gatcTAACTAACTGACCGCAGG
ATTGATTGACTGGCGTCCctag

iii) PEA1

PEA1 recognizes a sequence in the Py enhancer from nt 5114-5120 (Piette and Yaniv, 1987; Martin et al., 1988). This sequence, 'TGACTAA', is highly homologous to the consensus recognition motif, 'TGACTCA',for the human transcription factor AP1 (Lee et al., 1987a). PEA1 is probably the murine homolog of AP1 although this has not been shown directly. AP1 is a complex of several polypeptides encoded by multiple genes including c-jun, junB, junD, c-fos, and fra1 (reviewed in Curran and Franza Jr, 1988). The jun family of proteins can bind to the AP1 or PEA1 sequence as homodimers or heterodimers with the c-fos gene product, whereas c-fos on its own has no DNA binding ability. A common feature of these proteins is their 'leucine zipper' region which allows dimerization between the various members of the AP1 transcription complex.

c-jun, junB, and junD share significant sequence homology and are coexpressed in many tissues, although at different levels (Ryder et al., 1988, 1989). Moreover, in the presence or absence of c-fos, the jun proteins appear indistinguishable in their ability to bind to AP1 consensus oligonucleotides <u>in vitro</u> (Nakabeppu et al., 1988). However, at least two members of the jun family, junB and c-jun , exhibit distinct biological activities. Whereas c-jun is an efficient activator of promoters which contain a single AP1

site, junB is unable to trans-activate these promoters and inhibits their activation by c-jun. On the other hand, junB and c-jun are equally able to trans-activate a promoter which carries multiple AP1 sites (Chiu et al., 1989). The difference in activities exhibited by c-jun and junB is due to differences between their activation domains, because chimeric proteins composed of the N-terminal activation regions of c-jun and junB, fused to a heterologous DNA binding domain displayed the same properties as the wt proteins (Chiu et al., 1989). In the context of the Py enhancer, the PEA1 site is found in a single copy, therefore competition between junB and cjun for binding to this site may contribute to determining Py enhancer activity in cells where both factors are present.

PEA1 activity is highly regulated. DNase I footprinting and gel retardation experiments show that there is little or no PEA1 in undifferentiated EC cells whereas PEA1 binding activity is readily detectable in EC cells which have been induced to differentiate by treatment with retinoic acid (Kryszke et al., 1987; Wasylyk et al., 1988). PEA1 activity is also modulated by treatment with the phorbol ester TPA, and the products of several transforming oncogenes such as c-Ha-ras, v-src, Py middle T antigen, v-mos, and c-fos. By contrast, immortalizing oncogenes such as Py large T antigen,

myc, and Adenovirus E1A do not appear to affect PEA1 activity (Imler et al., 1988; Wasylyk et al., 1988).

PEA1 contributes significantly to Py enhancer activity in vivo. Viral mutants containing point mutations that abolish PEA1 binding in vitro are defective in replication and transcription. Enhancer function is regained by single point mutations which restore PEA1 binding (Tang et al., 1987; Martin et al., 1988). Furthermore, analysis of Py variant strains shows that the PEA1 site is often duplicated in the enhancers of these viruses, suggesting that PEA1 sites confer a positive activity (Ruley and Fried, 1983).

iv) PEA2 and PEBP4

PEA2 was first identified as a factor in 3T6 nuclear extracts which binds to the Py enhancer between nt 5122 and 5129 (Piette and Yaniv, 1987), immediately adjacent to the PEA1 site. Despite the close proximity of their recognition sites, in vitro binding studies have shown that PEA1 and PEA2 are capable of binding to their respective sites simultaneously, without any mutual inhibition. Each factor is also able to bind to its recognition motif independent of the other, although there is some evidence to suggest that PEA2 binding is enhanced by PEA1 (Piette and Yaniv, 1987; Satake et al., 1988).

The contribution of PEA2 to Py enhancer activity is unclear. Deletion of the PEA2 site is detrimental to enhancer activity with respect to both transcription and replication in mouse fibroblast cell lines, implying that PEA2 has a positive function (Mueller et al., 1988; Muller et al., 1988). By contrast, there is some evidence to suggest that PEA2 might be a repressor in undifferentiated F9 cells (Wasylyk et al., 1988). In undifferentiated EC cells, enhancer Element 2 of the Py enhancer is inactive (Herbomel et al., 1984; Wasylyk et al., 1988). This is interpreted to be the result of lack of active PEA1 and PEA2 in these cells since DNase I footprinting and gel retardation experiments show that there is negligible amounts of PEA2 and very low levels of PEA1 present in these cells (Kryszke et al., 1987). However, results from at least one lab suggest that Element 2 is repressed in undifferentiated cells and not merely inactive as was originally proposed. Wasylyk et al., (1988) showed that transcription of a reporter gene controlled by a promoter carrying a wt PEA1 site and mutated PEA2 site is four times greater than an analogous plasmid in which the wt PEA2 site was present. This implies that the inactivity of Element 2 in EC cells is the result of two factors; the low levels of positive <u>trans</u>-acting proteins in these cells and the presence of a repressor activity which is mediated through the PEA2

site.

If PEA2 is a repressor in undifferentiated cells, it is puzzling that no binding activity at the PEA2 site can be detected with F9 extracts. Wasylyk et al., (1988) propose that PEA2 is not detected in EC cells because it is a labile protein. This conclusion is based on the observation that cycloheximide treatment blocks PEA2-mediated repression.

It is also unclear how PEA2 could have properties of both an activator (in 3T3 and 3T6 cells) and repressor (in undifferentiated EC cells). One possibility is that two different factors recognize the PEA2 site; an activator in differentiated cells and a repressor in undifferentiated cells. Furukawa et al., (1990) demonstrate that there is a binding activity in F9 cells which recognizes the sequence between nt 5119 and 5138. The binding site for this factor, which they call PEBP4, encompasses and extends beyond the PEA2 recognition site. Functional assays show that the PEPB4 binding site has silencer activity in undifferentiated F9 cells, but acts positively to enhance transcription when the F9 cells are induced to differentiate. This change in activity correlates with the induction of PEA2 binding activity. Bandshift assays using partially purified proteins show that PEA2 and PEBP4 compete for binding to the PEBP4 sequence. Based on these results Furukawa et al., (1990)

propose that the change in activity of the Py enhancer in undifferentiated versus differentiated EC cells occurs, in part, by the induction of a positive acting factor, PEA2, which competes with the repressor, PEBP4, for binding to overlapping recognition sites in Element 2.

v) PEA3

Studies by several groups revealed that there is a third factor which binds to Element 2 (Martin et al., 1988; Yamaguchi et al., 1989). This factor, PEA3, recognizes the sequence 'AGGAAG' (nt 5108 to 5113) which is homologous to part of the adenovirus E1A enhancer core element (Hearing and Shenk, 1983). PEA3 appears to bind with relatively low affinity to its recognition site, or is present in 3T6 cells at low levels, because DNase I footprinting experiments do not reveal any protection at nt 5108 to 5113. Binding of a factor over this region is indicated by the presence of a hypersensitive site at nt 5108 (Martin et al., 1988). Gel retardation experiments confirm that there is indeed a factor in 3T6 cells whose binding site includes the sequence 'AGGAAG' (Yamaguchi et al., 1989).

Functional studies of Py enhancer activity suggest that PEA3 binding is important for enhancer function. Tang et al., (1987) show that a mutant B122, which carries multiple

transitions throughout the Py enhancer that severely impair viral trancription and replication, can have wt activity restored by the reversion of a single nucleotide (nt 5109) in the PEA3 site. Furthermore, experiments performed by Mueller et al., (1988) in 3T3 cells show that loss of the PEA3 site by linker insertion (nt 5109-5113) reduces the transcriptional activity of the mutant Py enhancer to 61% of the wt enhancer. These observations suggest that PEA3 is a positive trans-acting factor.

Like PEA1, the activity of PEA3 is also highly regulated. PEA3 transcriptional activity can be activated by serum, TPA, and the products of several oncogenes including v-src, Py middle T antigen, Ha-ras, v-mos, and v-raf. However, PEA3 activity is not affected by the expression of SV40 or Py large T antigen, Adenovirus E1A, myc, fos or jun (Martin et al., 1988: Satake et al., 1988; Wasylyk et al., 1989, Yamaguchi et al., 1989).

Recently it has been shown that the products of the ets-1 and ets-2 proto-oncogenes can bind to the PEA3 recognition motif and activate transcription from promoters carrying PEA3 sites (Wasylyk et al., 1990). This suggests that PEA3 may be a murine c-ets related protein. Relavent to this hypothesis, Xin et al., (manuscript in preparation) have isolated a partial cDNA for PEA3 by screening an expression

library with the PEA3 binding site. The predicted amino acid sequence of the polypeptide encoded by the cDNA shows that it is highly homologous to c-ets1 and other members of the ets family in a carboxy terminal region corresponding to the DNA binding domain. However, outside of this conserved region, PEA3 diverges significantly from all of the known ets and ets-related proteins.

E. Experimental design

At the time these experiments were started, studies from various labs had shown the Py enhancer to be comprised of multiple functionally redundant elements about 50-100 bp in length (Mueller et al., 1984, 1988; Muller et al., 1983, 1988; Veldman et al., 1985). However, it was clear from linker mutagenesis and deletion analysis that these elements were composed of smaller functional subunits. This was confirmed by DNase I footprinting and gel retardation assays which identified small 8-10 bp sequences in the Py enhancer corresponding to binding sites for individual cellular proteins. In particular, at least three distinct factors, PEA1, PEA2, and PEA3 were shown to bind to sequences 5108-5130 in the Py enhancer (Piette and Yaniv, 1987; Martin et al., 1988; Wasylyk et al., 1988; Yamaguchi et al., 1989). This

region corresponds to the alpha-core element defined by Muller et al., (1988) or element A defined by Veldman et al., (1985). Multimers of this 23 bp element have been shown to activate both replication and transcription in mouse fibroblast cell lines (Veldman et al., 1985; Muller et al., 1988).

We wished to be able to determine the contribution of individual Py enhancer sequence motifs to enhancer activity by assaying their activity in a heterologous system composed of a minimal number of well-defined elements. We reasoned that since the activation potential of individual sequence motifs might be small, it would be best to work with a simplified system so that these small effects would not be masked. To do this, I designed a reporter plasmid which includes the minimal sequence requirements for replication and transcription initiation, Py ori-core and a TATA box, respectively. In the absence of any enhancer binding sites, these minimal sequences direct low levels of replication and transcription but both these processes can be greatly stimulated by the presence of DNA motifs which are recognized by activator proteins. To study the activities of PEA1, PEA2, and PEA3, I cloned multiple copies of the recognition sites for these proteins into my reporter plasmid and assayed for their ability to independently activate replication and/or transcription <u>in vivo</u>. I also tested paired combinations of

these sites to determine if two different factors bound at adjacent sites could cooperate to activate replication and transcription.

MATERIALS AND METHODS

A. DNA restriction and modification

Restriction endonucleases and DNA modifying enzymes such as calf intestinal alkaline phosphatase, T4 polynucleotide kinase, Klenow, and T4 DNA ligase were obtained from Bethesda Research Laboratories, Pharmacia, or Boehringer Mannheim and were used according to manufacturers specifications. Radiolabeled isotopes were purchased from Amersham.

B. Construction of recombinant plasmids

Procedures for manipulating DNA were performed as described in Maniatis et al., (1982). Py sequences were numbered using the system described by Soeda et al., (1980). The plasmids p371 (C. Yong, manuscript in preparation), pTE1 (Edlund et al., 1985), and pdORI1.2 (Muller et al., 1983) which were used to constuct JSCAT-ORI are described in the references noted.

JSCAT-ORI was made in the following way (and see Figure 4). p371 was partially restricted with Sac I and then digested to completion with Bam HI. The fragments were separated by gel electrophoresis and the largest fragment was recovered by electroelution. This fragment was ligated to a fragment carrying the bacterial gene encoding chloramphenicol acetyl transferase (CAT) plus accompanying SV40 poly A

processing signals, which had been cut from the plasmid pTE1 by digestion with Sac I and Bam HI. A recombinant, JSCAT, was isolated which carried the TATA box from p371 juxtaposed to the CAT gene. To facilitate future cloning steps, the unique Nde I site in JSCAT was changed to a unique Cla I site. This was achieved by cleaving JSCAT with Nde I, treating the linearized plasmid with the klenow fragment of E. Coli DNA polymerase I to fill in the cohesive ends, followed by ligation with phosphorylated Cla I linkers. After cleavage with Cla I to remove tandem copies of the linker, the DNA fragment was gel purified and self-ligated to produce the recombinant JSCAT-Cla.

pdORI1.2 was restricted with Bam HI. The linearized plasmid was treated with klenow to fill in the cohesive ends and then ligated to phosphorylated Bgl II linkers. After digestion with Bgl II, the linearized plasmid was gel-purified and self-ligated to produce the recombinant pdORI-Bg. Note that the Bam HI site was regenerated during this procedure.

JSCAT-Cla was cleaved with Cla I and Bgl II. The DNA fragments from this reaction were separated by gel electrophoresis and the largest fragment was recovered by electroelution. This was ligated to a DNA fragment carrying the Py origin of replication (ori) released from pdORI-Bg by cleavage of this plasmid with Cla I and Bgl II. The recombinant JSCAT-ORI was isolated from this reaction. JSCAT-

ORI has a promoter, consisting of a TATA box only, driving transcription of the CAT gene. 5' to the TATA box is a unique Bgl II site, and an additional 10 bp upstream is Py ori (nt 5265-90) with its late border (nt 5265) closest to the Bgl II site. The JSCAT-ORI recombinant was initially identified by restriction analysis but the sequence over the region encompassing the junctions between Py ori, the TATA box and the 5' end of the CAT gene was confirmed by DNA sequence analysis using a commercial kit (Sequenase, United States Biochemical). See Figure 5.

Oligonucleotides were designed comprising the binding sites for AP1, PEA1, PEA2, PEA3, and PEBP4 individually, or in pairs of two sites as they appear in element 2 of the Py enhancer (see Figure 3 for sequences). Mutant versions including double base substitutions that block factor binding (Piette et al., 1987; Wasylyk et al., 1989; Satake et al., 1989) were also made. All oligonucleotides were synthesized and purified by Dins Gooden at the Central Facility of the Institute for Molecular Biology and Biotechnology, McMaster University. Plasmids were generated by cloning these oligonucleotides into the Bgl II site of JSCAT-ORI and named according to the nature of the oligonucleotide, with a number in brackets indicating the number of copies inserted. Copy number and orientation were confirmed by sequencing (Sequenase, United States Biochemical).

JSCAT-αORI was constructed in the following way. The α ori-core fragment consisting of the α repliction element (nt 5039-5130) plus Py ori-core (nt 5265-90) was isolated from pdPyVαc (Muller et al, 1988) by cleaving this plasmid with Bam HI and Cla I. This fragment was ligated to JSCAT-Cla which had been cleaved with Cla I and Bgl II. Ligation of the compatible Bam HI and Bgl II sticky ends eliminated the cleavage sites for both enzymes.

JSCAT/PyE(5') was constructed in the following way. The Py enhancer (nt 5039-5265) was isolated as a Cla I/Bgl II fragment and ligated to JSCAT-Cla which had been cleaved with Cla I and Bgl II. This placed the Py enhancer immediately adjacent to the TATA box and in the orientation which corresponds to the early transcription unit of Py. This plasmid, JSCAT-PyE(5'), does not carry the Py replication ori-core sequences and therefore could not be used in replication assays.

JSCAT-ORI/PyE(3') was constructed in the following way. JSCAT-ORI was partially restricted with Hind III. DNA fragments representing one-cut linear species were isolated by gel electrophoresis and then ligated to Py enhancer sequences (nt 5039-5265) which had been isolated as a Hind III fragment. The 3' position (relative to the CAT gene) was determined by cleavage with Pvu II. The Py enhancer is oriented with the late border (nt 5039) next to the 3' end of

the CAT gene. This construct does carry the Py ori-core sequences upstream of the CAT gene.

