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Activation of polyomavirus DNA replication by yeast GAL4 is dependent on its transcriptional activation domains

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The polyomavirus replication origin contains transcriptional regulatory sequences. To determine how these elements function in DNA replication, and to learn whether a common mechanism underlies the activation of transcription and DNA replication, we tested whether a well-characterized transcriptional activator, yeast GAL4, was capable of stimulating DNA replication and transcription in the same mammalian cell line. We observed that GAL4 activated polyomavirus DNA replication in mouse cells when its binding site was juxtaposed to the late border of the polyomavirus origin core. Synergistic activation of DNA replication was achieved by multimerization of the GAL4 binding site. Analysis of GAL4 mutant proteins, GAL4 hybrid proteins and mutants of the latter revealed that the activation domains of these transcriptional activators were required to stimulate DNA replication. In agreement with previously published data, the activation domains of GAL4 were also required to enhance transcription in the same mouse cell line. These observations implicate transcriptional activators in Py DNA replication and suggest that similar mechanisms govern the activation of transcription and DNA replication.

Key words: activation domains/DNA replication/GAL4/transcriptional activators

Introduction

Replication origins derived from a wide variety of sources (viruses, plasmids, organelles and cells) include transcriptional regulatory elements, which serve as binding sites for cellular proteins (reviewed by DePamphilis, 1988). In mammalian systems the first indication of this came from studies of polyomavirus (Py) and SV40, two members of the papovaviruses. The replication origins of these viruses comprise two functionally distinct components, an auxilliary component and a core component (Tyndall *et al.*, 1981; Bergsma *et al.*, 1982; Muller *et al.*, 1983; de Villiers *et al.*, 1984; DeLucia *et al.*, 1986; Hertz and Mertz, 1986; Lee-Chen and Woodworth-Gutai, 1986; Li *et al.*, 1986). The origin core (ori-core) is composed of ~65 base pairs (bp) and is the site of binding of large T antigen, a virus encoded protein required for the initiation of DNA replication (for reviews see Challberg and Kelly, 1989; Stillman, 1989). Although the core origin is sufficient for SV40 DNA replication, and to a much lesser extent for that of Py, the auxiliary elements stimulate replication dramatically (Tyndall *et al.*, 1981; Bergsma *et al.*, 1982; Muller *et al.*, 1983; Muller *et al.*, 1988). These auxilliary regions overlap the viral promoters and enhancers.

Several lines of evidence suggest that the same sequence elements that activate transcription also activate DNA replication. First, *cis*-acting mutations that affect one process also affect the other (Fujimura *et al.*, 1981; Fujimura and Linney, 1982; Tang *et al.*, 1987; Muller *et al.*, 1988; Mueller *et al.*, 1988). Second, reversion of these mutations result in gain of function with respect to both processes (Tang *et al.*, 1987). Finally, transcriptional regulatory sequences from heterologous sources, including those of cellular origin, are capable of activating papovavirus DNA replication (de Villiers *et al.*, 1984; Bennett *et al.*, 1989).

Because common DNA sequence motifs, and by inference the same regulatory factors, appear to activate both transcription and DNA replication, it has been suggested that the two processes could occur by a similar mechanism (Hassell *et al.*, 1986). However, there are differences between the requirements for transcription activation and DNA replication activation. For example, DNA replication can be activated by far fewer copies of a transcriptional regulatory sequence than can transcription (Veldman *et al.*, 1985; Muller *et al.*, 1988). And, while enhancers are capable of activating transcription from great distances relative to the promoter to which they are coupled, these same sequences must be closely positioned near the late border of ori-core to activate DNA replication (Innis and Scott, 1984; Hassell *et al.*, 1986; Hertz and Mertz, 1986; Lee-Chen and Woodworth-Gutai, 1986). Finally, whereas it appears that the same sequence motifs that activate transcription can also activate DNA replication, there is no direct evidence that these two processes are mediated by the same proteins. To clarify the purported role of transcriptional activators in Py DNA replication and to determine whether a common mechanism underlies the activation of transcription and DNA replication, we asked whether a known transcriptional activator could stimulate both processes in the same cell line, and whether the features of the activator required for one process were also required for the other.

The recent molecular cloning and analysis of cDNAs which encode eukaryotic transcriptional activators has provided significant insight into their functional organization (for recent reviews see Johnson and McKnight, 1989; Mitchell and Tjian, 1989; Struhl, 1989). These factors are composed of at least two domains that are required for their activity. One of these is necessary for DNA binding, whereas the other, the activation domain, is required in conjunction with the DNA binding domain to activate transcription. DNA binding domains of transcriptional activators confer specificity for interactions with DNA, whereas activation

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domains directly or indirectly contact and/or stabilize interactions with general transcription factors and perhaps RNA polymerse II (Ptashne, 1988).

One example of such a factor is *GAL4*, an 881 amino acid yeast transcriptional activator, which binds to four 17 bp sites in the galactose upstream activation region and stimulates divergent transcription of the *GAL1* and *GAL10* genes (Guarente *et al.*, 1982; West *et al.*, 1984; Yocum *et al.*, 1984). The DNA binding domain of GAL4 is located between residues 1 and 74 (Brent and Ptashne, 1985; Keegan *et al.*, 1986), whereas its two principal activation domains are positioned between amino acids 148 and 196, and between residues 768 and 881 (Ma and Ptashne, 1987). The activation regions of GAL4 are rich in acidic amino acids, but this characteristic alone is not sufficient for their activity. Apparently, a specific structure, an amphipathic helix with negative charges along one surface, is required to constitute an activation domain (Giniger and Ptashne, 1987). Interestingly, GAL4 and hybrid proteins formed between the DNA binding domain of GAL4 and the activating regions of other transcriptional regulatory proteins are able to activate transcription not only in yeasts, but also in mammalian (Kakidani and Ptashne, 1988; Webster *et al.*, 1988; Sadowski *et al.*, 1988), insect (Fischer *et al.*, 1988), and plant cells (Ma *et al.*, 1988).

GAL4 seemed an ideal choice with which to address the requirement for transcriptional activators for DNA replication, and the relatedness of their mechanisms of action in this process and in transcription. First, GAL4 functions in mammalian cells to activate transcription (Kakidani and Ptashne, 1988. Webster *et al.*, 1988). Second, a mammalian counterpart of GAL4 apparently does not exist (Kakidani and Ptashne, 1988; Webster *et al.*, 1988). And third, the functional domains of GAL4 and several hybrid proteins formed between its DNA binding domain and the activation domains of several viral transactivators have been defined, thereby providing a rich source of activators for analysis (Kakidani and Ptashne, 1988; Sadowski *et al.*, 1988; Lillie and Green, 1989). Here we report that GAL4 and GAL4 hybrid proteins activated Py DNA replication in mouse cells. This activation was dependent on the GAL4 binding site being juxtaposed to ori-core and was increased synergistically by multimerization of this site. These observations provide direct evidence that transcriptional activators are capable of stimulating Py DNA replication. Replication activation required the transcriptional activation domains of the proteins, implying that factor binding alone is insufficient to activate DNA replication. Because these same features of the GAL4 proteins were also required for transcription activation in the same cell line, these findings suggest that the activation of both processes occurs by similar mechanisms.

Results

GAL4 activates Py DNA replication

To determine whether GAL4 was capable of activating Py DNA replication, we constructed a reporter plasmid bearing a single near consensus GAL4 binding site, a 17-mer, at the late border of ori-core (Figure 1A).The 17-mer, 5′-CGGAGTACTGTCCTCCG-3′, differs from the perfect palindromic consensus sequence by a single base pair (Giniger *et al.*, 1985; Bram *et al.*, 1986). GAL4 binds to this sequence *in vitro* three times better than it does to the wild type element (Giniger *et al.*, 1985), and the same sequence efficiently activates GAL4-dependent transcription in mammalian cells *in vivo* (Webster *et al.*, 1988).