The replication-defective plasmids, JSCAT-ORI/PEA2(4)Apa and JSCAT-ORI/PEBP4(6)Apa, were made in the following way. JSCAT-ORI/PEA2(4) and JSCAT-ORI/PEBP4(6) were restricted with Apa I which cleaves at a unique site within the Py ori-core sequences. The linearized plasmids were treated with Klenow to remove the 3' overhangs and then blunt-end ligated. This procedure, which creates a 4 bp deletion in the Py large T antigen binding site in ori-core around nt 5235 (Py numbering system), has been shown by other researchers (Murakami et al., 1990) to impair replication of Py ori-core sequences. JSCAT-ORI/PEA2(4)Apa and JSCAT-ORI/PEBP4(6)Apa were sequenced to confirm the deletions.

JSCAT/PEBP4(6)ORI$^-$ was constructed from JSCAT-ORI/PEBP4(6) in the following way. JSCAT-ORI/PEBP4(6) was partially restricted with Bam HI and the one-cut linear species were isolated by gel electrophoresis. These fragments were then cleaved with Cla I, treated with klenow to blunt the ends, and ligated together. The recombinant, JSCAT/PEBP4(6)ORI$^-$, was recovered from this reaction. This plasmid lacks the entire Py ori-core element.

C. Isolation of plasmid DNA

E. coli strain DH5 was made competent for DNA transformation by the CaCl2/RbCl method (Maniatis et al., 1982). Bacteria were grown in broth containing 25 g/L bactotryptone and 7.5 g/L yeast extract and supplemented with 5 ml/L of a 40% stock of dextrose and 5 ml/L of a 20 mg/ml stock of ampicillin where appropriate. Small scale isolation of DNA for screening of recombinant plasmids was performed by the following method. Typically, five mls of a confluent overnight culture was centrifuged at 4000 RPM for five minutes. The bacterial pellet was resuspended in 200 ul glucose buffer (50 mM glucose, 25 mM Tris-HCl (pH 8), 10 mM EDTA (pH 8). 400 ul alkaline solution containing .2M NaOH and 1% SDS was added and the tubes were inverted gently and immediately placed on ice. After 10 minutes incubation on ice, 300 ul of 5M potassium acetate was added to the tube. After inverting the tube sharply several times, it was replaced on ice for an additional 10 minutes. To pellet cellular debris, the sample was centrifuged at 13000 RPM for 5 minutes at 4 C. 500 ul isopropanol was added to the supernatant to precipitate the DNA. After centrifugation, the pellet was resuspended in T.E. containing 50 ug/ml RNase. Restriction enzyme digestion was performed directly using this DNA. If the DNA was to be used for sequence analysis, it was further treated by one extraction with buffered-saturated

phenol, followed by one extraction with chloroform/isoamyl alcohol (24:1) and precipitation with ethanol. The precipitate was resuspended in 100ul water and 18ul of this sample was used in a sequencing reaction (Sequenase, United States Biochemical).

Large scale plasmid preparations were performed in essentially the same manner except for the following changes. Usually 50 ml bacterial cultures were grown overnight. After centrifugation, the cells were resuspended in 5 ml glucose buffer containing 10 mg/ml lysozyme. 10 ml alkaline solution consisting of .2N NaOH and 1% SDS was added and the samples were inverted gently and placed on ice for 10 minutes. 8 ml of 3M sodium acetate was added to the sample and the tubes were inverted sharply and replaced on ice for 20 minutes. After pelleting the cellular debris, the supernatant was poured into a clean tube. Nucleic acids were precipitated by the addition of 2 volumes of ethanol. The precipitate was resuspended in 10.5 ml TE. 10.5 g CsCl and 100ul of a 10 mg/ml stock of ethidium bromide were added to each sample and mixed. Samples were then transfered to Beckman heat-seal tubes and centrifuged at 55,000 RPM for 16 hours at 20C. The plasmid DNA band was recovered from the gradient and ethidium bromide was extracted with isobutanol. The DNA was precipitated twice with ethanol and resuspended in water. Concentration and purity of DNA was determined by spectrophotometry and gel analysis.

D. Cell culture

FM3A cells and FOP cells were grown in Dulbecco's Modified Eagle's Medium(DMEM) containing 20 ug/ml gentamycin and 1 ug/ml fugizone and supplemented with 10% calf serum (v/v). They were maintained at 37C in a humidified CO_2 atmosphere. FM3A cells are a mouse mammary carcinoma cell line (Nakano,N. 1966. Tohoku J. Exp. Med. 88: 69-84.). FOP cells were derived by transfection of FM3A cells with a plasmid called pSVE1-B1A (Muller et al., 1983) which encodes all three Py T antigens.

E. DNA transfection and measurement of CAT activity

CsCl gradient-purified plasmid DNAs were transfected into cells by a modification (Muller et al., 1983) of the DEAE-dextran technique (McCutchen et al., 1968). Cells were plated at $1x10^6$ cells per 60 mm plate 4 to 12 hours prior to transfection. The cells were rinsed once with PBS, once with serum-free DMEM and then incubated with DMEM containing 250 ug/ml DEAE-dextran and 3 ug test plasmid for 1.5 hours at 37C in a humidified CO_2 incubator. The cells were rinsed once with PBS, then treated with 2 ml of 10% dimethyl sulfoxide (DMSO) in PBS (v/v) for 2 minutes at room temperature. This was followed by two rinses with PBS, one rinse with serum-free DMEM and then 5 ml fresh medium containing serum was added back to the cells. 48 hours post-transfection, cells were

collected and transfered to a 15 ml tube. Cells were pelleted by centrifugation, rinsed once with 5 ml PBS, centrifuged a second time, resuspended in 1 ml PBS, transfered to an eppendorf tube, centrifuged again and finally resuspended in 100 ul of 250 mM Tris-HCl (pH 7.5). Cells were lysed by three cycles of freeze-thawing. The cellular debris was pelleted by centrifugation for 15 minutes at 4C and the supernatant transfered to a clean tube. Typically, the lysates contained the contents of approximately $5x10^6$ cells.

CAT assays were performed according to Gorman et al., (1982). In a typical reaction, 50 ul of cell extract was incubated at 37C for 1.5 hour with .1 to .2 uCi dichloroacetyl-1,2, ^{14}C-chloramphenicol (New England Nuclear) and 10 ul of a 10 mM stock of Acetyl CoA (Pharmacia) in a final volume of 180 ul made up with 250 mM Tris-HCl (pH 7.5). The reaction was terminated by the addition of 1 ml ethyl acetate. The samples were vortexed to mix and centrifuged for 5 minutes to separate the layers. The ethyl acetate phase was removed to a clean tube and allowed to evaporate overnight. The residue was resuspended in 25 ul ethyl acetate and spotted on a thin layer chromatogram (Kodak). The plate was developed in a chloroform:methanol (19:1) bath for 30 minutes and then exposed to X-ray film for 24 to 48 hours. The areas corresponding to the acetylated and nonacetylated forms were cut out and counted using liquid scintillation fluid. CAT

activity was assessed by the percentage of acetylated ^{14}C-chloramphenicol.

Relative CAT activities produced by the various recombinant plasmids were determined in the following way. Background activity obtained from an extract of mock transfected cells (always <1% conversion) was subtracted from the conversion values obtained for each of the test extracts. The CAT activity produced by the plasmid JSCAT-αORI was then set to a value of 100. The CAT activities of the other plasmids tested in this assay were subsequently adjusted to give relative CAT activities by multiplying by 100 and dividing by the original conversion value for JSCAT-αORI. Constructs were tested in this assay 2-4 times each and the results from different experiments were averaged. To facilitate comparisons between experiments, the positive control, JSCAT-αORI was always transfected in parallel with the test plasmids.

F. DNA transfection and measurement of DNA replication

CsCl gradient-purified, supercoiled plasmid DNAs were transfected into FOP cells by the DEAE-dextran method. Cells were cotransfected with test plasmids prepared from wild type (dam^+) E. Coli and control pUC 19 DNA prepared from a dam^- strain of bacteria.

Replication of test plasmids was measured by an assay

designed by Peden et al., (1980). This assay allows replicated DNA to be distinguished from unreplicated DNA on the basis of differential restriction of the two species of DNA. Transfected test DNAs, isolated from dam^+ bacteria, are methylated and therefore sensitive to the restriction endonuclease DpnI. By contrast, DNA which has replicated in mammalian cells and the control DNA which was propagated in dam^- bacteria are unmethylated and therefore are not cleaved by Dpn I. The assay was performed in the following way. Cells were plated at $5x10^5$ cells/60 mm plate, 4 to 12 hours prior to transfection. The cells were rinsed once with PBS, once with serum-free DMEM and then incubated with DMEM containing 250 ug/ml DEAE-dextran, 250 ng test plasmid, and 100-250 ng control pUC 19 dam unmethylated DNA which was included to monitor transfection efficiency and to allow comparisons of the replicative capacity of different test DNAs within the same experiment to be made. The DNA/DEAE-dextran mixture was left on the cells for 1.5 hours at 37C in a humidified CO_2 incubator. The cells were rinsed once with PBS, once with serum-free DMEM and then 5 ml medium containing serum was added back to the plates.

Low-molecular weight DNA was isolated by the Hirt extraction method (Hirt et al., 1967) 48 hours post transfection. Cells were lysed in Hirt's buffer (.6% SDS, 10 mM EDTA (pH 8), 10 mM Tris-HCl (pH 8)) and the cellular debris

and high-molecular weight DNA were pelleted by centrifugation. The cleared lysate was extracted once with buffer-saturated phenol and once with chloroform-isoamyl alcohol (24:1 v/v). Nucleic acids were precipitated by the addition of 2 volumes of ethanol. Precipitates were collected by centrifugation and suspended in 100 ul TE buffer. A 10-20ul aliquot of each sample was digested with Dpn I and a one cut restriction enzyme (Sst I) for the transfected DNAs. Dpn I cleaves only methylated input DNA and leaves both replicated DNA and unmethylated control plasmid intact. Sst I, which cleaves the test and control DNAs at one site only, generates linearized forms of both species. (Sst I actually cleaves the test plasmids twice, but the two cleavage sites are only 50 bp apart so the large fragment generated by Sst I digestion migrates in a manner indistinguishable from the full length linear plasmid in the agarose gels used in these experiments)

The products of the digestion reaction were separated by electrophoresis through a .8% agarose gel and transferred to a synthetic membrane (Gene Screen Plus, Dupont) by a modification of the Southern blotting technique (Southern et al., 1975). After electrophoresis, the agarose gel was soaked with gentle agitation, in .25M HCl for 15 minutes, and then in solutions containing .4M NaOH/.6M NaCl and 1.5M NaCl/.5M Tris-HCl (pH 7.5) for 30 minutes each. DNA was transferred from the gel to the nylon membrane by one of two methods. If

the transfer was to proceed overnight, the gel was placed on a piece of Whatman paper on a glass plate with the ends of the Whatmann paper sitting in a tray of 10X SSC (.5M NaCl/150mM sodium citrate). The nylon membrane was placed directly on top of the gel, followed by three pieces of Whatmann paper presoaked in 10X SSC, and then a stack of paper towels. A weight was placed on top of the blotting materials and the transfer was allowed to proceed overnight. The membrane was then rinsed for 30 seconds in .4 M NaOH and neutralized in .2M Tris (pH 7.5)/2X SSC(.3M NaCl/ 30mM sodium citrate) for 15 minutes. For more rapid transfer, a posiblot apparatus (Stratagene) was employed. This apparatus was used according to guidelines suggested by the manufacturer. 10X SSC was used as the transfer buffer and the transfer process was allowed to procede for 45 to 60 minutes. After the transfer process was complete, the membrane was treated as described above for the capilliary blot procedure.

Probe DNA was labelled by nick translation as described in Maniatis et al., (1982). In general, 200 ng of JSCAT-ORI and 10 uCi each ^{32}P-dNTP was used in the reaction. Radiolabeled DNA was separated from unincorporated dNTPs by putting the reaction mixture over a Sephadex G80 column. The specific activity of probes made by this protocol was generally 1-2x10^{8} cpm/ug. Just prior to use, the probe plus .5 mg of sheared calf thymus DNA were boiled for 10 minutes.

The membrane with the transfered DNA fragments was incubated with 15 ml of a hybridization solution composed of 50% formamide, 1% SDS, .8M NaCl, 20mM PIPES (pH6.5) and 100 ug/ml denatured, sheared calf thymus DNA, for at least 1 hour at 37C on a rotating platform. $5x10^6$ to $1X10^7$ cpm of nick translated probe plus .5 mg sheared calf thymus DNA were added to the hybridization solution and the membrane was allowed to hybridize overnight on a rotating platform at 37C. The next day, the membrane was washed twice in .5X SSC (75mM NaCl/1.5mM sodium citrate) at room temperature for 10 minutes each time and then twice in .5X SSC/1% SDS at 65C for 30 minutes each time. If necessary, an extra wash was performed with .1X SSC (15 mM NaCl/1.5mM sodium citrate) at 65-70C for 30 minutes to reduce background.

The membrane was exposed to Kodak X-ray film in a cassette containing intensifier screens for 4 to 24 hours at -80C. Relative amounts of replicated DNA and control DNA were quantitated by 2D laser densitometric scanning of autoradiograms. Replication activation of the test plasmids is expressed relative to JSCAT-αORI which was given a value of 100.

RESULTS

A. Construction of JSCAT-ORI

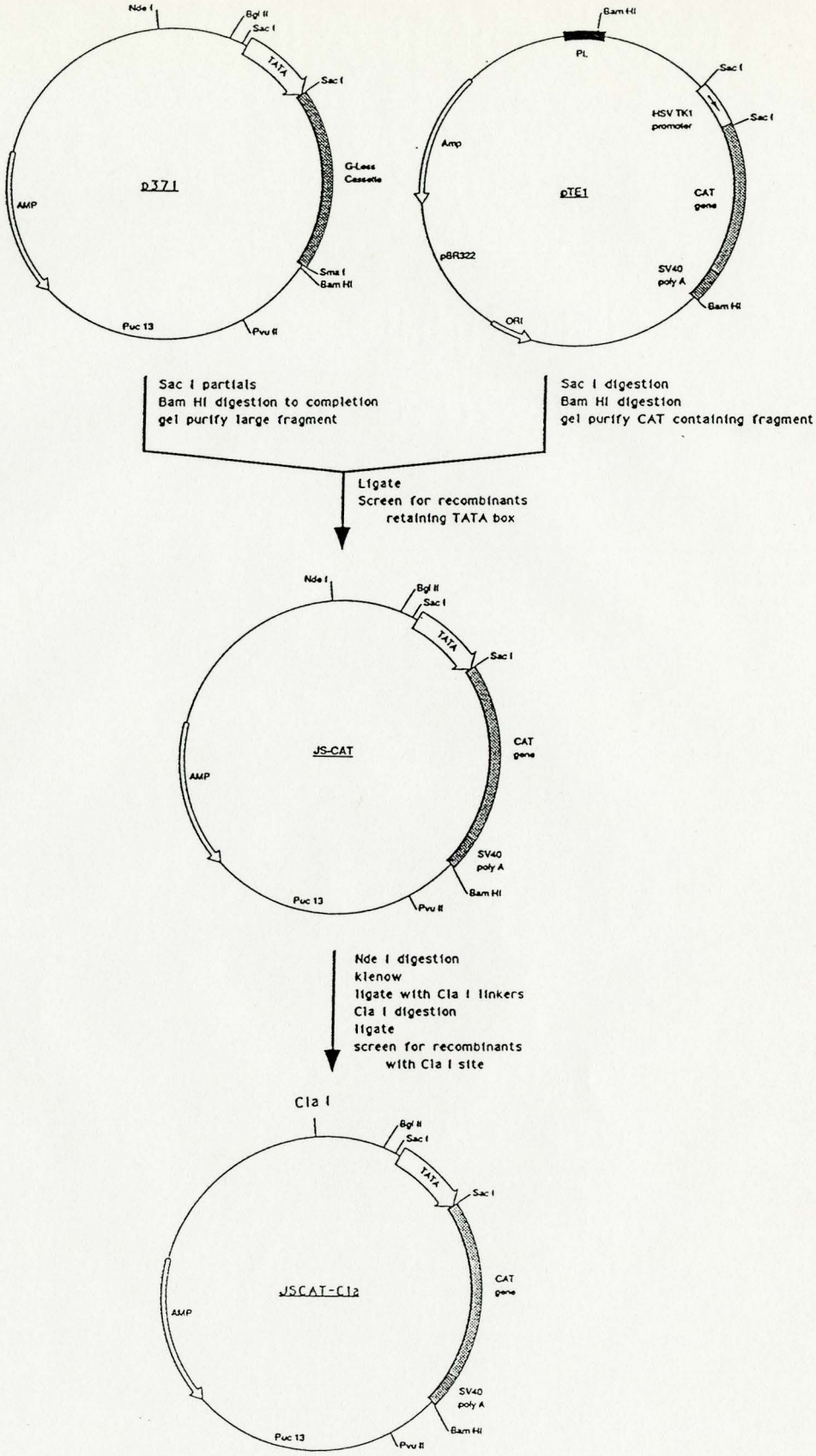
To assay enhancer elements for their ability to activate replication and transcription, I constructed a reporter plasmid with features which allows both activities to be tested. The cloning procedure followed to make this reporter plasmid, JSCAT-ORI, is shown in Figure 4 and described in detail in the Materials and Methods section.