The reporter was tested for its capacity to replicate in mouse FM3A cells that express functional Py large T antigen. These cells, named FOP cells, efficiently support the replication of plasmids bearing the natural and hybrid Py replication origins. The mammalian effector plasmid, pAG4 (Kakidani and Ptashne, 1988), was transfected with the reporter to provide GAL4 in FOP cells (Figure 1B). To determine whether the effect of GAL4 was specific to the reporters harbouring its binding site, we also cotransfected the effector plasmid with several other reporters bearing the natural Py auxiliary elements, named alpha (α) and beta (β) (Figure 1A). These sequences represent two of the elements of the Py enhancer (Muller *et al.*, 1983, 1988; Mueller *et al.*, 1988).

The extent of replication of the reporter plasmids was measured by the *Dpn*I assay (Peden *et al.*, 1980), which permits discrimination between plasmid DNA that has been taken up by the cell but not replicated (this DNA appears as a collection of *Dpn*I cleavage products at the bottom of the gel) from plasmid DNA which has replicated (replicated DNA is insensitive to *Dpn*I digestion). To visualize the replicated DNA as a single species, the products of *Dpn*I cleavage were digested with a unique site restriction endonuclease for the reporter DNA (Figure 1C, arrow).

A plasmid bearing only the Py ori-core (Figure 1A) replicated very poorly in FOP cells (Figure 1C, lane 2 and a longer exposure, lane 12; the replicated DNA is not visible in these exposures of the autoradiogram). This was expected because Py DNA replication requires auxiliary elements in addition to the core origin (Tyndall *et al.*, 1981; Muller *et al.*, 1983). By contrast, plasmids carrying the α, β, or both enhancer elements of Py positioned at the late border of ori-core replicated efficiently in FOP cells (Figure 1C, lanes 4, 6 and 8). Provision of GAL4 in *trans* did not improve or inhibit the capacity of these plasmids to replicate (Figure 1C, compare lanes 2, 4, 6 and 8 with lanes 3, 5, 7 and 9). (A longer exposure of lane 3 is represented by lane 13.) The reporter bearing the 17-mer did not replicate in the absence of the effector presumably because mouse cells, like other mammalian cells (Kakidani and Ptashne, 1988; Webster *et al.*, 1988), either lack a factor capable of acting at this sequence to enhance DNA replication or possess a repressor which binds to this site and blocks DNA replication (Figure 1C, lanes 10 and 12). However, addition of GAL4 to this reporter resulted in its replication, thereby demonstrating that GAL4, a yeast activator of transcription, activated Py DNA replication in mouse cells and this was dependent on the presence of its binding site near ori-core (Figure 1C, lanes 11 and 13). (Lanes 12–15 were exposed for five times longer than lanes 1–11.) Comparison of the ability of the 17-mer ori-core to replicate in the absence and presence of GAL4 revealed that GAL4 activated DNA replication by a factor of at least 10. The limited extent of replication of the 17-mer ori-core reporter in the absence of GAL4 in this experiment precluded accurate measurement of GAL4's capacity to stimulate DNA replication. The differential replicative capacity of the various ori-core constructs probably reflects the relative activity in FOP cells of the factors which interact with the auxiliary