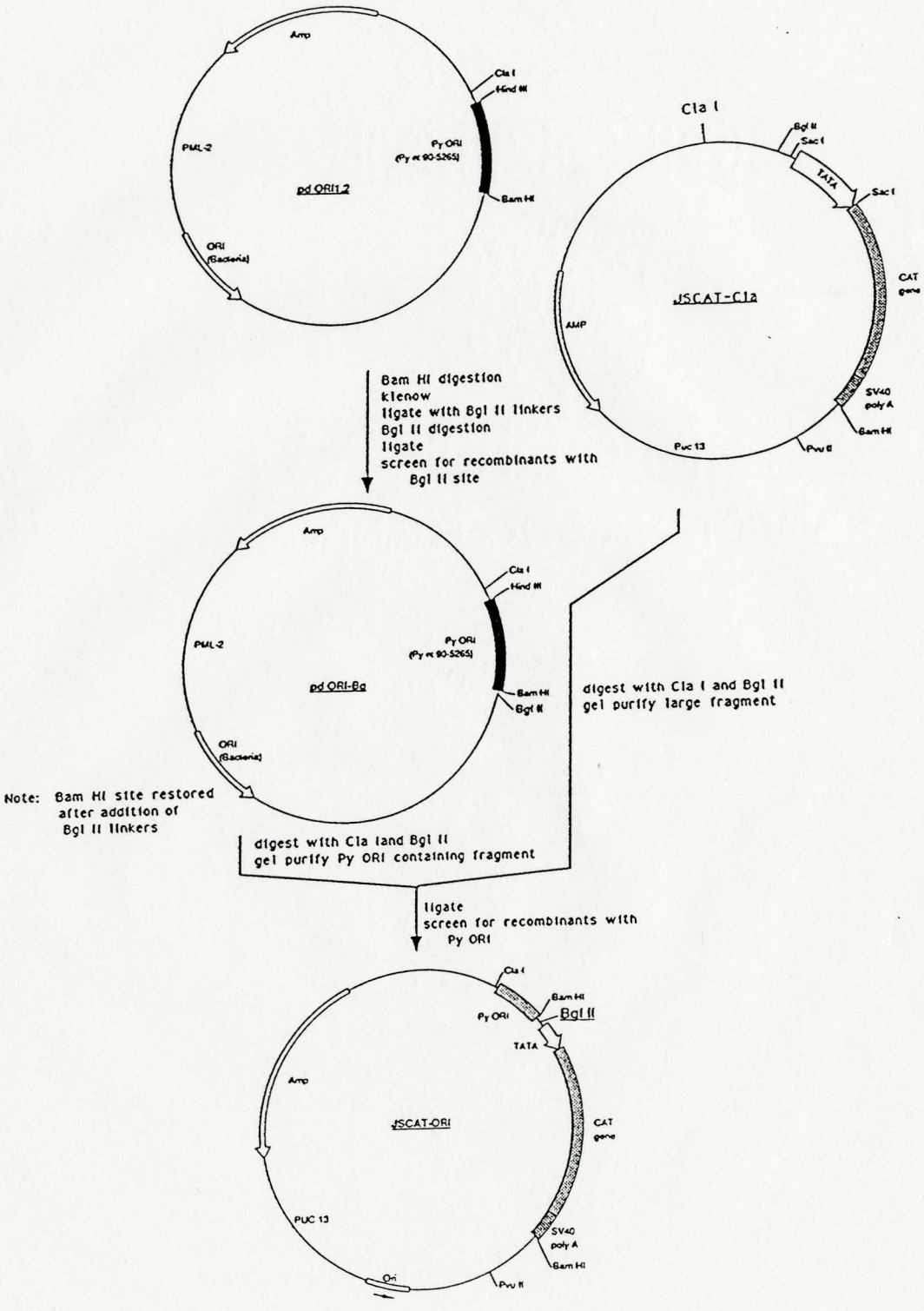
JSCAT-ORI has a minimal promoter, consisting of a TATA box only, to direct transcription of the bacterial gene encoding the chloramphenicol acetyl transferase (CAT) enzyme. The TATA box corresponds to that of the adenovirus 2 major late promoter. In vitro studies have shown that this sequence is both necessary and sufficient for directing basal levels of transcription from a linked gene, however transcription can be increased by the presence of enhancer or promoter elements upstream of the TATA box (Concino, et al., 1984; Sawadogo and Roeder, 1985b; Yong et al., manuscript in preparation). In JSCAT-ORI, transcription is presumed to initiate at an A residue 26 bp downstream of the centre of the TATA box.

In addition to the TATA box and CAT gene, JSCAT-ORI also includes the Py ori-core element (nt 5265-90). These sequences, which include several binding sites for Py large T antigen, comprise the minimal replication origin of Py

Figure 4. Construction of JSCAT-ORI

JSCAT-ORI has a minimal promoter comprised of the adenovirus 2 major late TATA box which is present in front of the bacterial gene encoding the chloramphenicol acetyl transferase (CAT) enzyme. Also contained within JSCAT-ORI is the Py ori-core element (nt 5265-90; numbered according to Soeda et al., 1980). In between the ori-core element (Py ori) and the TATA box is a unique Bgl II site which serves as a useful cloning site for putative enhancer and promoter elements. JSCAT-ORI was constructed from the plasmids p371 (C. Yong et al., manuscript in preparation), pdORI1.2 (Muller et al., 1983), and pTE1 (Edlund et al., 1985) as shown here and outlined in detail in the materials and methods section of this thesis.


Nde I
Bgl II
Sac I
TATA
Sac I
G-Less Cassette
Sma I
Bam HI
Pvu II
Puc 13
AMP
p371
Bam HI
PL
Sac I
HSV TK1 promoter
Sac I
Amp
pTE1
CAT gene
pBR322
SV40 poly A
Bam HI
ORI
Sac I partials
Bam HI digestion to completion
gel purify large fragment
Sac I digestion
Bam HI digestion
gel purify CAT containing fragment
Ligate
Screen for recombinants retaining TATA box
Nde I
Bgl II
Sac I
TATA
Sac I
CAT gene
JS-CAT
AMP
SV40 poly A
Bam HI
Puc 13
Pvu II
Nde I digestion
klenow
ligate with Cla I linkers
Cla I digestion
ligate
screen for recombinants with Cla I site
Cla I
Bgl II
Sac I
TATA
Sac I
CAT gene
JSCAT-Cla
AMP
SV40 poly A
Bam HI
Puc 13
Pvu II


Amp
Cla I
Hind III
PML-2
Py ORI
(Py nt 90-5265)
pd ORI1.2
Bam HI
ORI
(Bacterial)
Cla I
Bgl II
Sac I
TATA
Sac I
CAT
gene
pJSCAT-Cla
AMP
SV40
poly A
Bam HI
Puc 13
Pvu II
Bam HI digestion
klenow
ligate with Bgl II linkers
Bgl II digestion
ligate
screen for recombinants with
Bgl II site
Amp
Cla I
Hind III
PML-2
Py ORI
(Py nt 90-5265)
pd ORI-Bg
Bam HI
Bgl II
ORI
(Bacterial)
digest with Cla I and Bgl II
gel purify large fragment
Note: Bam HI site restored
after addition of
Bgl II linkers
digest with Cla I and Bgl II
gel purify Py ORI containing fragment
ligate
screen for recombinants with
Py ORI
Cla I
Bam HI
Py ORI
Bgl II
TATA
Amp
pJSCAT-ORI
CAT
gene
PUC 13
SV40
poly A
Bam HI
Ori
Pvu II

(Muller et al., 1983, 1988). In between the Py ori-core element and the TATA box is a unique Bgl II site which serves as a useful cloning site for the insertion of putative enhancer or promoter elements. In this location, inserted elements are within 20 bp of both the TATA box and Py ori-core, where they should be in a position to modulate both replication and transcription activation under the appropriate conditions.

A schematic diagram illustrating the important features of JSCAT-ORI is shown in Figure 5A. The sequence across the region encompassing the junctions between Py ori-core, the TATA box and the 5' end of the CAT gene was confirmed by DNA sequence analysis (Figure 5B).

B. Characterization of JSCAT-ORI

i) Transcription

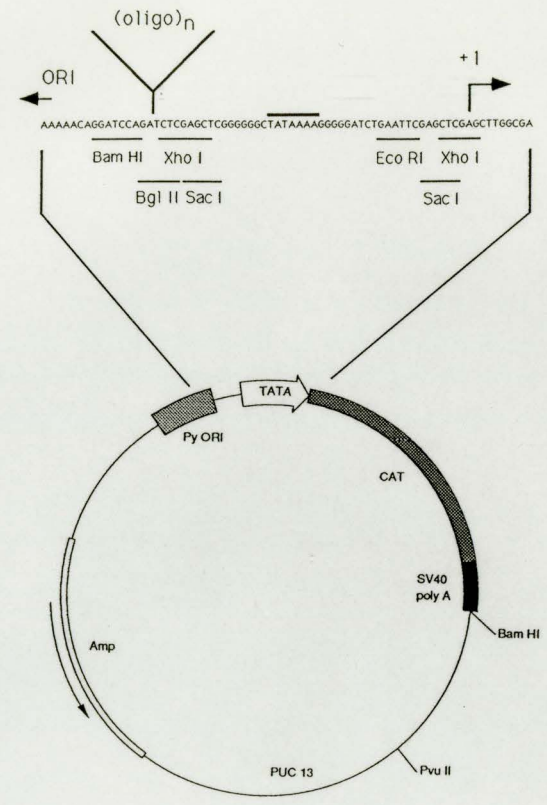
To determine whether JSCAT-ORI would be useful for testing transcription activation by enhancer elements, I constructed plasmids which contained the entire Py enhancer (nt 5039-5265), only the alpha element (equivalent to enhancer elements 1 plus 2 (Muller et al., 1988; Mueller et al., 1988) nt 5039-5130), or 3 copies of the binding site for the transcriptional activator AP1 (Lee et al., 1987a) upstream of

Figure 5. JSCAT-ORI

A) Structure of JSCAT-ORI. The sequence across the region encompassing the junctions between the Py ori-core element, the TATA box, and the 5' end of the CAT gene is shown, with selected restriction sites (underlined), TATA element (overlined), and putative transcription start site (+1) indicated. Enhancer or promoter elements can be inserted at the unique Bgl II site. The CAT gene plus accompanying poly A signals are derived from the plasmid pTE1 (Edlund et al., 1985). Py ori-core (nt 5265-90; numbered according to Soeda et al., 1980) was taken from the plasmid pdORI1.2 (Muller et al., 1983). The TATA box corresponds to that of the adenovirus 2 major late promoter. The rest of the plasmid, including the ampicillin resistance gene is derived from pUC 13.

B) Autoradiogram showing the sequence illustrated in A). The TATA box is marked for reference. Sequence analysis of JSCAT-ORI, and derivatives of this plasmid containing enhancer elements cloned in at the Bgl II site, was done using a commercial kit (Sequenase, United States Biochemical).

A


(oligo)n
ORI
+1
AAAAACAGGATCCAGATCTCGAGCTCGGGGGGCTATAAAAGGGGGATCTGAATTCGAGCTCGAGCTTGGCGA
Bam HI
Xho I
Bgl II
Sac I
Eco RI
Xho I
Sac I
TATA
Py ORI
CAT
SV40
poly A
Bam HI
Amp
PUC 13
Pvu II

B


A T G C
TATAAAA

the TATA box. These plasmids were transfected into FM3A cells, an established mouse cell line, and extracts were tested for CAT activity.

No CAT activity was detected in extracts from cells transfected with JSCAT-ORI, suggesting that the TATA box alone functions poorly, or not at all, to activate transcription in vivo. However, the presence of either the entire Py enhancer, or enhancer elements 1 plus 2, upstream of the TATA box significantly activated transcription (at least 60 fold greater than JSCAT-ORI) as observed by the high levels of CAT activity in extracts from cells transfected with the plasmids JSCAT-PyE(5') and JSCAT-αORI(Figure 6A, compare lanes 4 and 5 to lane 3). Transcription from the minimal promoter was also increased by upstream AP1 sites in the plasmid JSCAT-ORI/AP1(3) (at least 20 fold above JSCAT-ORI; Figure 6A, compare lane 6 to lane 3), suggesting that JSCAT-ORI would be useful for detecting transcription activation by small enhancer motifs.

To determine whether I could detect low levels of transcription from JSCAT-ORI, I tested the equivalent of five times as much extract from cells transfected with this plasmid (Figure 6A, lane 2). Because no CAT activity was detected, even under these conditions, I conclude that the minimal promoter in JSCAT-ORI has little or no activity in vivo in the

Figure 6. Characterization of JSCAT-ORI: Transcription

60 mm plates of FM3A cells were transfected with 3 ug of reporter plasmid by the DEAE-dextran method. Cells were harvested 48 hours post-transfection and CAT activities were assayed as described in Materials and Methods. Plasmids used in these experiments are indicated above each lane.
5X indicates that the equivalent of 5 times the normal amount (50 ul) of extract was assayed.

A) Activation of transcription by enhancer elements in JSCAT-ORI
B) Effect of TATA box deletions on transcription from JSCAT-ORI
C) Distance-dependent activation of transcription by enhancer elements in JSCAT-ORI

A

1
2
3
4
5
6
MOCK (5X)
JSCAT-ORI (5X)
JSCAT-ORI
JSCAT/PyE(5')
JSCAT- α ORI
JSCAT-ORI/AP1(3)

B

1
2
3
4
JSCAT/PyE(5')
JSCAT/PyE(5')TATA⁻
JSCAT-ORI/AP1(3)
JSCAT-ORI/AP1(3)TATA⁻

C

1
2
JSCAT/PyE(5')
JSCAT-ORI/PyE(3')

absence of upstream enhancer elements.

To test whether transcription could be activated by upstream enhancer elements in the absence of a promoter, I removed the TATA box from JSCAT-PyE(5') and JSCAT-ORI/AP1(3). This was accomplished by cleaving these plasmids with the restriction enzyme Sac I, which cleaves at sites immediately 5' and 3' of the TATA box, and religating the linear fragments. Extracts from cells transfected with the promoterless constructs JSCAT-PyE(5')TATA$^-$ and JSCAT-ORI/AP1(3)TATA$^-$ had significantly lower CAT activity than extracts from cells transfected with corresponding plasmids containing the TATA box (Figure 6B, compare lane 2 to lane 1 and lane 4 to lane 3). Deletion of the TATA box reduced activation of transcription by the Py enhancer at least 15 fold, however, this level was still at least 6 fold greater than measured for JSCAT-ORI. By constrast, deletion of the TATA box from the plasmid carrying the AP1 sites reduced CAT activity to basal levels.

To determine whether transcription activation in JSCAT-ORI is distance-dependent, I cloned the Py enhancer downstream (3') of the CAT gene. By comparison to JSCAT-PyE(5'), the CAT activity of JSCAT-ORI/PyE(3') was reduced approximately 15 fold (Figure 6C, compare lanes 1 and 2). It seems, therefore, that enhancer elements must be positioned

close to the TATA box in JSCAT-ORI for maximal stimulation of transcription. This finding agrees with results obtained by Kuhl et al. (1987) who showed that the level of transcription activation by an enhancer in the context of a minimal promoter (TATA box only) decreases the farther away the enhancer is moved.

ii) Replication

To test replication activation, plasmids bearing the Py ori-core element were transfected into mouse FM3A cells that express functional Py large T antigen. These cells, named FOP cells, efficiently support the replication of plasmids carrying natural or hybrid Py replication origins (Bennett-Cook and Hassell, 1991; D. Dufort, personal communication). The extent of replication of reporter plasmids can be determined by the Dpn I assay (Peden et al., 1980). This assay allows replicated DNA to be distinguished from unreplicated, transfected DNA on the basis of differential methylation of the two species of DNA.

Transfected test DNAs, isolated from dam^+ bacteria, are methylated and can be cleaved by the restriction endonuclease Dpn I. By contrast, DNA which has replicated at least once in mammalian cells is insensitive to Dpn I cleavage because mammalian cells lack dam methylase activity. FOP

cells are cotransfected with test plasmids and a control plasmid (pUC 19) which had been isolated from a dam$^-$ strain of E. coli. 48 hours after transfection, low molecular weight DNA was isolated and digested with Dpn I and Sst I, an enzyme which cleaves once in both the test and control plasmids. When the products of this reaction are run through an agarose gel, the plasmid DNA which had been taken up by the cells but not replicated appears as a series of Dpn I cleavage products at the bottom of the gel, whereas the plasmid DNA which had replicated, and the control DNA, appear at the top of the gel in single discrete bands. The control DNA, which does not replicate in mammalian cells (because it lacks a mammalian replication origin), serves as a useful marker to monitor transfection efficiency and allows comparisons of the replicative capacity of different test DNAs within the same experiment to be made.

To determine whether replication of JSCAT-ORI could be activated by enhancer elements, I tested JSCAT-αORI and JSCAT-ORI/AP1(3) in replication assays. Whereas JSCAT-ORI replicated only to a small extent in FOP cells, the plasmids bearing the α element or three copies of the AP1 site replicated very well (Figure 7A, compare lanes 2 and 3 to lane 1). Replication of these plasmids was dependent upon Py large T antigen because JSCAT-ORI and JSCAT-ORI/AP1(3) were unable

Figure 7. Characterization of JSCAT-ORI: Replication

60 mm plates of FOP cells were cotransfected with an equal amount of reporter plasmid and internal control (500 ng each, panels A, B, C; 250 ng each, panel D). The reporter plasmids used in these experiments are indicated above each lane. The internal control is pUC 19, isolated from a dam$^-$ strain of bacteria. Cells were harvested 48 hours post-transfection and low molecular weight DNA was recovered as described in Materials and Methods. Replicated DNA appears as the top band in each panel (R) and the internal control (C) appears immediately below. Bands found at the bottom of each panel represent Dpn I cleavage products. Panels A, B, and C were obtained from the same experiment, however lanes were taken from different exposures of the same autoradiogram in order to show weakly replicating species. Panel D was taken from a separate experiment.

A) Activation of replication by enhancer elements in JSCAT-ORI.

B) Replication of JSCAT-ORI is dependent upon the presence of Py large T antigen.

C) The TATA box in JSCAT-ORI does not affect replication.

D) Replication activation in JSCAT-ORI is distance-dependent.

C
R
A
1
2
3
JSCAT-ORI
JSCAT-α ORI
JSCAT-ORI/AP1(3)
B
1
2
3
4
FM3A
FOP
JSCAT-ORI
FM3A
FOP
JSCAT-ORI/AP1(3)
C
1
2
3
4
JSCAT-ORI
JSCAT-ORI/TATA⁻
JSCAT-ORI/AP1(3)
JSCAT-ORI/AP1(3)TATA⁻
D
1
2
3
JSCAT-ORI
JSCAT-ORI/PyE(3')
JSCAT- α ORI

to replicate in FM3A cells, which lack T antigen (Figure 7B).

Because both promoter and enhancer elements have been shown to activate replication, I wanted to assess whether the TATA box in JSCAT-ORI played any role in replication activation. By comparing the replicative ability of JSCAT-ORI and JSCAT-ORI/AP1(3) with equivalent plasmids lacking the TATA box, no difference was observed (Figure 7C). This result shows that the TATA element does not contribute to replication activation in these plasmids.

Activation of replication in JSCAT-ORI requires that enhancer elements be placed close to the Py ori-core sequence because the plasmid, JSCAT-ORI/PyE(3'), which has the Py enhancer located 3' of the CAT gene (approximately 1.6 kb from Py ori-core) failed to replicate in FOP cells (Figure 7D). This is consistent with results obtained by other researchers (Hassell et al., 1986; Murakami et al., 1991) who have also shown that activation of replication by enhancer elements is distance-dependent.

C. Transcription and replication activation by individual enhancer elements

i) AP1

To further evaluate the usefulness of JSCAT-ORI as a reporter for studying the activity of individual enhancer binding proteins, I assessed the activation properties of AP1 in greater detail. The AP1 DNA motif has been shown by others to confer transcriptional activation both in vivo (Angel et al., 1987; Lee et al., 1987a) and in vitro (Lee et al., 1987b; Bohmann and Tjian, 1989; Yong et al., manuscript in preparation) in the context of a variety of different promoters. To determine the requirements for transcription and replication activation by AP1 in the context of JSCAT-ORI, an oligonucleotide comprising the consensus AP1 binding motif (Figure 3) was cloned as a monomer or as tandem repeats of up to six copies into the Bgl II site between the TATA box and the Py ori-core sequences in JSCAT-ORI. The ability of AP1 to activate replication and transcription was tested by transfection of these plasmids into FOP or FM3A cells respectively.

Whereas a single AP1 motif was unable to activate transcription, two or more tandem copies of this element upstream of the TATA box in JSCAT-ORI efficiently activated

transcription as determined by the increased CAT activity in extracts of cells transfected with these plasmids (Figure 8A, compare lanes 2, 3, and 4 to lane 1). Because basal levels of transcription from JSCAT-ORI were too low to be accurately determined, the transcriptional activities of all plasmids derived from JSCAT-ORI were expressed relative to that of JSCAT-αORI, which was arbitrarily assigned a value of 100. Plasmids bearing two, three, or six copies of the AP1 binding site were transcribed at levels equal to 6, 22, or 56% (respectively) that of JSCAT-αORI. By comparing the relative transcriptional activities of the plasmids bearing different numbers of AP1 motifs, it seems that a minimum of two copies of this element are required for activation of transcription and the presence of additional copies of the AP1 site result in a synergistic (greater than additive) increase in transcription (Figure 8C and Table 1).

Replication was also activated synergistically by multimers of the AP1 site. JSCAT-ORI/AP1(1), bearing a single AP1 motif, replicated approximately 15% as well as JSCAT-αORI (Figure 8B, compare lane 2 to lane 6). Two, three, or six copies of the AP1 oligomer enhanced replication an additional 4-, 7-, and 12-fold over the levels observed for JSCAT-ORI/AP1(1) (Figure 8B, lanes 3 to 5 and Table 1).

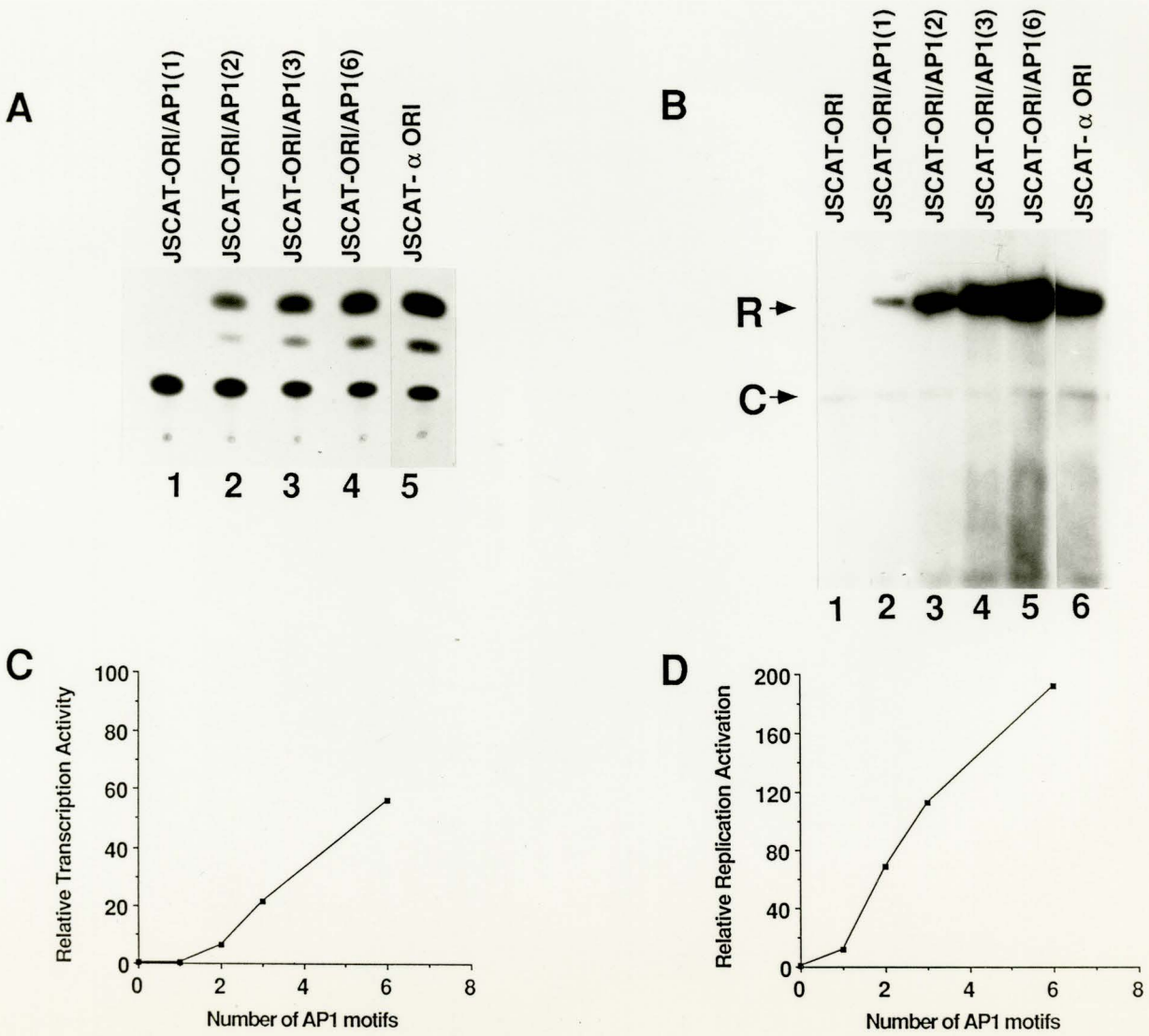
Figure 8. Transcription and replication activation by AP1 sequence motifs.

A) FM3A cells were transfected with 3 ug of reporter plasmid by the DEAE-dextran method. Reporter plasmids used in this experiment are shown above each lane. Cell extracts were incubated with ^{14}C-chloramphenicol for 1.5 hours at 37C and analyzed by thin layer chromatography as described in Materials and Methods.

B) FOP cells were cotransfected with an equal amount (250 ng) of reporter plasmid (indicated above each lane) and internal control, pUC 19 (isolated from a dam$^-$ strain of E.coli). Replicated DNA appears as the top band (R) in the panel, and the internal control (C) appears immediately below.

C) Graphic representation of the relative CAT activities of the reporter plasmids tested in panel A. CAT activities for each reporter plasmid were initially determined as the percentage of ^{14}C-chloramphenicol which became acetylated. These values were then normalized to that of JSCAT-αORI, defined as 100. Plasmids were tested 3-5 times in separate experiments; the results presented here represent the average of these experiments. Quantitative data from these experiments is also shown in Table 1 and Appendix A.

D) Densitometric quantitation of the replicating species in panel B. Values for reporter plasmids were normalized to that of JSCAT-αORI, defined as 100. These values also appear in Table 1.


A
JSCAT-ORI/AP1(1)
JSCAT-ORI/AP1(2)
JSCAT-ORI/AP1(3)
JSCAT-ORI/AP1(6)
JSCAT-α ORI
1 2 3 4 5
B
JSCAT-ORI
JSCAT-ORI/AP1(1)
JSCAT-ORI/AP1(2)
JSCAT-ORI/AP1(3)
JSCAT-ORI/AP1(6)
JSCAT-α ORI
R
C
1 2 3 4 5 6
C
Relative Transcription Activity
100
80
60
40
20
0
0 2 4 6 8
Number of AP1 motifs
D
Relative Replication Activation
200
160
120
80
40
0
0 2 4 6 8
Number of AP1 motifs

Comparing the levels of activation mediated by AP1 with respect to transcription and replication, it is apparent that there are quantitative differences in the activation of these processes. Whereas one AP1 binding site was sufficient to activate replication, at least two sites appeared to be necessary to activate transcription to any measurable extent. It seems, therefore, that in the context of JSCAT-ORI, replication is more sensitive to activation by enhancer elements, such as the AP1 motif, than is transcription. This agrees with results described by Veldman et al. (1985) who showed that only two copies of the minimal α element from the Py enhancer (nt 5108-5130) were needed to activate replication to wild type levels, whereas equivalent activation of transcription required five copies.

ii) PEA1

PEA1 is identified as a cellular protein that binds to the DNA sequence 'TGACTAA' (nt 5113-5120) within the Py enhancer. This recognition element is highly homologous to the AP1 consensus binding site 'TGACTCA'. c-jun and jun-B, major components of the AP1 transcription complex, have been shown to bind to both the AP1 motif and the Py PEA1 site as heterodimers with c-fos (Hirai et al., 1989; A. Cowie, personal communication). Moreover, both the AP1 and PEA1

sites mediate regulation by serum, TPA and the H-ras oncoprotein (Angel et al., 1987; Lee et al., 1987a; Ryder and Nathans, 1988; Imler et al., 1988; Yamaguchi et al., 1989). Together, these observations suggest that murine PEA1 may be identical or highly related to human AP1.

The activity of the PEA1 element has been studied in vivo, both in the context of the entire Py enhancer, or as part of a smaller region termed α-core, or the minimal α element (nt 5108-5130) (Tang et al., 1987; Muller et al., 1988; Martin et al., 1988; Imler et al., 1988; Wasylyk et al., 1988; Yamaguchi et al., 1989, Murakami et al., 1990). These studies have shown that mutations within the PEA1 site dramatically reduce the ability of the remaining sequences to activate either transcription or replication, emphasizing the importance of this factor in both these processes.

When multimers of the Py PEA1 motif (Figure 3B) were tested for their ability to activate replication and transcription in JSCAT-ORI, the results obtained were very similar to the results from the experiments with AP1. PEA1, like AP1, efficiently activated both replication and transcription (Figure 9). Furthermore, while a single copy of the PEA1 site was able to activate replication (Figure 9B, compare lane 2 to lane 1), two copies were required for transcription activation (Figure 9A, compare lane 2 to lane

Figure 9. Transcription and replication activation by PEA1 sequence motifs.

A) FM3A cells were transfected with 3 ug of reporter plasmid by the DEAE-dextran method. Reporter plasmids used in this experiment are shown above each lane. Cell extracts were incubated with ^{14}C-chloramphenicol for 1.5 hours at 37C and analyzed by thin layer chromatography as described in Materials and Methods.

B) FOP cells were cotransfected with an equal amount (250 ng) of reporter plasmid (indicated above each lane) and internal control, pUC 19 (isolated from a dam$^-$ strain of E. coli). Replicated DNA appears as the top band (R) in the panel, and the internal control (C) appears immediately below.