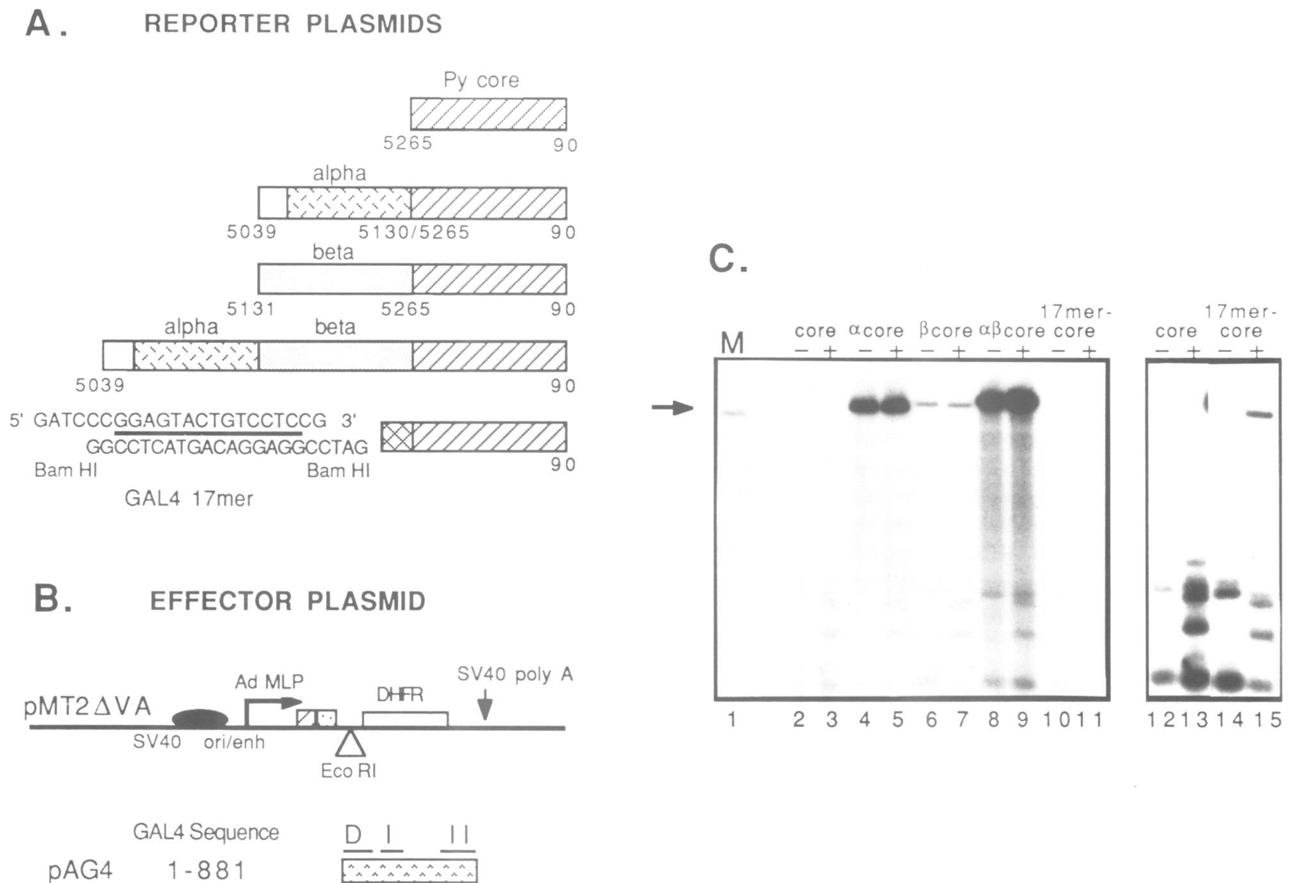


Fig. 1. Activation of Py DNA replication by GAL4 in FOP cells. (**A**) Structure of various configurations of the Py ori that were cloned in the reporter plasmids. DNA fragments bearing the Py ori-core (nucleotides 5265–90) by itself or fused to either one or both Py enhancer elements, α (nucleotides 5039–5130) and β (nucleotides 5131–5265), or to the GAL4 binding site (17-mer) were cloned in the same orientation in pML-2 between its *Hind*III and *Bam*HI sites. The sequence of the 17-mer oligonucleotide is shown; the underlined region corresponds to the GAL4 binding site. The oligonucleotide is represented by the cross-hatched box next to the Py ori-core. In each case the auxiliary elements were juxtaposed to the late border (nucleotide 5265) of the Py cori-core. (**B**) Structure of the effector plasmid capable of expressing GAL4 in mammalian cells. Expression of GAL4 is driven by the adenovirus major late promoter (Ad MLP) and the SV40 enhancer (Kakidani and Ptashne, 1988). Other regulatory sequences within the plasmid include the SV40 replication origin (filled circle), the adenovirus late mRNA tripartite leader (hatched box), splice donor and acceptor sequences from an immunoglobulin heavy chain gene (stippled box), and the dihydrofolate reductase (DHFR) gene (open rectangle). The sequences that encode GAL4 were cloned into the *Eco*RI site of the effector plasmid to derive pAG4. D denotes the DNA binding domain (residues 1–74); I and II represent the two activation domains of GAL4 (residues 148–238 and 768–881). (**C**) Analysis of the extent of replication of the various reporter plasmids in the absence or presence of GAL4. DNA replication was measured by the *Dpn*I assay. The arrow marks the position of replicated DNA. The array of different sized fragments at the bottom of the autoradiogram represents DNA that did not replicate after transfection. The intensity of these bands is an approximate measure of the recovery of effector and to a lesser extent reporter DNA from the transfected cells. M represents a marker of 10^{-5} μg of linear 17-mer–ori-core DNA (lane 1). The name of each reporter plasmid is designated above the corresponding lane. The '+' and '–' refer to the presence or absence of exogenous GAL4 provided by the effector plasmid. The membrane representing lanes 1–11 was exposed to film for one-fifth as long as that representing lanes 12–15. Lanes 12 and 13 represent longer exposures of lanes 2 and 3, whereas lanes 14 and 15 represent longer exposures of lanes 10 and 11. All lanes were originally part of the same autoradiogram, which was cropped to remove those lanes unimportant to the conclusions drawn from this experiment.

sequences, and it may be influenced by the number and arrangement of factor binding sites within this region (see below).

Transcriptional activation domains are required for DNA replication

To determine whether the activation domains of GAL4 were required for replication activation we made use of two ori-core reporter plasmids, one bearing the 17-mer and one lacking this sequence (Figure 2A), and five effector plasmids, which encode activator proteins whose structures are shown in Figure 2B. The effectors were capable of encoding the entire GAL4 protein (pAG4); the first 147 amino acids of GAL4 (pAG147), which contains the DNA binding domain but not the two principal activation regions; and two versions of GAL4, which contain one (pAG236) or both (pAG242) activation regions of GAL4 as well as the DNA binding domain (Kakidani and Ptashne, 1988). Because the GAL4 protein composed of the first 147 amino acids was shown to harbour a cryptic activation domain between residues 74 and 147, which was demonstrable in *in vitro* transcription assays, we also employed another effector plasmid, pSGλ, which encodes only the first 73 amino acids of GAL4 (the DNA binding domain) fused to the dimerization domain of the bacteriophage λ repressor (Lin *et al.*, 1988). These effectors were cotransfected separately with the reporter plasmids shown in Figure 2A.

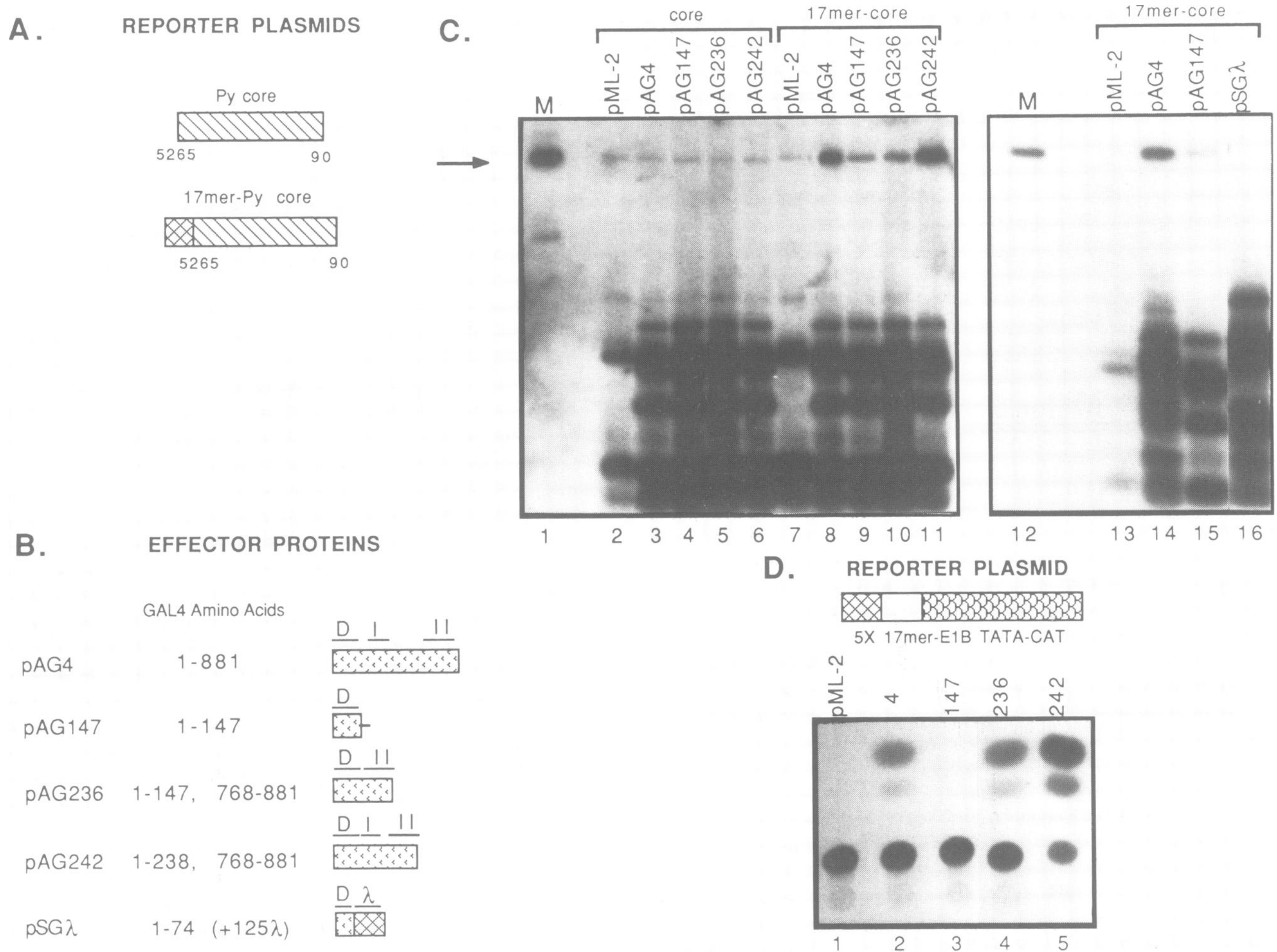


Fig. 2. Transcriptional activation domains are required to activate Py DNA replication. (**A**) Structure of reporter plasmids. Two reporter plasmids bearing the Py ori-core, or ori-core linked to the 17-mer GAL4 binding site (represented by the cross-hatched box) were cotransfected individually with expression plasmids that encode each of the effector proteins shown in panel **B**. (**B**) Structure of effector proteins comprising the DNA binding domain of GAL4 and various GAL4 activation domains. The names of the plasmids that encode the effector proteins appear to the left of the GAL4 derivative (Kakidani and Ptashne, 1988; Lin *et al.*, 1988; Carey *et al.*, 1989). The GAL4 residues found in each of the effector proteins are indicated. pSGλ comprises GAL4 amino acids 1–74 and 125 residues bearing a dimerization domain from bacteriophage λ. The box diagrams to the right depict the approximate relative size and composition of each effector protein. D represents the DNA binding domain; I and II depict the transcriptional activation domains of GAL4; (**C**) Activation of Py DNA replication by GAL4 derivatives. The arrow shows the position of replicated reporter DNA. The reporters (core and 17-mer–core) were individually cotransfected with each of the effector plasmids indicated in panel B. Lanes 1 and 12 each contained 10^{-5} μg of Py ori-core reporter DNA, which was linearized by cleavage with *Bam*HI, as a marker. Undigested circular DNA and supercoiled DNA appear as minor species above and below the major species of linear DNA in lane 1. The name of each effector plasmid cotransfected with the indicated reporter is shown above each lane. Lanes denoted pML-2 were cotransfected with this plasmid DNA as a control. Lanes 12–16 are from a separate autoradiogram and hence include some of the same controls represented in lanes 1–11. (**D**) Analysis of CAT expression effected by GAL4 and its derivatives in FOP cells. FOP cells were cotransfected with a reporter bearing five GAL4 binding sites (5 × 17-mer) and the Ad5 E1B TATA box located usptream of the CAT gene, and the effector plasmids shown in panel B. The effector plasmids cotransfected with the reporter plasmid were as follows: lane 1, pML-2; lane 2, pAG4; lane 3, pAG147, lane 4, pAG236; and lane 5, pAG242. The extent of conversion of chloramphenicol to its acetylated derivatives was measured as described in materials and methods.