C) Graphic representation of the relative CAT activities of the reporter plasmids tested in panel A. CAT activities for each reporter plasmid were initially determined as the percentage of ^{14}C-chloramphenicol which became acetylated. These values were then normalized to that of JSCAT-αORI, defined as 100. Plasmids were tested 3-5 times in separate experiments; the results presented here represent the average of these experiments. Quantitative data from these experiments is also shown in Table 1 and Appendix A.

D) Densitometric quantitation of the replicating species in panel B. Values for reporter plasmids were normalized to that of JSCAT-αORI, defined as 100. These values also appear in Table 1.

A
1
2
3
4
5
6
JSCAT-ORI/PEA1(1)
JSCAT-ORI/PEA1(2)
JSCAT-ORI/PEA1(3)
JSCAT-ORI/PEA1(4)
JSCAT-ORI/PEA1(7)
JSCAT- α ORI
B
C
R
1
2
3
4
5
6
7
JSCAT-ORI
JSCAT-ORI/PEA1(1)
JSCAT-ORI/PEA1(2)
JSCAT-ORI/PEA1(3)
JSCAT-ORI/PEA1(4)
JSCAT-ORI/PEA1(7)
JSCAT- α ORI
C
Relative Transcription Activity
0
20
40
60
80
100
Number of PEA1 motifs
0
2
4
6
8
D
Relative Replication Activity
0
40
80
120
160
200
Number of PEA1 motifs
0
2
4
6
8

1). For both replication and transcription, the presence of multiple PEA1 sites worked synergistically to activate these processes (Figure 9A and B, lanes 3-6 and panels C,D), however the levels of activation achieved were slightly lower than that observed for an equivalent number of AP1 sites (Table 1). Plasmids bearing two, three, four, or seven copies of the PEA1 motif were transcribed at levels equal to 4, 11, 37, or 67% that of JSCAT-αORI. In replication assays, the plasmid JSCAT-ORI/PEA1(1) replicated to a small extent (approximately 5% of JSCAT-αORI) and the presence of an additional one, two, three, or six copies of the PEA1 site further enhanced replication to levels 18, 56, 120, and 180% relative to JSCAT-αORI.

iii) PEA2 and PEBP4

PEA2 was first identified as a factor in 3T6 nuclear extracts which binds to the Py enhancer between nt 5122 and 5129, immediately adjacent to the PEA1 site (Piette and Yaniv, 1987; Figure 3). The contribution of PEA2 to Py enhancer activity is unclear. Deletion of the PEA2 site from the Py enhancer impairs the activity of element 2 with respect to both transcription and replication <u>in vivo</u> in mouse fibroblast cell lines (Mueller et al., 1988; Muller et al., 1988), implying that PEA2 has a positive function. Deletion of the

PEA2 site has also been shown to negatively affect enhancer activity in mouse myeloma MPC-11 cells (Imler et al., 1988). By contrast, Wasylyk et al., (1988) have reported that PEA2 functions as a repressor of PEA1 in mouse L cells and in F9 embryo carcinoma cell lines. It has also been proposed that the repressor activity associated with the PEA2 site is mediated by a distinct factor, called PEBP4, which binds to a sequence that encompasses but extends beyond the PEA2 recognition site (Furukawa et al., 1990; see Figure 3A).

To investigate the activities of PEA2 and PEBP4, I cloned binding sites (Figure 3B) for these factors into JSCAT-ORI and tested for their capacity to activate replication and transcription in vivo. Neither PEA2 nor PEBP4 activated transcription, even when as many as six copies of the binding site were present upstream of the TATA box (Figure 10A). By contrast, PEA2 and PEBP4 activated replication, however, the levels of activation were low in comparison to activation observed in the presence of AP1 or PEA1 sites (Table 1). By comparison to JSCAT-αORI, plasmids bearing one, two, three, four or six copies of the PEA2 motif replicated 1, 5, 10, 20 and 25% as well, respectively (Figure 10B, compare lanes 2-6 to lane 9; and Table 1). A plasmid carrying six copies of the PEBP4 oligomer replicated to approximately the same extent as JSCAT-ORI/PEA2(6) (Figure 10B, compare lanes 6 and 8).

Figure 10. Transcription and replication activation by PEA2 and PEBP4 sequence motifs

A) FM3A cells were transfected with 3 ug of reporter plasmid by the DEAE-dextran method. Reporter plasmids used in this experiment are shown above each lane. Cell extracts were incubated with ^{14}C-chloramphenicol for 1.5 hours at 37C and analyzed by thin layer chromatography as described in Materials and Methods. 5X indicates that the equivalent of 5 times the normal amount (50 ul) of cell extract was assayed. B) FOP cells were cotransfected with an equal amount (250 ng) of reporter plasmid (indicated above each lane) and internal control, pUC 19 (isolated from a dam$^-$ strain of E. coli). Replicated DNA appears as the top band (R) in the panel, and the internal control(C) appears immediately below. The lane labeled M contains 10^{-4} ug of JSCAT-ORI DNA which was linearized by cleavage with a one-cut enzyme for this plasmid. C) Densitometric quantitation of replicating species in panel B. Values for reporter plasmids were normalized to that of JSCAT-αORI, defined as 100. These values also appear in Table 1.

A
1 2 3 4 5 6 7 8 9 10 11 12 13
5X MOCK
JSCAT-ORI
5X JSCAT-ORI
JSCAT-ORI/PEA2(1)
JSCAT-ORI/PEA2(2)
JSCAT-ORI/PEA2(3)
JSCAT-ORI/PEA2(4)
JSCAT-ORI/PEA2(6)
5X JSCAT-ORI/PEA2(6)
JSCAT-ORI/PEBP4(1)
JSCAT-ORI/PEBP4(6)
5X JSCAT-ORI/PEBP4(6)
JSCAT- α ORI
B
R
C
1 2 3 4 5 6 7 8 9
M
JSCAT-ORI
JSCAT-ORI/PEA2(1)
JSCAT-ORI/PEA2(2)
JSCAT-ORI/PEA2(3)
JSCAT-ORI/PEA2(4)
JSCAT-ORI/PEA2(6)
JSCAT-ORI/PEBP4(1)
JSCAT-ORI/PEBP4(6)
JSCAT- α ORI
C
Relative Replication Activation
0 10 20 30 40 50
Number of PEA2 motifs
0 2 4 6 8

iv) PEA3

PEA3 binds to the purine-rich sequence 'AGGAAG' (nt 5108-5113) within Element 2 of the Py enhancer. Mutation or deletion of the PEA3 element in the Py enhancer significantly impairs both replication and transcription activation suggesting that PEA3, like PEA1, is a positive trans-acting factor (Martin et al., 1988; Muller et al., 1988; Wasylyk et al., 1989; Yamaguchi et al., 1989, Murakami et al., 1990). The PEA3 motif is recognized by Ets-related proteins (Wasylyk et al., 1990; Xin et al.,manuscript in preparation; S. Bowman, personal communication) and is responsive to activation by serum, TPA, and the products of several oncogenes (Martin et al., 1988; Satake et al., 1988; Wasylyk et al., 1989; Yamaguchi et al., 1989).

To investigate the activity of PEA3 independent of other Py enhancer elements, an oligonucleotide comprising the PEA3 binding site was constructed. In addition, an oligonucleotide bearing a mutated version of the PEA3 site (PEA3mB) was made. This oligonucleotide has two point mutations in the PEA3 recognition site and these mutations have been shown to block factor binding in vitro (A. Cowie, personal communication). Both the wt and mutated PEA3 sites (Figure 3B) were cloned separately into JSCAT-ORI and tested in replication and transcription assays.

Consistent with the results obtained for AP1 and PEA1, a single PEA3 motif was unable to activate transcription from the minimal promoter in JSCAT-ORI (Figure 11A, lane 1). Two copies of the PEA3 binding site upstream of the TATA box gave weak, but reproducible, levels of transcription activation by comparison to JSCAT-ORI/PEA3(1) (Figure 11B, compare lane 2 to lane 1), and the presence of additional PEA3 sites further stimulated transcription; JSCAT-ORI/PEA3(5), with five copies of the PEA3 site was transcribed at 10% the level of JSCAT-αORI as judged by relative CAT activities in cells transfected with these plasmids (Figure 11A, compare lanes 5 and 7; Table 1). No activation of transcription was observed from the template bearing four copies of the mutated PEA3 element (Figure 11A, lane 6).

For replication activation, a single copy of the PEA3 site was sufficient, however the presence of additional PEA3 sites significantly increased the level of replication (Figure 11B, lanes 2-6). Templates bearing 2, 3, 4, or 5 copies of the PEA3 oligimer were replicated at least 5-, 8-, 18-, and 20-fold better than the template containing a single PEA3 site (Table 1). By contrast, the plasmid carrying four copies of the mutant PEA3 site replicated poorly or not at all in FOP cells (Figure 11B, lane 7).

Figure 11. Transcription and replication activation by PEA3 sequence motifs.

A) FM3A cells were transfected with 3 ug of reporter plasmid by the DEAE-dextran method. Reporter plasmids used in this experiment are shown above each lane. Cell extracts were incubated with ^{14}C-chloramphenicol for 1.5 hours at 37C and analyzed by thin layer chromatography as described in Materials and Methods.

B) FOP cells were cotransfected with an equal amount (250 ng) of reporter plasmid (indicated above each lane) and internal control, pUC 19 (isolated from a dam$^-$ strain of E. coli). Replicated DNA appears as the top band (R) in the panel, and the internal control (C) appears immediately below. The lane labeled M contains 10^{-4} ug of JSCAT-ORI which was linearized by cleavage with a one-cut enzyme for this plasmid.

C) Graphic representation of the relative CAT activities of the reporter plasmids tested in panel A. CAT activities for each reporter plasmid were initially determined as the percentage of ^{14}C-chloramphenicol which became acetylated. These values were then normalized to that of JSCAT-αORI, defined as 100. Plasmids were tested 3-5 times in separate experiments; the results presented here represent the average of these experiments. Quantitative data from these experiments is also shown in Table 1 and Appendix A.

D) Densitometric quantitation of the replicating species in panel B. Values for reporter plasmids were normalized to that of JSCAT-αORI, defined as 100. These values also appear in Table 1.

A
1 2 3 4 5 6 7
JSCAT-ORI/PEA3(1)
JSCAT-ORI/PEA3(2)
JSCAT-ORI/PEA3(3)
JSCAT-ORI/PEA3(4)
JSCAT-ORI/PEA3(5)
JSCAT-ORI/PEA3mB(4)
JSCAT- α ORI
B
R
C
M
JSCAT-ORI
JSCAT-ORI/PEA3(1)
JSCAT-ORI/PEA3(2)
JSCAT-ORI/PEA3(3)
JSCAT-ORI/PEA3(4)
JSCAT-ORI/PEA3(5)
JSCAT-ORI/PEA3mB(4)
JSCAT- α ORI
1 2 3 4 5 6 7 8
C
Relative Transcription Activity
0 10 20 30 40 50
Number of PEA3 motifs
0 2 4 6 8
D
Relative Replication Activity
0 10 20 30 40 50
Number of PEA3 motifs
0 2 4 6 8

Table 1. Relative transcription and replication activation values for JSCAT-ORI plasmids bearing single and tandem repeats of individual PEA sequence motifs.

Representative results are shown for transcription and replication of the plasmids listed in the far-left column. Transcription was measured by CAT assay and replication was measured by the Dpn I assay as described in Materials and Methods. Activation values are expressed relative to JSCAT-αORI defined as 100. CAT values represent the average of three to five separate experiments; CAT values from inivididual experiments are shown in Appendix A.

Table 1 - Relative Transcription and Replication Activation values for JSCAT-ORI plasmids bearing single and tandem repeats of individual PEA sequence motifs

Plasmid	Transcription Activitation [a]	Replication Activation [a]
JSCAT- α ORI	100	100
JSCAT-ORI	<1	.5
JSCAT-ORITATA$^-$	<1	.5
JSCAT/PyE(5')	117	ND
JSCAT/PyE(5')TATA$^-$	8	ND
JSCAT-ORI/PyE(3')	7	.5
JSCAT-ORI/AP1(1)	<1	16
AP1(2)	6	69
AP1(3)	22	113
AP1(6)	56	192
AP1(3)TATA$^-$	<1	108
JSCAT-ORI/PEA1(1)	<1	5
PEA1(2)	4	18
PEA1(3)	11	56
PEA1(4)	37	120
PEA1(7)	67	181
JSCAT-ORI/PEA2(1)	<1	1
PEA2(2)	<1	5
PEA2(3)	<1	9
PEA2(4)	<1	19
PEA2(6)	<1	23
JSCAT-ORI/PEBP4(1)	<1	3
PEBP4(6)	<1	24
JSCAT-ORI/PEA3(1)	<1	2
PEA3(2)	2	10
PEA3(3)	6	17
PEA3(4)	9	36
PEA3(5)	10	42
PEA3mB(4)	<1	.5

[a] activation values are expressed relative to the activity of JSCAT- α ORI which was given a value of 100

ND not determined

D. Comparison of AP1, PEA1, PEA2, PEBP4, and PEA3 activities in different cell lines

To further characterize the enhancer binding proteins AP1, PEA1, PEA2, PEBP4, and PEA3, I compared the activities of these factors in a variety of different cell lines including mouse C127 and 3T3 cells, monkey CV1 cells and human HeLa cells. The results obtained were qualitatively similar to those obtained in FM3A cells. Whereas plasmids bearing AP1, PEA1, or PEA3 sites upstream of the TATA box in JSCAT-ORI were efficiently transcribed, I could not detect any activation of transcription by factors binding to either the PEA2 or PEBP4 sites in any of the cell lines tested (Table 2).

There were some quantitative differences in the relative activities of AP1, PEA1, and PEA3 in different cell lines. In particular, the activity of the PEA3 element in all of the cell lines tested was considerably higher than in FM3A cells. This may reflect a higher abundance of the PEA3 factor in these cells in comparison to FM3A cells. Alternatively, a distinct factor, with different activation properties, may mediate transcription activation through the PEA3 element in these other cell lines. Although there is no data on the relative levels of PEA3 protein in the different cell lines tested, Northern analysis of RNA from these cells shows that

Table 2. Relative transcription activities of JSCAT-ORI plasmids in different cell lines.

Cells were transfected with 3 ug of the reporter plasmids indicated in the far-left column. Transfections were performed by the calcium-phosphate precipitation technique for C127, 3T3, CV1, and HeLa cells and by the DEAE-dextran method for FM3A cells. Cell extracts were incubated with ^{14}C-chloramphenicol for 1.5 hours at 37C and analyzed by thin layer chromatography as described in Materials and Methods. Activation values are expressed relative to JSCAT-αORI, defined as 100. The values in this table were derived from a single experiment only.

Table 2 - Relative transcription activities of JSCAT-ORI plasmids in different cell lines [a]

Plasmids	C127	3T3	CV1	HeLa	FM3A
JSCAT- α ORI	100	100	100	100	100
JSCAT-PyE(5')	100	115	144	ND	106
JSCAT-ORI	<1	<1	11	<1	1.5
JSCAT-ORI/AP1(6)	70	88	244	>1400	57
JSCAT-ORI/PEA1(7)	120	127	111	83	85
JSCAT-ORI/PEA2(4)	<1	<1	<1	<1	1.5
JSCAT-ORI/PEA3(4)	140	169	222	117	12
JSCAT-ORI/PEBP4(6)	<1	<1	<1	<1	<1
pSV_2CAT	310	138	467	>1600	87

[a] activation values are expressed relative to the activity of JSCAT- α ORI which was given a value of 100

ND not determined

values are derived from a single experiment only

PEA3 mRNA is present in the highest amount in FM3A cells (M. McWilliams, J-H. Xin, unpublished results) which does not correlate with the relatively weak PEA3 activity detected in FM3A cells. This observation lends support to the hypothesis that other proteins, perhaps related members of the c-ets family, may be mediating transcription activation through the PEA3 element in different cell lines (Bhat et al., 1987).

Comparisons between the activation properties of enhancer motifs in different cell lines revealed another interesting point. Levels of transcription, as measured by CAT activity, are approximately equal for plasmids bearing either six copies of the AP1 binding site or seven copies of the PEA1 binding site in most of the cell lines tested. This is not surprising because the AP1 and PEA1 elements are thought to interact with the same factor(s) based on the sequence similarity of these motifs and the fact that they possess many of the same properties including the ability to mediate activation by serum, TPA, and H-ras. It is interesting to note, therefore, that the PEA1 and AP1 motifs display significantly different activities in HeLa cells. Whereas JSCAT-ORI/AP1(6) and JSCAT-ORI/PEA1(7) show similar levels of transcription in most of the cell lines tested, in HeLa cells, the plasmid bearing six copies of the AP1 motif is transcribed almost 20-fold better than the plasmid carrying

seven copies of the PEA1 motif (Table 2). While it is possible that two distinct factors (or subsets of factors) recognize the PEA1 and AP1 sites in HeLa cells, it is also possible that the human AP1 factor has more stringent sequence requirements and perhaps recognizes the PEA1 site with low affinity only. By contrast, the mouse factor appears to recognize both sites (cAP1 and PEA1) equally well.

E. Transcription and replication activation by paired, heterologous enhancer motifs

Single copies of either the PEA1 or PEA3 motifs were not able to activate transcription from JSCAT-ORI, even though they occur as single sites in the Py enhancer. Futhermore, even though it has been shown by deletion and mutational analysis that the PEA2 element contributes positively to Py enhancer function, in JSCAT-ORI the PEA2 motif was unable to activate transcription even when it was present in up to six copies.

With reference to the enhanson model described for the SV40 enhancer (Ondek et al., 1988), it seemed feasible that in the context of the Py enhancer, single copies of the PEA1, PEA2, and PEA3 elements may cooperate with each other to activate transcription and DNA replication. To test this

hypothesis, paired combinations of Py enhancer motifs were cloned into JSCAT-ORI and assayed for their ability to activate transcription and replication.

i) Paired PEA3 and PEA1 motifs

To examine whether PEA1 and PEA3 could activate transcription in conjunction with one another (and in the absence of PEA2), I used an oligonucleotide comprising the binding sites for both these factors (PEA3+PEA1, Figure 3B) as they occur naturally within the Py enhancer. Similar oligonucleotides described by other researchers have been shown to activate transcription (Wasylyk et al., 1988; Yamaguchi et al., 1989) and replication (Murakami et al., 1990) when present in multiple copies in the appropriate reporter system.

A single copy of the PEA3+PEA1 oligonucleotide was unable to activate transcription from the minimal promoter in JSCAT-ORI because no CAT activity was detected in cells transfected with JSCAT-ORI/PEA3+PEA1(1) (Figure 12A, lane 3). However, plasmids bearing two or three copies of this oligomer were transcribed to levels equal to 17 and 46% the activity of JSCAT-αORI (Figure 12A, compare lanes 4 and 5 to lane 14; Table 3). Significantly, two copies of the paired PEA3+PEA1 element activated transcription better than two copies of

Figure 12. Transcription and replication activation by plasmids bearing paired, heterologous PEA sequence motifs.

A) FM3A cells were transfected with 3 ug of reporter plasmid by the DEAE-dextran method. Reporter plasmids used in this experiment are shown above each lane. Cell extracts were incubated with ^{14}C-chloramphenicol for 1.5 hours at 37C and analyzed by thin layer chromatography as described in Materials and Methods.

B) FOP cells were cotransfected with an equal amount (250 ng) of reporter plasmid (indicated above each lane) and internal control, pUC 19 (isolated from a dam$^-$ strain of E. coli). Replicated DNA appears as the top band (R) in the panel, and the internal control (C) appears immediately below.

A
1 2 3 4 5 6 7 8 9 10 11 12 13 14
MOCK
JSCAT-ORI
JSCAT-ORI/PEA3+PEA1(1)
JSCAT-ORI/PEA3+PEA1(2)
JSCAT-ORI/PEA3+PEA1(3)
JSCAT-ORI/mPEA3+PEA1(8)
JSCAT-ORI/PEA3+mPEA1(6)
JSCAT-ORI/PEA1+PEA2(1)
JSCAT-ORI/PEA1+PEA2(2)
JSCAT-ORI/PEA1+PEA2(3)
JSCAT-ORI/PEA1+PEA2(5)
JSCAT-ORI/PEA1+mPEA2(5)
JSCAT-ORI/mPEA1+PEA2(5)
JSCAT- α ORI
B
1 2 3 4 5 6 7 8 9 10 11 12 13
JSCAT-ORI
JSCAT-ORI/PEA1+PEA2(1)
JSCAT-ORI/PEA1+PEA2(2)
JSCAT-ORI/PEA1+PEA2(3)
JSCAT-ORI/PEA1+PEA2(5)
JSCAT-ORI/PEA1+mPEA2(5)
JSCAT-ORI/mPEA1+PEA2(5)
JSCAT-ORI/PEA3+PEA1(1)
JSCAT-ORI/PEA3+PEA1(2)
JSCAT-ORI/PEA3+PEA1(3)
JSCAT-ORI/PEA3+mPEA1(6)
JSCAT-ORI/mPEA3+PEA1(8)
JSCAT- α ORI

either the PEA3 or PEA1 motif alone (compare data in Tables 1 and 3) suggesting that PEA3 and PEA1 can work cooperatively to enhance transcription.

To determine whether PEA3 and PEA1 could activate transcription independently of each other in the context of the double site oligonucleotide, I tested mutant versions, PEA3+mPEA1 and mPEA3+PEA1 (Figure 3B), in which double point mutations abolish binding of PEA1 or PEA3, respectively (Piette and Yaniv, 1987; Wasylyk et al., 1989; A. Cowie, personal communication). Whereas multiple copies of the mPEA3+PEA1 oligomer were able to efficiently activate transcription from the minimal promoter in JSCAT-ORI, no transcription activation by the PEA3+mPEA1 oligomer was detected even when as many as six copies were present (Figure 12A, lanes 6 and 7).

The PEA3+PEA1 combinations were also tested for their ability to activate replication. Plasmids bearing one, two, or three copies of the PEA3+PEA1 oligonucleotide replicated at levels equal to 29, 76, and 143% that of JSCAT-αORI (Figure 12B, compare lanes 8-10 to lane 13). Multimers of the mPEA3+PEA1 oligomer also activated replication (Figure 12B, lane 12). However, consistent with the results obtained for transcription activation, the ability of PEA3 to activate replication from the PEA3+mPEA1 double site element was

considerably reduced by comparison to PEA3 binding sites in the context of the single site oligonucleotide (Figure 12B, lane 11 and compare data in Tables 1 and 3). Whereas a plasmid carrying five copies of the PEA3 monomer oligonucleotide replicated to levels equal to 42% JSCAT-αORI activity, JSCAT-ORI/PEA3+mPEA1(6), bearing six PEA3 sites, only replicated to 7% that of JSCAT-αORI.

Since earlier experiments using single site oligonucleotides had shown PEA3 to be capable of independently activating transcription and replication, its reduced ability to do so in the context of the PEA3+mPEA1 element is somewhat puzzling. One explanation is that the PEA3 motif, like the enhansons described for the SV40 enhancer, may have strict spacing requirements, such that adjacent PEA3 factors can only interact cooperatively when they are spaced the appropriate distance apart. In tandem arrays of the double site oligonucleotide PEA3+mPEA1, the presence of the mutated PEA1 motif results in an additional 5 bp (equal to one half helical turn in the DNA) between adjacent PEA3 binding sites ie. by comparison to the spacing which occurs between multimers of the PEA3 single site oligonucleotide. Inappropriate spacing may explain why the PEA3 motifs, which can activate transcription from multimers of the single site oligomer, fail to do so in the context of the PEA3+mPEA1 elements.

ii) Paired PEA1 and PEA2 motifs

Even though PEA2 was unable to activate transcription on its own, it was possible that it might do so in conjunction with PEA1 because binding sites for these factors are juxtaposed in the Py enhancer. Alternatively, if PEA2 is a repressor as Wasylyk et al.,(1988) have proposed, then PEA2 might block the ability of PEA1 to activate transcription. To test these theories, an oligonucleotide comprised of binding sites for both PEA1 and PEA2 (PEA1+PEA2, Figure 3B) was cloned into JSCAT-ORI. As controls, the oligonucleotides PEA1+mPEA2 and mPEA1+PEA2, which carry double point mutations in either the PEA2 or PEA1 site respectively (Figure 3B), were also cloned into JSCAT-ORI. These plasmids were transfected into FOP or FM3A cells and assayed for replication and transcription activation.

Transcription from the minimal promoter in JSCAT-ORI was only weakly activated by multimers of the PEA1+PEA2 element. No CAT activity was detected in cells transfected with plasmids carrying one or two copies of the PEA1+PEA2 oligonucleotide, and the presence of three or five copies of this oligomer only activated transcription to levels equivalent to 1 and 3% that of JSCAT-αORI (Figure 12A, compare lanes 8-11 and lane 14).

By contrast, plasmids bearing multiple copies of the PEA1+mPEA2 element upstream of the TATA box were transcribed very well; CAT activity in cells transfected with JSCAT-ORI/PEA1+mPEA2(5) is approximately 75% that of JSCAT-αORI (Table 3). These results suggest that PEA2 functions as a repressor in FM3A cells since the presence of wt PEA2 sites inhibits activation of transcription by PEA1 (Table 3, compare activities of JSCAT-ORI/PEA1+PEA2(5) and JSCAT-ORI/PEA1+mPEA2(5)). JSCAT-ORI/mPEA1+PEA2(5) had no activity in FM3A cells (Figure 12A, lane 13), confirming results obtained with the monomer PEA2 oligonucleotide, indicating that PEA2 is not an independent activator of transcription.

Plasmids bearing PEA1+PEA2 sites were efficiently replicated in FOP cells. The presence of one, two, three, or five copies of the PEA1+PEA2 oligomer activated replication to levels equal to 38, 97, 181, and 215% that of JSCAT-αORI (Figure 12B, compare lanes 2, 3, 4, and 5 to lane 13; Table 3). Plasmids carrying five copies of either the PEA1+mPEA2 or mPEA1+PEA2 oligonucleotides also replicated, but to a considerably lesser extent than the corresponding plasmid, JSCAT-ORI/PEA1+PEA2(5) in which both the wt PEA1 and PEA2 sites were present (Figure 12B, compare lanes 6 and 7 to lane 5; Table 3). These results show that, in contrast to its role as a repressor of transcription in FM3A cells, PEA2 can

Table 3. Relative transcription and replication activation values for JSCAT-ORI plasmids bearing paired, heterologous PEA sequence motifs.

Representative results are shown for transcription and replication of the plasmids listed in the far-left column. Transcription was measured by CAT assay and replication was measured by the Dpn I assay as described in Materials and Methods. Activation values are expressed relative to JSCAT-αORI, defined as 100. CAT values represent the average of two to three separate experiments; CAT values from individual experiments are shown in Appendix A.

Table 3 - Relative transcription and replication activation values for JSCAT-ORI plasmids bearing paired, heterologous PEA sequence motifs

Plasmids	Transcription Activation [a]	Replication Activation [a]
JSCAT-α ORI	100	100
JSCAT-ORI	<1	.5
JSCAT-ORI/PEA1+PEA2(1)	<1	38
PEA1+PEA2(2)	<1	97
PEA1+PEA2(3)	1	181
PEA1+PEA2(5)	3	215
JSCAT-ORI/PEA1+mPEA2(5)	74	68
JSCAT-ORI/mPEA1+PEA2(5)	<1	13
JSCAT-ORI/PEA3+PEA1(1)	<1	29
PEA3+PEA1(2)	17	76
PEA3+PEA1(3)	46	143
JSCAT-ORI/mPEA3+PEA1(8)	68	105
JSCAT-ORI/PEA3+mPEA1(6)	<1	7

[a] activation values are expressed relative to the activity of JSCAT-α ORI which was given a value of 100

function either independently, or in cooperation with PEA1 to activate replication in FOP cells.

F. Activation of transcription by PEA2 and PEBP4 in FOP cells

PEA2 was unable to independently activate transcription in FM3A cells. Moreover, PEA2 inhibited the ability of PEA1 to activate transcription in these cells. By contrast, PEA2, which could weakly activate replication on its own, appeared to work cooperatively with PEA1 to enhance replication in FOP cells. I was interested in trying to determine whether the different behavior of PEA2 in the two systems was a consequence of underlying differences in the mechanisms of replication and transcription activation, or the result of the particular properties of the two cell lines used for these studies. FOP cells are the same as FM3A cells except that they express all three Py T antigens. Because middle and large T antigens have both been implicated in modulating Py enhancer activity (Kern et al., 1986; Wasylyk et al., 1988) it is possible that PEA2 has a different activity in FOP cells in comparison to FM3A cells. To examine this possibility, plasmids carrying PEA2 sites upstream of the TATA box in JSCAT-ORI were transfected into FOP cells and tested for transcription activation. For comparison, plasmids bearing multiple copies of the AP1, PEA1, PEA3, or PEBP4 motifs were

also tested in FOP cells. Figure 13 shows the results of these experiments.

By contrast to results obtained in FM3A cells, both PEA2 and PEBP4 activated transcription in FOP cells (Figure 13,lanes 5 and 7). Although the levels of transcription mediated by PEA2 and PEBP4 were low by comparison to plasmids containing AP1, PEA1, or PEA3 sites (Figure 13, compare lanes 5 and 7 to lanes 3, 4, and 6), these levels are significant since basal transcription from JSCAT-ORI was not detected in FOP cells. (Figure 13, lane 1).

The level of transcription activation mediated by all factors tested was considerably higher in FOP cells than in FM3A cells (note that for the experiment shown in Figure 13, only one-tenth the usual amount of extract was assayed ie. by comparison to experiments performed with extracts from FM3A cells). Because earlier studies had already shown that AP1, PEA1, PEA2, and PEPB4, and PEA3 could all activate replication through the JSCAT-ORI Py ori-core element in FOP cells, increased copy number provides one explanation for the high levels of transcription observed in FOP cells. This makes it difficult to determine whether PEA2 and PEBP4 truly have the ability to activate transcription in FOP cells. The levels of transcription detected using JSCAT-ORI/PEA2(4) and JSCAT-ORI/PEBP4(6) in FOP cells could be the result of basal

Figure 13. Transcription of plasmids bearing PEA sequence motifs in FOP cells.

FOP cells were transfected with 3 ug of reporter plasmid by the DEAE-dextran method. Reporter plasmids used in this experiment are shown above each lane. Cell extracts were incubated with ^{14}C-chloramphenicol for 1.5 hours at 37C and analyzed by thin layer chromatography as described in Materials and Methods except that only one-tenth the normal amount (ie. in comparison to experiments in FM3A cells) of extract was assayed. The percentage of ^{14}C-chloramphenicol which became acetylated is shown below each lane.

	1	2	3	4	5	6	7
% Acetylated chloramphenicol	.3	95	98	72	5.5	75	6.2

transcription from the TATA box, which may be elevated in relation to JSCAT-ORI because of the increased copy number resulting from replication of the plasmids carrying either PEA2 or PEBP4 motifs. Alternatively, transcription of JSCAT-ORI/PEA(2) and JSCAT-ORI/PEPB4(6) in FOP cells may represent real transcription activation by PEA2 and PEBP4 in these cells. To try to distinguish between these two possibilities, I constructed replication-defective plasmids from JSCAT-ORI/PEA2(4) and JSCAT-ORI/PEBP4(6). JSCAT-ORI/PEA2(4)Apa and JSCAT-ORI/PEBP4(6)Apa have a 4 bp deletion in one of the large T binding sites in Py ori-core which impairs replication (Murakami et al., 1990); JSCAT/PEBP4(6)ORI^{-} has the entire Py ori-core element removed. Figure 14A shows that none of these plasmids replicate in FOP cells.