The results of this analysis are shown in Figure 2C. The reporter lacking the 17-mer GAL4 binding site replicated very poorly in the absence or presence of the various effectors (Figure 2C, lanes 2–6). Hence, like GAL4, each of its derivatives failed to activate DNA replication in the absence of a binding site in the reporter plasmid. Cotransfection of the reporter carrying a GAL4 binding site with the various effectors yielded the results shown in Figure 2C, lanes 8–11. Each of the effectors that encode GAL4 proteins with activation domains functioned to augment Py DNA replication, albeit to different extents (lanes 8–11). The effector encoding the first 147 amino acids of GAL4 (pAG147) activated Py DNA replication poorly, but reproducibly (compare lane 7 with lane 9). In several independent repetitions of this experiment this effector was on average ~10–15% as active as the wild type activator (pAG4), whereas the effectors encoding truncated GAL4 species carrying one (pAG236), or both activation regions (pAG242) were ~50% and 110% as active as wild type. Cotransfer of pML-2 DNA or an effector lacking the coding sequences for GAL4, but otherwise isogenic with pAG4, failed to activate Py DNA replication (Figure 2C, compare lanes 2 and 7; data not shown).

To determine whether the activity of the GAL4 1–147 protein in activating Py DNA replication was due to its cryptic activation domain we compared the relative activity

of pAG4 and pAG147 with pSGλ, which lacks all known activation regions of GAL4 including the cryptic activation domain. Because the deletion of the cryptic activation domain of GAL4 (amino acids 74 – 147) removes a domain required for dimerization, the dimerization domain of the phage λ repressor was substituted for that of GAL4. The results are shown in Figure 2C, lanes 12 – 16. The wild type GAL4 derivative activated Py DNA replication efficiently (lane 14), whereas the 1 – 147 protein functioned poorly (lane 15), and the GAL4 fusion protein lacking all known activation surfaces failed to activate Py DNA replication (lane 16). Increasing the amount of the pSGλ effector cotransfected with the reporter did not change the outcome of the experiment. The inability of the pSGλ encoded protein to activate Py DNA replication was probably not due to its failure to bind to its sites in the reporter plasmid because Lin *et al.* (1988) have reported that like the 1 – 147 version of GAL4 (Keegan *et al.*, 1986), the protein encoded by pSGλ binds to the 17-mer as well if not better than does wild type GAL4 and nuclear extracts from mammalian cells do not affect the binding of either version of GAL4 to its sites in DNA (Lin *et al.*, 1988). Moreover, we have observed that co-transfection of increasing quantities of pSGλ with a fixed amount of pAG4 and the reporter bearing the 17-mer reduced and ultimately blocked replication of the reporter (data not shown). Presumably overexpression of the pSGλ encoded protein resulted in the preferential binding of this inactive molecule rather than GAL4 to the reporter. Therefore, these experiments suggest that efficient GAL4-mediated replication of Py DNA requires the activation domains of the protein.

To determine whether GAL4 and its derivatives were expressed to the same extent after transfection of the reporters, we analysed the abundance of GAL4-related proteins after transfection of COS-1 cells with the various effector plasmids by immunoprecipitation of radiolabelled cell lysates with an antibody directed against GAL4. Each of the GAL4 proteins, except the 1 – 147 protein, was expressed to about the same extent (data not shown). The GAL4 protein composed of the first 147 amino acids (and four terminal amino acids encoded by linker DNA in pAG147) was 5- to 10-fold more abundant than any of the others. Results similar to these have been reported earlier (Kakidani and Ptashne, 1988; see also Lillie and Green, 1989).

To ensure that the transcriptional phenotype of GAL4 and its derivatives as assayed in Chinese hamster ovary cells (Kakidani and Ptashne, 1988) and human HeLa cells (Webster *et al.*, 1988) was the same in mouse FOP cells, we measured the capacity of the GAL4 activator proteins to enhance transcription in mouse FOP cells of a reporter CAT plasmid, whose expression is governed by the adenovirus E1B promoter and five repeats of the GAl4 binding site (Figure 2D). The results of this experiment are shown in Figure 2D. In agreement with previous data, we observed that the reporter plasmid was relatively inactive in the absence of GAL4 (lane 1). As expected the wild type GAL4 protein (lane 2) and the deleted versions bearing one (lane 4) or both (lane 5) activation domains enhanced transcription, whereas the GAL4 derivative composed of the first 147 amino acids was inactive (lane 3). Therefore, it appears that the same activation domains are required for transcription enhancement in different mammalian cell lines. Furthermore, comparison of the transcriptional and replicational activation potential of the various GAL4 derivatives revealed that they were roughly equivalent (compare panel C with D). The fact that DNA replication was more responsive to the cryptic activation domain of GAL4 than was transcription we believe reflects a difference in sensitivity between the two processes. DNA replication may be more sensitive to activation domains than is transcription. This contention is supported by the observation that a single GAL4 binding site is sufficient to activate DNA replication (Figure 1) whereas a minimum of three sites are required to activate transcription (Carey *et al.*, 1990).

GAL4 hybrid proteins activate Py DNA replication

To determine whether the activation domains of other transcriptional activators could also stimulate Py DNA replication, we measured the capacity of several GAL4 hybrid proteins to function in this regard. Each of these hybrid proteins had been shown previously to enhance transcription in mammalian cells (Kakidani and Ptashne, 1988; Sadowski *et al.*, 1988; K.Martin, J.Lillie and M.Green, manuscript submitted). We independently transfected the reporter plasmid bearing a single 17-mer and a control lacking this sequence (Figure 3A) with effector plasmids (Figure 3B) that express wild type GAL4, or the DNA binding domain of GAL4 (amino acids 147) fused to: the first 661 amino acids of the pseudorabies virus immediate early gene protein (GAL4 D-PRVIE); and the 88 amino acid activation domain of the herpesvirus VP16 or Vmw65 protein (GAL4 D-VP16). In addition, we included two control effector plasmids. One of these, pAG147, is isogenic with pAG4 and expresses the first 147 amino acids of GAL4 tagged with four extra amino acids at its carboxy terminus encoded by a linker sequence, and the other, GAL4 D, is isogenic with the effector plasmids that express the hybrid proteins and encodes GAL4 amino acids 1 – 147 only. The results are shown in Figure 3C. Cotransfection of a reporter carrying only the Py core element (lane 2) or one bearing the 17-mer linked to the core (lane 3) with an inert reporter, pML-2, did not result in significant levels of replication of the ori-bearing construct. However, replication of the 17-mer ori-core construct was boosted significantly by effector plasmids that encoded the native GAL4 protein (lane 4), the GAL4 – pseudorabies virus immediate early hybrid protein (lane 5), and the herpes virus VP16 hybrid protein (lane 6). The two 1 – 147 versions of GAL4 activated replication very poorly or not at all (compare lane 3 with lanes 7 and 8). None of the activator proteins effected the replication of the control reporter plasmid lacking the 17-mer auxiliary element implying that these hybrid activators, like GAL4, must bind to the reporter to effect DNA replication activation (data not shown).