When examined for transcription activation in FOP cells, the levels of CAT activity in extracts from cells transfected with any of the replication defective plasmids were considerably lower than for their replicative competent counterparts, JSCAT-ORI/PEA2(4) and JSCAT-ORI/PEBP4(6) (Figure 14B, compare lanes 5, 6, and 7 to lanes 3 and 4). It seems, therefore, that PEA2 and PEBP4 are, at best, weak activators of transcription since transcription activation by these factors can only be detected when plasmid amplification occurs as a result of replication.

Figure 14. Analysis of replication defective plasmids bearing PEA2 and PEBP4 sequence motifs for transcription activation in FOP cells.

A) FOP cells were cotransfected with an equal amount (250 ng) of reporter plasmid (indicated above each lane) and internal control, pUC 19, isolated from a dam$^-$ strain of E. coli. Replicated DNA appears as the top band (R) in the panel, and the internal control (C) appears immediately below.
B) FOP cells were transfected with 3 ug of reporter plasmid by the DEAE-dextran method. Reporter plasmids used in this experiment are shown above each lane. CAT activity was tested as described in Materials and Methods except only half the normal amount of cell extract (ie. in comparison to experiments using FM3A cell extracts) was assayed. The percentage of ^{14}C-chloramphenicol which became acetylated is shown below each lane.

A

1 2 3 4 5
JSCAT-ORI/PEA2(4)
JSCAT-ORI/PEBP4(6)
JSCAT-ORI/PEA2(4)Apa
JSCAT-ORI/PEBP4(6)Apa
JSCAT/PEBP4(6)ORI⁻

B

%Acelated Chloramphenicol
98
1
62
41
1.5
<1
<1
1 2 3 4 5 6 7
JSCAT- αORI
JSCAT-ORI
JSCAT-ORI/PEA2(4)
JSCAT-ORI/PEBP4(6)
JSCAT-ORI/PEA2(4)Apa
JSCAT-ORI/PEBP4(6)Apa
JSCAT/PEBP4(6)ORI⁻

DISCUSSION

Expression of pol II transcribed genes in eukaryotes is controlled through the action of cellular trans-acting proteins bound to short cis-acting DNA sequence elements within promoters and enhancers. Enhancer and promoter elements also participate in the regulation of DNA replication suggesting that the processes of replication and transcription activation may share some common features. To simplify the analysis of enhancer and promoter elements for their ability to activate both replication and transcription, I constructed a vector, JSCAT-ORI, which can be used to assay both processes.

A. Characterization of JSCAT-ORI

i) Transcription

The promoter of JSCAT-ORI consists of a TATA box only. Although this TATA box can direct low basal levels of transcription in vitro (Sawadogo and Roeder, 1985b; Yong et al., manuscript in preparation), I was unable to detect transcription from this element in vivo. Other researchers have also shown that promoters comprised of only a TATA box have very little activity in vivo (Pelham, 1982; Wasylyk et al., 1984; Treisman and Maniatis, 1985; Kuhl et al., 1987). The different activity of the TATA element in vitro and in vivo likely reflects the different conditions which exist in

the two environments. In _in vitro_ assays where there is no competition from other promoters, the TATA element may be able to recruit sufficient quantities of the general initiation factors for basal level transcription. By contrast, in the context of the cellular environment, the TATA box is likely too weak to efficiently compete with cellular promoters for limiting quantities of these transcription factors. Other features which could influence the behavior of promoter elements _in vivo_ and _in vitro_ include differences in template configuration, chromatin structure, composition of the aqueous environment, and the presence or absence of the nuclear membrane.

The use of a minimal promoter to direct transcription in JSCAT-ORI was considered desirable because of the possibility that a strong promoter might mask activation of transcription by small enhancer elements, should the activities of these elements be relatively weak. As shown from experiments in this study, the promoter in JSCAT-ORI is indeed responsive to transcription activation by individual enhancer motifs. However, in the absence of detectable basal levels of transcription from JSCAT-ORI, it is difficult to determine the magnitude of transcription activation by these motifs. Moreover, it is possible that elements that only weakly activate transcription would not be detected using

JSCAT-ORI. It would be worth investigating the use of a more sensitive reporter such as the luciferase gene (de Wet et al., 1987) or perhaps expanding the promoter in JSCAT-ORI to include an UPE such as a CCAAT box or G/C rich element. This may increase basal transcription to measurable levels and still maintain the sensitivity of JSCAT-ORI to activation by weak enhancer motifs.

Transcription from the minimal promoter in JSCAT-ORI is dramatically increased by the presence of either the entire Py enhancer (elements 1, 2, and 3; nt 5039-5265) or the alpha (α) element (equivalent to enhancer elements 1 and 2; nt 5039-5130). The alpha element has only slightly weaker activity than the entire enhancer, illustrating the redundancy which is inherent to the Py enhancer. This observation also agrees with results obtained by Mueller et al., (1988), who tested different combinations of Py enhancer elements for their ability to activate transcription from the HSV tk-1 promoter in mouse 3T3 cells. In particular, they found that elements 1 plus 2 could activate transcription almost as well as the complete enhancer, although this effect was dependent upon these elements being oriented with their early border (nt 5039) closest to the promoter. In the opposite orientation, they found that elements 1 plus 2 had only one-tenth the activity of the complete enhancer. In JSCAT-αORI the alpha

element is oriented with its early border closest to the TATA box.

Although the TATA box in JSCAT-ORI has no activity on its own, this element is important for transcription activation by upstream elements. With respect to the entire Py enhancer, deletion of the TATA box reduces activation considerably but does not completely abolish transcription. Deletion of the TATA box also impairs activation by AP1 motifs and in this context, transcription is reduced to basal levels. The absence of a TATA box may have more severe consequences for transcription activation by enhancer motifs because these elements are, themselves, relatively weak. By contrast, the presence of a strong enhancer close to the transcription initiation site may be able to partially compensate for the loss of the TATA box (Benoist and Chambon, 1980; Fromm and Berg, 1982; Hen et al., 1982). It is also possible that the residual levels of transcription that occur in the absence of the bona-fide TATA box are the result of initiation at sites located within the Py enhancer. Other researchers have shown that viable deletion mutants of Py that lack the TATA box from the viral early promoter make use of heterologous initiation sites (Kamen et al., 1982; Mueller et al., 1984). RNase or S1 protection assays on the CAT mRNA produced using the JSCAT/PyE(5') TATA^{--} template could be used to determine if

alternate initiation sites within the Py enhancer are being utilized in the absence of a TATA box in this plasmid.

Activation of transcription in JSCAT-ORI is distance-dependent because the Py enhancer is at least 15 fold less active from a position 1.6 kb away from the initiation site than from a position immediately adjacent to the TATA box. Studies by Kuhl et al., (1987) have also shown that activation of a minimal promoter (TATA box only) by enhancer elements is distance-dependent, with activation levels decreasing the farther away the enhancer elements are moved. This observation contrasts with the distance-independent property of enhancers in the context of complete promoters, which include upstream elements such as G/C or CCAAT boxes in addition to the TATA sequence.

These results support a model based on the idea that activation of transcription by factors bound at enhancers located far away from the transcription initiation site is mediated by proteins bound at proximal promoter elements (Ptashne, 1986; Courey et al., 1989). The mechanism underlying synergistic activation by promoter proximal factors and enhancer binding proteins is unknown, although it is considered unlikely that this effect occurs as a result of cooperative DNA binding, due to the large distances which often separate enhancers and promoters. Instead, synergy is

thought to take place at the level of transcription activation. One way this might occur is if proteins bound at promoter proximal and enhancer elements interact to form a complex which is able to efficiently recruit or activate some component of the general transcription machinery. This is shown schematically in Figure 2C.

ii) **Replication**

When JSCAT-ORI was constructed it was not clear whether this vector would be useful for studying replication in mammalian cells. The reason for this is that some sequences present in plasmids of bacterial origin inhibit replication in mammalian cells. These 'poison sequences' were originally defined in pBR322 by Lusky and Botchan (1981). Consequently, reporter plasmids to be utilized for replication studies in mammalian systems are often based on a vector called pML2, which was constructed from pBR322 by deletion of these inhibitory sequences (nt 1120-2490).

JSCAT-ORI is derived from a vector containing a pUC 13 background. pUC plasmids include some of the same sequences as pBR322 and, in particular, the pUC 13 fragment present in JSCAT-ORI contains a portion (nt 2348-2490) of the 'poison' element described in pBR322. However, when tested in replication assays, JSCAT-αORI, and an equivalent pML2-

based plasmid carrying the α element, replicated to approximately equivalent levels (data not shown). The pUC 13 sequences in JSCAT-ORI, therefore do not appear to have any inhibitory effect on replication in mammalian cells.

Considering the variety of promoter and enhancer elements which have been shown to activate papovavirus replication origins, it is somewhat surprising that the TATA element in JSCAT-ORI has no effect on replication activation. The presence of a TATA box within the SV40 ori-core has been postulated to account for the reduced dependence of the SV40 ori-core on auxiliary sequence elements for replication, in comparison to the Py ori-core which lacks a TATA box and functions only poorly in the absence of activator sequences (Hassell et al., 1986; DePamphilis et al., 1987; Bennett et al., 1989). This hypothesis is not supported by the observation that the TATA box, either alone or in cooperation with AP1 elements, has no effect on replication of JSCAT-ORI. The TATA box may therefore be one example of a transcription element which can not activate replication. Relevant to this is the finding that overexpression of the TATA binding factor TFIID, by transfection of the cDNA encoding a mammalian TFIID protein, has no effect on replication of JSCAT-ORI (C. Graham, personal communication). It is possible however, that the

TATA box in JSCAT-ORI is positioned inappropriately to activate replication. Also, by analogy to other transcription elements, the TATA element may need to be present in several copies in to efficiently activate replication.

The ability of enhancer elements to activate replication from the Py ori-core element is distance-dependent. In the viral genome, the α and ß auxiliary domains efficiently activate replication from their position immediately adjacent to the late border (nt5265) of ori-core. By contrast, these elements can not activate replication when they are moved 200 bp away from the late border or 50 bp from the early border (nt 90) of the Py ori-core (Hassell et al., 1986). In JSCAT-ORI, the Py ori-core element is oriented with its late border within 10 bp of the Bgl II site which is used as a cloning site for enhancer motifs. In this position, all of the enhancer motifs tested in this study were able to activate replication, however, consistent with the results described by Hassell et al., (1986), no replication activation is observed by the Py enhancer when it is located at a distance (1.6kb away) from the ori-core element.

The distance-dependent interaction between ori-core and auxiliary elements is also intrinsic to the SV40 and adenovirus replication units. By contrast, in viruses such as Bovine Papillomavirus (BPV) and Epstein-Barr virus (EBV),

the enhancer and ori-core units function independently of their relative orientation and distance from one another (DePamphilis, 1988 and references therein). However, the ori-core elements of these viruses include a promoter which may mediate interactions with factors bound at a distally located enhancer. If this is true, it would imply that long distance activation of replication and transcription by a remote enhancer is mediated in both cases by upstream promoter elements (UPE) because, as previously discussed, transcription activation is also distance-dependent in the context of a minimal promoter lacking UPE. To test this hypothesis, it would be simple to clone one or more UPE into the Bgl II site of JSCAT-ORI/PyE(3') and ask whether this would enable the Py enhancer to activate replication from a distance.

B. Transcription and replication activation by polyomavirus enhancer binding proteins

The region between nt 5108 and 5140 of the Py enhancer contains juxtaposed binding sites for at least three, and possibly four, proteins named PEA1, PEA2, PEA3, and PEBP4. The activity of these factors has been studied in the context of the entire enhancer or in smaller regions from within the enhancer, however the individual contributions of these

factors to Py enhancer activity is still not well understood. I attempted to define their properties by cloning the sequence recognition motifs for these factors into JSCAT-ORI and assessing their ability to activate replication and transcription *in vivo*.

The PEA1 recognition site is highly homologous to the binding site for the human transcription factor AP1. Moreover, PEA1 and AP1 share several functional properties including the ability to mediate transcription activation by TPA, serum, and the H-ras oncoprotein. Consistent with the hypothesis that PEA1 may be the murine homolog of AP1, the transcription and replication activation properties of the PEA1 motifs are qualitatively very similar to those conferred by the AP1 motifs. However, the PEA1 element displays slightly lower activity in both assays suggesting that it may represent a lower affinity binding site for the PEA1/AP1 factor.

A single copy of the PEA1 motif is unable to activate transcription but weakly activates replication. For both replication and transcription activation, multiple copies of the PEA1 motif function better than would be expected from the additive effect of single copies, indicating that some sort of synergistic interaction occurs between adjacently bound factors.

The AP1 and PEA1 motifs are recognized by the jun family of proteins which can bind to these elements as homodimers or heterodimers with c-fos (Hirai et al., 1990; A. Cowie, personal communciation). Although the various jun proteins appear to bind indistinguishably to AP1/PEA1 sites, at least two members of the jun family, junB and c-jun, have been shown to exhibit distinct biological activities. Whereas c-jun is an efficient activator of promoters which contain a single AP1 site, junB is unable to trans-activate these promoters and inhibits their activation by c-jun. By contrast, junB and c-jun are equally able to trans-activate a promoter which carries multiple AP1 sites (Chiu et al., 1990). It is not clear which species of jun protein is activating transcription and replication through the PEA1 motif in FM3A cells although both c-jun and junB are thought to be present since mRNAs for both proteins have been isolated from these cells (A. Cowie, personal communication).

PEA3 also activates both replication and transcription, although the levels of activation demonstrated by this factor in FM3A cells are modest in comparison to AP1 and PEA1. For example, templates bearing four copies of the PEA1 site are transcribed at least 4-fold better than comparable templates bearing a tetramer of the PEA3 site. This difference may be a reflection of the relative amounts

of these factors in FM3A cells, differences in the strengths of the activation domains of these factors, or differences in the relative affinities of the factors for their respective sites. Like the PEA1 element, a single copy of the PEA3 binding site could weakly activate replication, whereas two copies of the PEA3 motif were required to activate transcription to any measurable extent. The presence of additional PEA3 motifs resulted in synergistic increases in replication and transcription activation.

The inability of a single copy of either the PEA1 or PEA3 motif to activate transcription may reflect a requirement of these factors to bind cooperatively to adjacent sites. Furthermore, cooperative binding may explain the synergistic activation observed when multiple copies of the binding sites for these factors are present. These questions were addressed by C. Yong et al. (manuscript in preparation), who showed through bandshift assays, that neither PEA1 nor PEA3 bind cooperatively to multiple copies of their recognition site. Furthermore, in <u>in vitro</u> transcription assays, they showed that, although a dimer of either the PEA1 or PEA3 motif was required to activate transcription, the presence of oligonucleotides carrying a single binding site in the reaction mixture inhibited transcription from templates bearing multimers of the corresponding site. Since,

presumably inhibition occured as a result of competition for factor binding, these findings suggest that while a dimer of either the PEA1 or PEA3 motif is required for transcription activation, monomers of these elements are sufficient for factor binding. Furthermore, because PEA1 and PEA3 do not bind cooperatively to adjacent sites, the synergy displayed by these factors likely reflects interaction at the level of transcription activation.

Synergistic activation by transcription elements has been reported in many systems. In some instances, synergy occurs as a result of the cooperative binding between two factors to adjacent sites. This effect has been described for the protein TEF-1 which binds cooperatively to two copies of either the Sph I or GTIIC motifs from the SV40 enhancer (Davidson et al., 1988). The B cell-specific Oct2 protein has also been reported to bind cooperatively to adjacent octamer motifs (LeBowitz et al., 1989). By contrast, synergy has also been demonstrated to occur in the absence of cooperative binding. For example, the yeast GAL4 protein and the rat glucocorticoid receptor can activate transcription synergistically from a mammalian promoter containing binding sites for both proteins. This effect can be demonstrated <u>in vitro</u> under conditions in which all of the binding sites are saturated (Lin et al., 1988; Carey et al., 1990). In this

situation, cooperative binding effects are irrelevant and synergy is presumed to result from cooperative interaction between the activation domains of these factors. One model, which has been proposed to explain this phenomenon, is based on the idea that the activation domains of DNA bound transcription factors may work cooperatively because they are able to simultaneously contact a target in the general transcription complex. In studying this phenomenon with the GAL4 transcription factor, Carey et al., (1990) suggest that their data is compatible with up to 5 to 10 molecules of this factor being able to contact the target simultaneously.