To ensure that the hybrid proteins were active in transcription, we tested their capacity to activate expression of the CAT gene from an appropriate reporter in the same cell line in which we had measured Py DNA replication (Figure 3D). The wild type GAL4 protein (lane 2), the pseudorabies hybrid protein (Tlane 4), and the herpes VP16 hybrid protein (lane 5), all activated transcription in FOP cells, whereas the GAL4 1 – 147 derivatives did not (lanes 1 and 3).

The capacity of promoter and enhancer elements to activate DNA replication is augmented by reiteration of the element (Veldman *et al.*, 1985; Muller *et al.*, 1988). To determine

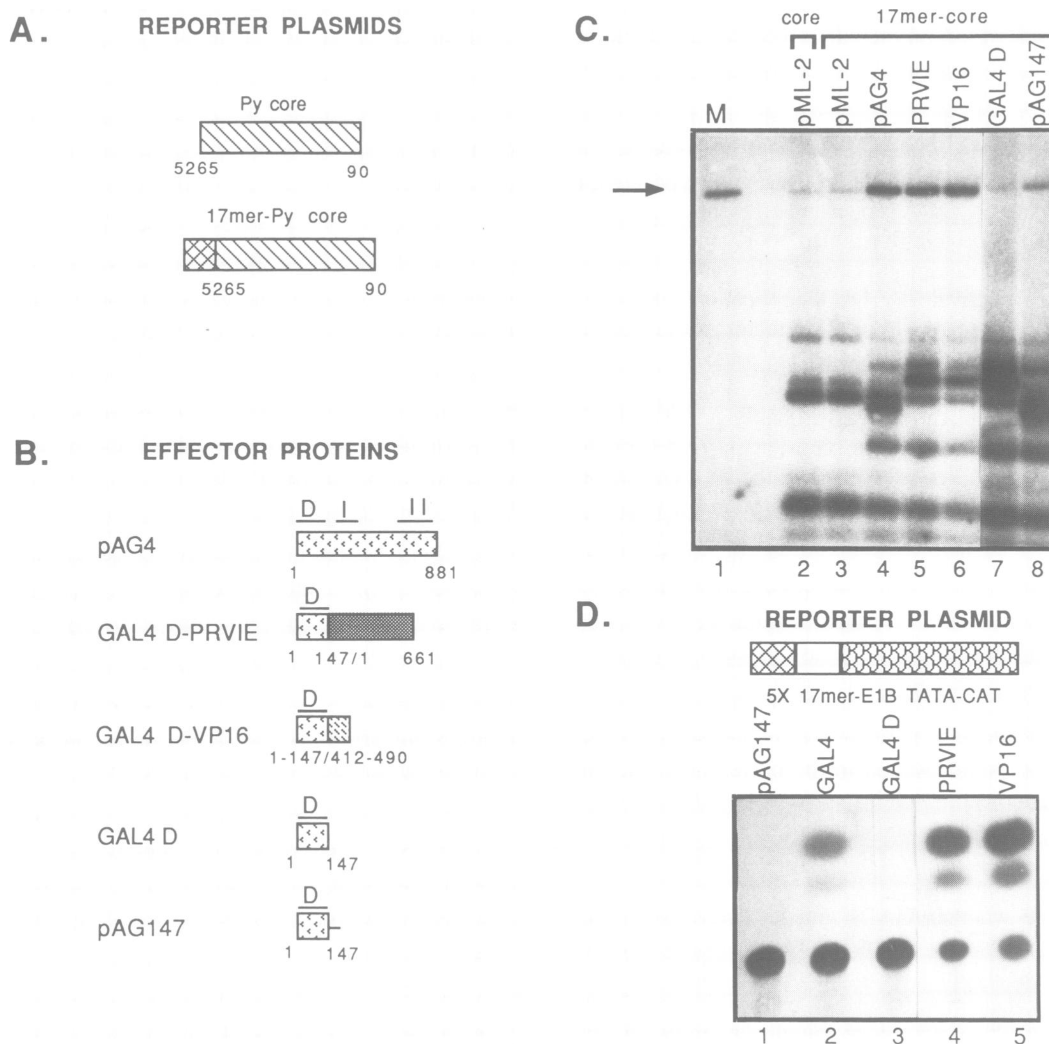
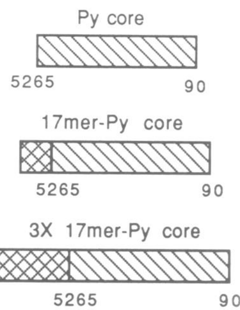


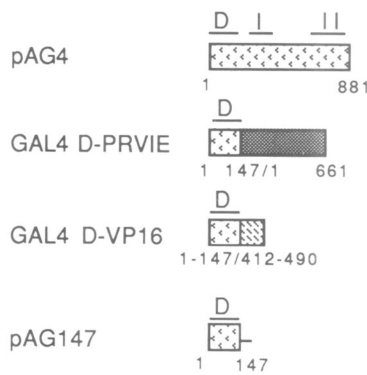
Fig. 3. Activation domains of herpesvirus transcriptional activators stimulate DNA replication and transcription in FOP cells. (**A**) Structure of the reporter plasmids bearing the Py ori-core only or one with a GAL4 binding site (17-mer). (**B**) Structure of effector proteins composed of the GAL4 DNA binding domain and the activation domains of herpesvirus transactivators of transcription. GAL4 residues 1−147, bearing the DNA binding domain(D) were joined to either PRVIE residues 1−661 (GAL4 D-PRVIE), or VP16 residues 412−490 (GAL4 D-VP16). Expression of GAL4D, GAL4D-RPVIE, and GAL4 D-VP16 was effected by the SV40 early promoter and enhancer, whereas expression of GAL4 and GAL4 1−147 (pAG147) was driven by the adenovirus major late promoter and the SV40 enhancer. The plasmids encoding the hybrid activators and GAL4 D are isogenic with the exception of their coding sequences. The same is true of those plasmids that encode GAL4 (pAG4) and GAL4 1−147). (**C**) Analysis of the capacity of GAL4 hybrid activators to stimulate Py DNA replication in FOP cells. The reporter plasmid was cotransfected with each of the effector plasmids as designated above each lane. All the other symbols and designations are the same as those in the legend to Figure 2. (**D**) Analysis of the capacity of the GAL4 hybrid activators to stimulate CAT gene expression in FOP cells. The structure of the reporter plasmid is shown; its promoter comprises five GAL4 binding sites (5 × 17-mer) and the Ad 5 E1B TATA box. The reporter plasmid was cotransfected with each of the effector plasmids marked above each lane as described in Materials and methods.

whether this was true of the 17-mer, we constructed a reporter plasmid bearing three copies of the 17-mer and compared its replicative capacity with that of a reporter carrying one copy of the GAL4 binding site (Figure 4A) after cotransfection with several effector plasmids (Figure 4B). The results are shown in Figure 4C. The native GAL4 protein and both hybrid proteins activated Py DNA replication (Figure 4C, lanes 4−6), and the reporter bearing three copies of the 17-mer replicated much better than that bearing a single site when cotransfected with these same activators (Figure 4C lanes 9−11). Several repetitions of this experiment showed that three copies of the 17-mer activated DNA replication from 6- to 8-fold better than a single copy did (data not shown). The GAL4 1−147 protein activated very poorly (lanes 8 and 14). The effect of three GAL4 binding sites on DNA replication was more than twice as great as expected if their activity was additive, suggesting that GAL4 and the hybrid proteins acted synergistically to activate DNA replication.

Transcription and DNA replication activation domains are coincident

Analysis of the capacity of GAL4 and its deleted versions to activate Py DNA replication revealed a correspondence between the presence of transcription activation domains and DNA replication activity. Proteins possessing transcription activation domains activated DNA replication, whereas those lacking these domains failed to activate DNA replication.





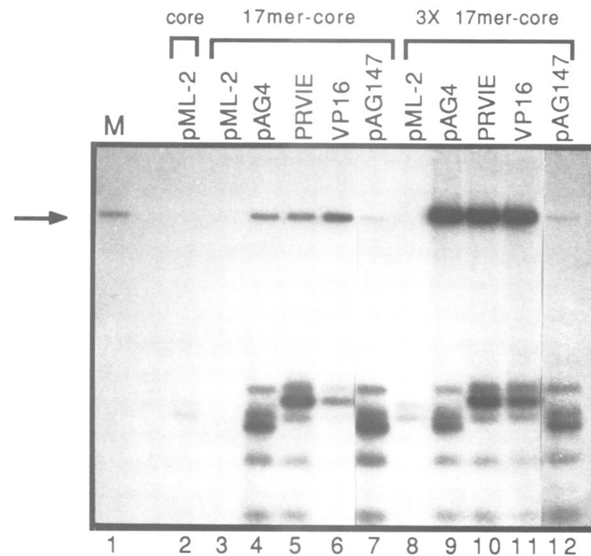


Fig. 4. Multiple GAL4 binding sites activate Py DNA replication synergistically. (**A**) Structure of reporter plasmids bearing zero, one, or three GAL4 binding sites juxtaposed to the late ori-core border. The 17-mer is represented by the cross-hatched box joined to the late border of ori-core. (**B**) Structure of effector proteins. The symbols and designations are the same as those used in the legend to Figure 3. (**C**) Analysis of replication of the various reporter plasmids after cotransfection with effector plasmids that encode each of the proteins shown in panel B. DNA replication was measured as described in Materials and methods.

However, large regions of GAL4 were removed to derive the mutants, and few mutants were analysed. Hence the formal possibility remained that the deletions adventitiously removed two domains, one required for DNA replication and another required for transcription activation. Therefore we sought independent confirmation of the requirement for transcriptional activation domains for Py DNA replication. To this end we used the reporter plasmids described previously (Figure 5A and D) and employed a set of unidirectional, carboxy-terminal deletion mutants of the GAL4-PRVIE hybrid protein (K.Martin, J.Lillie and M.Green, manuscript submitted; Figure 5B), and measured their capacity to activate transcription and DNA replication in the same mouse cell line (Figure 5C and D).

As observed earlier, reporter plasmids carrying the Py ori-core or the 17-mer linked to ori-core replicated very poorly in the absence of an activator (panel C, lanes 2 and 3). Provision of GAL4 or the hybrid activator GAL4-PRVIE in *trans* from the effectors (panel B) resulted in efficient replication of the reporter bearing the 17-mer and ori-core sequences (panel C, lanes 4 and 5). The four carboxy-terminal truncated derivatives of the hybrid protein activated Py DNA replication to differing extents (panel C, lanes 6–9). Removal of 81 amino acids from the carboxy terminus of the hybrid protein only marginally reduced its activity (compare lanes 5 and 6). However, deletion of another 64 amino acids reduced the activity of the hybrid protein to ~20% of the level of the wild type control (compare lanes 5 and 7). Therefore, amino acids between residues 516 and 580 in the PRVIE protein are required for Py DNA replication activation. Deletion of additional carboxy-terminal amino acids further impaired the capacity of the hybrid activator to stimulate DNA replication to the level of ~7–10% of the control (compare lanes 7 and 8). A slight improvement (to 15% of the control) in activation potential was achieved by removal of additional amino acids (compare lanes 8 and 9). It is noteworthy that the most truncated mutant (lane 9) was more active than the GAL4 DNA binding domain encoded by the GAL4 D effector (lane 10). The GAL4 D effector is isogenic with those effectors that encode the hybrid proteins. This suggests that there are other activation regions in the PRVIE protein between residues 1 and 421.

The wild type hybrid activator and each of its mutants were also tested for their capacity to enhance transcription of the CAT reporter, whose structure is shown in panel D. The wild type protein and the Δ3 mutant efficiently activated transcription of the reporter (lanes 2 and 3). By contrast, each of the other mutants was debilitated in transcriptional activity to essentially the same extent (20–30% of the wild