Synergistic activation by transcription factors may have evolved as a way to limit non-productive interactions between transcription factors and their targets when these proteins are not bound to DNA. Ptashne (1988) has proposed that the presence of strong activation domains in the cell would inhibit transcription because these domains could interact with their target proteins indiscriminately, making them unavailable for the formation of active transcription complexes at promoters. This problem would be avoided if transcription factors evolved with activation domains which individually are too weak to interact with their targets. However, these same weak activation domains could efficiently activate transcription if they work cooperatively to contact

a target. This may occur when activation domains are concentrated through the binding of transcription factors to adjacent sites in promoters and enhancers.

Multimers of an oligonucleotide comprising adjacent PEA3 and PEA1 sites are also able to interact cooperatively to activate transcription and replication in JSCAT-ORI. This occurs in the absence of cooperative binding between these factors (Wasylyk et al. 1988; Yong et al., manuscript in preparation). A survey of promoters and enhancers shows that PEA3 and AP1/PEA1 motifs are frequently found close together (Wasylyk et al., 1989, 1990; Gutman and Wasylyk, 1990), suggesting that the interaction of these factors may have biological significance. Relavent to this hypothesis, it has recently been shown that PEA3 acts synergistically with AP1 to enhance transcription from the collagenase promoter induced by either TPA or non-nuclear oncoproteins such as v-src (Gutman and Wasylyk, 1990). Furthermore, full induction of Py enhancer activity by the expression of oncogenes such as H-ras, v-mos, v-src, and Py middle T antigen requires both the PEA1 and PEA3 motifs (Wasylyk et al., 1989). This suggests that PEA1 and PEA3 activity may be coordinately induced by some of the same regulatory signals.

PEA2 does not activate transcription from the minimal promoter in JSCAT-ORI, even when as many as six copies of the

binding site for this factor are present upstream of the TATA box. This suggests that PEA2 can not independently activate transcription. Furthermore, in agreement with results described by Wasylyk et al., (1988), the presence of a wt PEA2 site adjacent to the PEA1 motif inhibits PEA1-activated transcription, indicating that PEA2 can act as a repressor in this context. The inhibitory effect of PEA2 requires the presence of wt PEA2 sites since a template bearing multiple copies of the PEA1+mPEA2 oligonucleotide is efficiently transcribed.

In contrast to its role as a repressor of PEA1-activated transcription in FM3A cells, PEA2 appears to function cooperatively with PEA1 to activate replication in FOP cells. Furthermore, multimers of the PEA2 element can independently activate replication, albeit to relatively low levels only.

There are several possible explanations for the different behavior of PEA2 with respect to transcription and replication activation. The FOP cells in which the replication assays are performed express all three Py T antigens. In addition to its role in initiating DNA replication through the Py ori-core element, large T antigen has also been implicated as a <u>trans</u>-activator of promoter function. Kern et al., (1986) show that Py large T antigen

greatly stimulates transcription from the Py late promoter in a manner which is independent of DNA replication, but dependent upon large T binding sites and the presence of the viral enhancer. They, and others (Kingston et al., 1986; M. Featherstone, Ph.D. thesis 1986, McGill University, Montreal, Quebec) show that Py large T antigen can also trans-activate promoters in the absence of binding sites for this protein. Although no particular target has been identified for transactivation by Py large T antigen, it is possible that the activity of the PEA2 factor is somehow altered through interactions with large T, and this may account for the different behavior of PEA2 in FOP and FM3A cells.

Py middle T antigen has also been reported to potentiate the activity of some transcription factors. In particular, both the Py PEA1 and PEA3 elements mediate transcription activation by this virally-encoded protein (Wasylyk et al., 1988). These authors did not comment on whether the expression of middle T antigen has any effect on the activity of the PEA2 element so this remains open to speculation.

The observation that PEA2 can activate transcription in FOP cells can be interpreted in several ways. It may confirm that PEA2 does indeed have a different activity in FOP cells, by comparison to FM3A cells in which the PEA2

element acted to repress transcription. If this is the case, the altered activity of PEA2 is linked to its ability to activate replication, because the replication defective plasmid JSCAT-ORI/PEA2(4)Apa was not transcribed in FOP cells to any significant level.

An alternate explanation can be proposed to explain the behavior of PEA2 in these assays. It is possible that PEA2 is, in fact, a weak activator of transcription in both FOP and FM3A cells. This activity may be detected in FOP cells because replication of plasmids bearing PEA2 sites results in increased copy number of these plasmids which in turn may result in the amplification of a weak transcription signal. In FM3A cells, where replication does not take place, PEA2 may function as a repressor of PEA1 activity because the binding of PEA2 to sites adjacent to PEA1 may disrupt the cooperative interaction which has been postulated to occur between PEA1 activation domains. Since, in this scenario, PEA2 only contributes a very weak activation domain, the disruption of the synergistic interaction between adjacent PEA1 proteins would dramatically reduce PEA1-activated transcription.

While any or all of these possibilities are possible, the activity of PEA2 still presents some contradictions. Although PEA2 seems to be a repressor of transcription in some

situations (this study; Wasylyk et al., 1988), it is clear from other studies that deletion of the PEA2 element from the Py enhancer (or smaller regions from within the enhancer) can have a deleterious effect on transcription, implying that PEA2 contributes a positive function (Mueller et al., 1988; Imler et al., 1988). Furthermore, while I show in this study, that PEA2 can independently activate replication, this contradicts results reported by Murakami et al., (1990) who found that PEA2 motifs could not activate replication in COP cells (mouse C127 cells which express Py large T antigen). However, in this same study, they show that deletion of the PEA2 site from an element encompassing Py sequences 5107-5130 results in a 10 fold decrease in replication activation.

It is clear that the activity of the PEA2 element needs to be investigated more thoroughly. If PEA2 is indeed a repressor in FM3A cells, this could be tested in a competition experiment, in which increasing amounts of a plasmid carrying PEA2 sites could be cotransfected with the PEA2 reporter plasmid to see if the repressor activity could be titrated away. Alternatively, the plasmid carrying multiple copies of the PEA1+PEA2 oligonucleotide could be tested for transcription in the presence of the protein synthesis inhibitor, cycloheximide, since Wasylyk et al., (1988) have proposed that PEA2 is a labile repressor based on

their experiments using a similar approach. Also, it would be interesting to investigate whether PEA2 could inhibit PEA1-activated transcription when the recognition sites for these factors are separated, rather than located adjacent to each other as they were in experiments described in this study. Finally, to determine whether any of the Py T antigens affect the activity of PEA2, one could cotransfect vectors expressing each of these proteins independently with the PEA2 reporter plasmid into FM3A cells and assay for the ability of PEA2 to activate transcription under these conditions.

The PEBP4 element, which encompasses and extends beyond the PEA2 binding site (Figure 3A), behaved similarly to the PEA2 motif in both replication and transcription assays. In particular, multiple copies of the PEBP4 element were unable to activate transcription in FM3A cells but could weakly activate replication in FOP cells. Gel retardation assays show that the PEBP4 element is recognized by a factor(s) present in FM3A nuclear extract. However, most of this binding activity can be competed away using the PEA2 oligonucleotide (A. Cowie, personal communication). This result, together with the results from the transcription and replication assays, suggests that the same factor or factors recognize both the PEA2 and PEBP4 element in FM3A cells.

C. Replication activation by transcription elements

There is a considerable amount of evidence to suggest that the processes of replication and transcription activation occur via similar mechanisms in eukaryotic cells. In particular, it has been shown that many of the same cis-acting sequences which activate transcription also activate replication (DePamphilis, 1988 and references therein). More detailed investigations have shown that the same protein domains of transcription factors which are required for transcription activation (ie. both a DNA binding domain and an activation domain) are also essential for activation of DNA replication (Bennett-Cook and Hassell, 1991). Furthermore, transcription factors with a wide variety of different activation domains (acidic, gln-rich, pro-rich etc.) can activate DNA replication (C. Coulber, personal communication). Results presented in this thesis show that the polyomavirus enhancer binding proteins PEA1, PEA2, and PEA3 can function individually, or in paired combinations, to activate replication from the Py ori-core.

The mechanism by which transcription factors activate DNA replication is not clear. Because the interaction between the activation domains of transcription factors and their target in the general transcription complex does not appear to involve highly specific interactions (reviewed by

Guarentes, 1988), it is feasible that non-specific interactions may allow these same proteins to activate the cellular replication machinery in a similar manner. Alternatively, the involvement of transcription factors in activation of DNA replication may imply that a component(s) of the general transcription complex also participates in some aspect of DNA replication. However, the process of DNA replication is clearly independent of transcription since α-amanitin, which blocks RNA pol II activity, has no effect on papovavirus DNA replication (Decker et al., 1987). Furthermore, as shown in this study, deletion of the TATA box from JSCAT-ORI/AP1(3) significantly reduces transcription but has no effect on the level of replication of this plasmid.

The binding of transcriptional activators to enhancer elements may affect replication indirectly by perturbing the local distribution of nucleosomes which otherwise might block the interaction of T antigen or other replication factors with ori-core. In support of this latter possibility, it has been demonstrated that SV40 replication is inhibited by the addition of histones to a cell-free system, but inhibition can be relieved by the prior binding of CTF/NF1, a transcription activator, to its recognition site near ori-core (Cheng and Kelly, 1989). However, as was the case for transcription activation, the simple exclusion of nucleosomes from the ori-

core can not be sufficient for replication activation because derivatives of transcription factors comprised only of a DNA binding domain can exclude nucleosomes (Workman et al., 1991), yet do not activate replication. The results of Bennett-Cook and Hassell (1991) show that replication activation specifically requires the involvement of the activation domains of transcription factors.

It is possible that transcription factors activate replication by promoting or stabalizing the binding of large T antigen to ori-core. Since there is only one documented example of a direct interaction between a transcription factor, AP2, and SV40 T antigen (Mitchell et al., 1987) such interactions, if they occur, would most likely be mediated through an intermediary protein.

If transcription factors are involved in promoting or stabilizing the T-antigen initiation complex to ori-core, then this might explain the stronger dependence of Py ori-core on cis-acting transcription elements than SV40 ori-core. Whereas the major T-antigen binding sites for SV40 are found within ori-core, the strongest affinity sites for Py large T antigen lie outside the ori-core. Consequently, efficient binding of Py large T antigen to the relatively weak sites within ori-core may be more dependent upon assistance from the transcription factors bound at enhancer or promoter elements

located close by.

Recently it has been suggested that the activation domains of transcription factors may not contact their target in the general transcription complex directly; instead it has been proposed that another class of proteins, called 'coactivators' may mediate these interactions (Smale et al., 1990; Pugh et al., 1990; Peterson et al., 1991). This hypothesis is based, in part, on the inability of purified recombinant forms of TFIID to respond to activation by sequence-specific transcription factors. For example, although recombinant forms of yeast, drosophila or human TFIID can bind to TATA sequences and function in basal transcription in a reconstituted system, they are unable to respond to transcription activation by upstream regulators such as SP1 (Peterson et al., 1991). By contrast, partially purified fractions of TFIID from human and drosophila cells can mediate both basal and activated transcription (Pugh et al., 1990). It has been proposed, therefore, that partially purified TFIID fractions contain coactivators that are dispensable for basal transcription, but are required to bridge interactions between trans-activators and the general initiation machinery. Furthermore, experiments by Pugh et al., (1990) show that TFIID interactions with coactivators are species-specific since purified yeast or drosophila TFIID can not utilize

coactivators present in human nuclear extracts to mediate transcription activation by SP1.

Because the activation of DNA replication requires that the activation domains of transcription factors be present, this may imply that coactivators are also important for mediating interactions between transcription factors and their targets in the replication initiation complex. This idea is attractive because it might explain why Py and SV40 are unable to replicate in the same cells types, despite the similarity in sequence and functional properties between the large T antigens and replication origins of these two viruses. Whereas SV40 replicates most efficiently in primate (monkey) cells, Py replication is restricted to murine cells. The cell tropism displayed by these viruses has been attributed to the action of species-specific permissive factors but the nature of these factors has not been determined. If coactivators mediate activation of DNA replication by transcription factors, then the same species specificity which controls the interaction of these factors with TFIID may also restrict their interaction with large T antigen. The development of cell free systems to study SV40 and Py replication <u>in vitro</u> will be important for determining whether the coactivators associated with transcription activation are the species-specific permissive factors which are required for papovavirus

DNA replication.

The significance of the observation that one copy of each of the PEA1, PEA2, or PEA3 binding sites can activate replication, whereas two copies are required to activate transcription is not clear. Studies by several groups have shown that the number of transcription factor binding sites required to activate replication is fewer than is needed for activation of transcription (Veldman et al., 1985; Muller et al., 1988; DePamphilis, 1988). This may be indicative of differences in the mechanisms by which transcription factors participate in the two processes, or it may simply reflect a higher sensitivity of replication to activation by these factors.

APPENDIX A

CAT ACTIVITIES - RELATIVE VALUES[a]

EXPT	1	2	3	4	5	6	Avg/SD[b]
JSCAT- αORI	100	100	100	100	100	100	100
JSCAT-ORI	<1	<1	<1	<1	<1	<1	<1
JSCAT-ORI/TATA⁻				<1			
JSCAT/PyE(5')	129	115	114	109	121	120	116/7.4
JSCAT/PyE(5')TATA⁻	7.9	9	7.5	5.2		8.5	7.6/1.5
JSCAT-ORI/PyE(3')			9.2		7	6	7.4/1.6
JSCAT-ORI/AP1(1)	<1	<1	<1			<1	<1
AP1(2)	6.5	5	7			7	6.4/.9
AP1(3)	23	11	25	21		26	22/5.0
AP1(6)	58	45	61			60	56/7.4
AP1(3)TATA⁻			<1	1.4		<1	<1
JSCAT-ORI/PEA1(1)	<1	1.6	<1			<1	<1
PEA1(2)		3.4	4.2			3.5	3.7/.4
PEA1(3)	9	7	13			14	11/3.3
PEA1(4)	29	38	40			40	37/5.3
PEA1(7)	62	65	68			71	66/3.9
JSCAT-ORI/PEA2(1)		<1			<1	<1	<1
PEA2(2)	<1	<1			<1	<1	<1
PEA2(3)						<1	<1
PEA2(4)	<1	<1			<1	<1	<1
PEA2(6)						<1	<1
JSCAT-ORI/PEBP4(1)		<1			<1	<1	<1
PEBP4(6)		<1			<1	<1	<1
JSCAT-ORI/PEA3(1)	<1	<1		<1	<1	<1	<1
PEA3(2)	2	1		3.2	2.1	3	2.3/.9
PEA3(3)					7.3	5.6	6.3/1.2
PEA3(4)	8	10.7		10.3	9	9.1	9.4/1.1
PEA3(5)					9.5	11.1	10.4/.8
PEA3mB(4)				1	<1	<1	<1

	EXPT	7	8	9	AVG/SD[b]
JSCAT- α ORI		100	100	100	100
JSCAT-ORI		<1	<1		<1
JSCAT-ORI/PEA1+PEA2(1)		<1	<1		<1
PEA1+PEA2(2)		<1	<1		<1
PEA1+PEA2(3)		1	2		1.5/.5
PEA1+PEA2(5)		2	4	4	3.3/.9
JSCAT-ORI/PEA1+mPEA2(5)			72	77	74.5/2.5
JSCAT-ORI/mPEA1+PEA2(5)		<1	<1	<1	<1
JSCAT-ORI/PEA3+PEA1(1)		<1	<1		<1
PEA3+PEA1(2)		15	19		17/2
PEA3+PEA1(3)		51	42		46.5/4.5
JSCAT-ORI/mPEA3+PEA1(8)		65	71		68/3
JSCAT-ORI/PEA3+PEA1(6)		<1	<1		<1

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a CAT activities are expressed relative to the activity of JSCAT- αORI which was given a value of 100

b Average/Standard Deviation

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