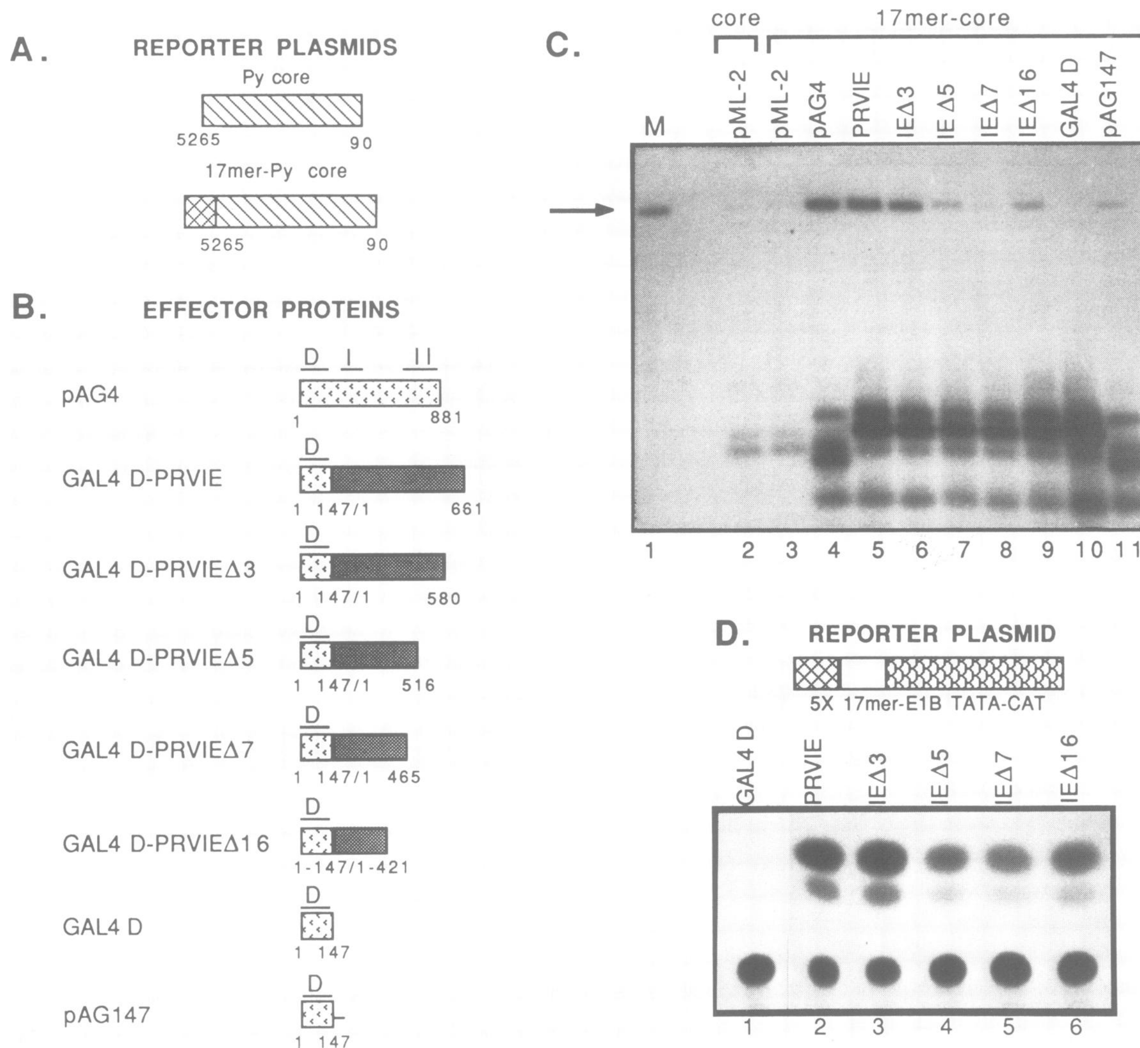


Fig. 5. Transcriptional activation domains and replication activation domains are affected similarly by mutation. (**A**) Structure of the reporter plasmids carrying the Py ori-core and the ori-core with a single GAL4 binding site. (**B**) Structure of the effector proteins encoded by the various effector plasmids. Those expression vectors that encoded GAL4 1–881 and GAL4 1–147 are isogenic, and those that encode the hybrid proteins and GAL4 D are isogenic. GAL4 1–147 differs from GAL4 D for four carboxy-terminal amino acids encoded by linker DNA. The symbols are the same as those used in the legend to Figure 2. (**C**) Analysis of the replication of the reporters after cotransfection with each of the effector plasmids encoding the proteins shown in panel B. DNA replication was measured as described in Materials and methods. (**D**) Analysis of *CAT* gene expression effected by the proteins described in panel B. The reporter plasmid bearing the *CAT* gene and accompanying promoter made up of five copies of the GAL4 binding site (17-mer) and the E1B promoter was cotransfected with effector plasmids that encoded GAL4 D, the GAL4 D-PRVIE full length protein, and each of its carboxy-terminal deletion derivatives. *CAT* gene expression was assessed as described in Materials and methods.

type control) (lanes 4–6). As was the case for DNA replication activation, the drop in transcriptional activity of the hybrid activator corresponded to the deletion of 64 amino acids between residues 580 and 516 in the PRVIE protein (compare lanes 6 and 7 in panel C with lanes 3 and 4 in panel D). Moreover, as in its activity in DNA replication activation, the largest deletion mutant, Δ16, was more active than Δ7, which carries a smaller deletion. Furthermore, Δ16 retained 20–30% of the activity of the wild type hybrid protein, implying that this activator possesses another transcriptional activation domain elsewhere in the protein. Similar observations have been made by Martin *et al.* (K.Martin, J.Lillie, and M.Green, manuscript submitted) who constructed these mutants and measured their transcriptional activity in Chinese hamster ovary cells. These data show that mutations which affect transcriptional activation also affect DNA replication activation, implying that the same function of the activator mediates both processes.

Discussion

Transcriptional activators stimulate DNA replication

We have shown that GAL4 and GAL4 hybrid activators of transcription activated Py DNA replication in mouse cells that express Py large T antigen. This represents the first direct demonstration that transcriptional activators are capable of activating Py DNA replication *in vivo*. Replication activation required a binding site for GAL4 near ori-core, implying that DNA binding is necessary to effect replication. GAL4 activated DNA replication at least 10-fold over basal levels if a single copy of its binding site was present in the

reporter plasmid. The replicative capacity of such reporters could be boosted another 6- to 8-fold, if the number of GAL4 binding sites was increased to three. Whereas we have not rigorously investigated the quantitative relationship between the number of GAL4 binding sites and the extent of replication of the plasmids carrying them, the fact that three sites functioned >6 times better than one site suggests that activation by Py DNA replication was synergistic. Reporter plasmids with three GAL4 binding sites replicated as efficiently as plasmids bearing the Py β enhancer element, which probably naturally activates Py DNA replication in the context of the viral genome (Hassell *et al.*, 1986). Therefore, GAL4 is a very effective activator of Py DNA replication.

The ability of GAL4 and its derivatives to activate Py DNA replication was dependent on the presence of an activation domain in the activator protein. The complete GAL4 protein activated Py DNA replication but deletion mutants of GAL4, which possessed its DNA binding domain but lacked activation regions defined in mammalian cells, failed to activate Py DNA replication. Similarly, fusion proteins between the DNA binding domain of GAL4 and the activation domains of other transcriptional regulatory proteins also activated Py DNA replication. Finally, analysis of the ability of a set of carboxy-terminal deletion mutants of a GAL4–PRVIE fusion protein to augment transcription and DNA replication in the same cell line revealed that both processes were affected similarly by the mutations. Because the mutations affected the activation potential of the proteins these findings suggest that transcriptional and replicational activation domains are synonymous.

The source of the activation domain was unimportant for replication activation. GAL4 activation domains as well as those of VP16 and the pseudorabies virus immediate early gene product functioned well to activate Py DNA replication. Both GAL4 and VP16 contain acidic activation surfaces. The nature of the activation domain of the pseudorabies virus protein is not known. However, the region is this hybrid protein whose deletion leads to a reduction in activation potential for both transcription and DNA replication is not rich in acidic amino acids or α helical structure (Vlcek *et al.*, 1988).

The binding sites for mammalian transactivators such as Spl and CTF/NF-1 are also able to activate Py DNA replication (C.Coulber and J.A.Hassell, manuscript in preparation). These activators contain glutamine-rich and proline-rich activation domains, respectively (Courey and Tjian, 1988; Santoro *et al.*, 1988). Therefore, DNA replication activation may be mediated by the same repertoire of activation domains as is transcription.

The requirement for activation domains to effect DNA replication appears to be contradicted by the observations that the 1–147 GAL4 derivative completely failed to enhance transcription but activated Py DNA replication, albeit very poorly. Our data suggest that the cryptic activation domain between amino acids 74 and 147, which augments transcription *in vitro*, also functions to activate Py DNA replication *in vivo* because the GAL4 derivative containing the DNA binding domain and the λ repressor dimerization domain failed to activate DNA replication. We suspect that DNA replication is more sensitive to activation domains than is transcription. This contention is bolstered by the observation that in general fewer factor binding sites, and by inference fewer activation domains, are required to augment Py DNA replication than are required to effect transcription activation (Veldman *et al.*, 1985; Muller *et al.*, 1988). However, more rigorously controlled experiments with weak and strong activators will have to be performed to validate this explanation. It is curious that increasing the number of 17-mers from one to three did not increase the replicative potential of the reporter when the 1–147 GAL4 derivative was provided in *trans*. Our expectation, confirmed recently by Carey *et al.* (1990), was that the activation potential of a weak activator would be increased by reiteration of its binding site. Perhaps more than three copies of the 17-mer are required to manifest this effect of GAL4 1–147.

Comparison with other viruses

Replication of adenovirus DNA, which occurs by a protein priming mechanism, also requires the participation of transcriptional activators (Challberg and Kelly, 1989. Stillman, 1989). Both CTF/NF-1 and Oct-1/NF-III are required for efficient adenovirus DNA replication *in vitro* (Jones *et al.*, 1985, 1987; Santoro *et al.*, 1988; Pruijn *et al.*, 1986; Rosenfeld *et al.*, 1987). However, in this system the activation domains of each activator can be individually deleted without affecting the initial stages (formation of the preterminal-dCMP complex) of adenovirus DNA replication *in vitro* (Mermod *et al.*, 1989; Verrijzer *et al.*, 1990). Therefore, despite a common requirement for transcriptional activators for DNA replication, it appears that the mechanism of action of these factors differs in the two viruses. We anticipate that studies with other systems will reveal the diversity of mechanisms that underlie the activation of DNA replication by transcriptional activators.

Mechanism of DNA replication activation

Large T antigen catalyses the initiation of papovavirus DNA replication by binding to sequences within ori-core and through its intrinsic helicase activity it unwinds and untwists sequences adjacent to its recognition sites (see Borowiec *et al.*, 1990 for references). It is thought that this facilitates access of the DNA polymerase α-primase complex to the template and results in the synthesis of RNA primers required for DNA replication. Large T antigen also binds to the DNA polymerase α-primase complex and presumably thereby recruits the polymerase to its site of action. We presume that transcriptional activators affect one or more of these presynthesis reactions because their requirement for DNA replication is not absolute, even in the case of Py. Consequently, in thinking about the likely mechanisms whereby they act, we imagine that activators facilitate a reaction that can occur in their absence.

The binding of transcriptional activators near ori could activate DNA replication by promoting or stabilizing the binding of large T antigen or components of the replication machinery to the ori-core. This possibility is supported by the observation that Py DNA replication is more dependent on transcription regulatory sequences than is SV40 DNA replication, and correspondingly the affinity of Py large T antigen for its binding sites in ori is much less than that of SV40 large T antigen for its ori-core. This hypothesis predicts that transcriptional activators interact either directly or indirectly with the large T antigens of papovaviruses thereby stabilizing the binding of the latter to ori. While there

is evidence that the activator Ap-2 binds directly to SV40 large T antigen (Mitchell *et al.*, 1987) there are no other examples which support the idea that large T antigens or any of the factors required for DNA replication are able to associate directly with the activators that are thought to activate papovavirus DNA replication. Consequently, if the binding of factors such as large T antigen or other components of the replication apparatus to ori-core is affected by transcriptional activators, then it seems more likely that this occurs indirectly by interaction with a common target.

Another means whereby activators could enhance DNA replication would be to effect changes in the structure of ori DNA that facilitated the binding or action of the replicational apparatus, including large T antigen. For example, activators may directly, or more likely indirectly, unwind DNA near ori-core and thereby facilitate the initiation of DNA replication. Alternatively, activators could function by displacing or antagonizing the binding of repressors of DNA replication such as histones to ori-core, which would otherwise block the interaction of T antigen or other replication factors with the ori-core. The latter possibility is supported by the recent observation that the replication of SV40 DNA *in vitro* is inhibited by assembly of the template into chromatin, and this inhibition can be relieved by prior binding of CTF/NF-1, a transcriptional activator, to its cognate site near ori-core (Cheng and Kelly, 1989). Whatever the mechanisms of DNA replication activation, our results indicate that the activation domains of transcriptional activators play an important role in this process.

The molecular mechanism of transcription activation and the role of activation domains have not been clearly delineated. It has been suggested that activation surfaces are domains for interaction with the general transcription machinery of the cell (Ptashne, 1988). Indeed there is evidence that the activation domains of some activators interact directly with TFIID, a general transcription factor which binds to TATA boxes (Sawadogo and Roeder, 1985; Abmayr *et al.*, 1988. Horikoshi *et al.*, 1988a and b; Stringer *et al.*, 1990). There is also data suggesting that transcriptional activators bind to RNA polymerase II (Brandl and Struhl, 1989). However, not all activation regions may contact TFIID or RNA polymerase directly (reviewed by Ptashne and Gann, 1990). For example, the glutamine-rich activation domain of Sp1 (Pugh and Tjian, 1990) and the activation domain of VP16 (Berger *et al.*, 1990. Kelleher *et al.*, 1990) appear to interact with an adaptor molecule or co-activator, which bridges interactions between regulatory factors bound to upstream elements and general transcriptional factors, like TFIID, bound to the TATA box.

If TFIID is a common target of activation domains, then this factor may be involved in papovavirus DNA replication. In this regard it may be noteworthy that the SV40 ori-core contains a bona fide TATA box that directs the start sites for the early region RNAs. By contrast, Py lacks a TATA box within its ori-core. Interestingly, the ori-core of SV40 functions relatively well in the absence of auxiliary transcriptional elements, whereas that of Py functions very poorly by comparison. Whereas there are several explanations for this, one possibility is that TFIID is able to bind directly to the SV40 ori, but can only interact indirectly with that of Py. Perhaps transcription activators are required to recruit TFIID to the ori-core of Py. Apparently the interaction of TFIID and activation domains of transcriptional activators can take place in the absence of TATA elements on the DNA template (Peterson *et al.*, 1990). It will be interesting to learn whether the effects of transcriptional activators in DNA replication can be reproduced *in vitro* and whether components of the general transcriptional machinery such as TFIID and RNA polymerase II are involved in this process.

Materials and methods

Mammalian cells

FOP cells were derived by cotransfection of mouse FM3A cells with an expression vector capable of effecting the expression of all three Py T antigens and pSV2neo as described previously (Muller *et al.*, 1984). After selection for cells which could grow in the presence of G418, several colonies were isolated and cloned. One of these, which expresses functional Py large T antigen, was used for the experiments described here. The cells were grown on plastic dishes with Dulbecco's modified Eagle medium (DMEM) supplemented with 10% calf serum and antibiotics and maintained at 37°C in a humidified CO_2 atmosphere.

Preparation of DNA and its modification

Recombinant plasmid DNAs were isolated from bacteria and purified by CsCl density gradient centrifugation. Reaction of these DNAs with restriction endonucleases and modifying enzymes were performed in accordance with the conditions specified by their manufacturers, or as described in Sambrook *et al.* (1989).

Construction of recombinant plasmids

All of the Py ori-core plasmids were cloned in pML-2 (Lusky and Botchan, 1981). A synthetic oligonucleotide harbouring the GAL4 17-mer recognition site was annealed to a complementary strand to yield *Bam*HI overhangs and ligated as a monomer into the unique *Bam*HI site of a plasmid carrying only the Py ori-core, pdPyVc, (Bennett *et al.*, 1989) to create 17-mer-Py core. The sequence of the oligonucleotide was 5′-GATCCCGGAGTACTG-TCCTCCGG-3′, where the underlined region corresponds to the GAL4 recognition site. (The first T in the recognition sequence was changed from a G in the authentic site to create a *Sca*I site to aid in the identification of plasmids bearing the GAL4 binding site.) Potential recombinants were identified by colony hybridization and analysed by both restriction enzyme digestion and dideoxy sequencing.

The recombinant plasmid carrying three copies of the 17-mer was made by ligating the phosphorylated oligonucleotide to itself followed by ligation to pdPyVc which had been cleaved with *Bam*HI and treated with calf alkaline phosphatase to remove the 5′ phosphate groups. Recombinants were screened and characterized as described above.

The effector plasmids pAG4, pAG147, pAG236, pAG242, pSGλ and that encoding the GAL4–VP16 fusion protein were generously provided by Mark Ptashne and Ivan Sadowski and have been described elsewhere (Kakadani and Ptashne, 1988; Carey *et al.*, 1989. Sadowski *et al.*, 1988). The GAL4-PRVIE recombinant plasmids and the deletion mutants thereof were kindly provided by Michael Green and Jim Lillie (K.Martin, J.Lillie, and M.Green, manuscript submitted). The constructs bearing only the Py core ori or the α and β auxiliary elements linked to ori-core have been described previously (Bennett *et al.*, 1989). The numbering system of Soeda *et al.* (1980) was used to describe Py DNA sequences.

Assay for DNA replication

Transfection of reporter DNA (0.2 μg) and effector DNA (2.0 μg) into FOP mouse cells were carried out as described previously (Muller *et al.*, 1988). In all the experiments reported here the effector DNA was present in quantities at least 4-fold greater than that required to maximally stimulate DNA replication. After autoradiography, the bands representing replicated DNA were quantified by laser densitometric scanning of autoradiographs exposed for various periods (2–12 h) to ensure that the band intensity was directly proportional to the number of labelled DNA molecules in each band. The replication assays were repeated at least three times with the same result.

CAT assays

Each reporter plasmid and effector plasmid (2 μg of each) was cotransfected into FOP cells as described above with some modifications. The DEAE–dextran–DNA solution was left on the cells for 2 h and maintained at 37°C. The cells were subsequently washed once with phosphate buffered

saline (PBS), and incubated with 2 ml of dimethyl sulphoxide (DMSO) (10% in PBS) for 2 min at room temperature. Cells were quickly washed twice with PBS, followed by incubation with 5 ml of DMEM containing 10% calf serum and 0.1 mM chloroquine for 2 h at 37°C. Thereafter, the cells were washed twice with DMEM and incubated with fresh medium supplemented with 10% calf serum at 37°C. 48 h post-transfection the cells were lysed by freeze-thawing three times, and CAT assays were performed as described elsewhere (Gorman *et al.*, 1982; Mueller *et al.*, 1988). Transfections were repeated a minimum of three times with two different preparations of effector and reporter DNAs.

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