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# Three Functional Classes of Transcriptional Activation Domains

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**We have studied the abilities of different transactivation domains to stimulate the initiation and elongation (postinitiation) steps of RNA polymerase II transcription in vivo. Nuclear run-on and RNase protection analyses revealed three classes of activation domains: Sp1 and CTF stimulated initiation (type I); human immunodeficiency virus type 1 Tat fused to a DNA binding domain stimulated predominantly elongation (type IIA); and VP16, p53, and E2F1 stimulated both initiation and elongation (type IIB). A quadruple point mutation of VP16 converted it from a type IIB to a type I activator. Type I and type IIA activators synergized with one another but not with type IIB activators. This observation implies that synergy can result from the concerted action of factors stimulating two different steps in transcription: initiation and elongation. The functional differences between activators may be explained by the different contacts they make with general transcription factors. In support of this idea, we found a correlation between the abilities of activators, including Tat, to stimulate elongation and their abilities to bind TFIIH.**

Stimulation of eukaryotic gene expression requires sequence-specific factors with DNA binding domains and activation domains that interact with the general transcription factors (GTFs) and recruit RNA polymerase II (pol II) to the promoter (3, 65). In vivo the transcriptionally active form of pol II is probably a holoenzyme complex which contains a number of the GTFs as well as other polypeptides (36). Different activation domains interact with different GTFs, including TFIIB (44), TFIID (17, 19, 63), and TFIIF (76). These interactions are thought to recruit, stabilize, and/or modify the activity of the pol II holoenzyme.

We recently demonstrated that the activation domains of p53, VP16, and E2F1 bind directly to TFIIH (50a, 72). TFIIH is a multisubunit factor, different forms of which are required for both transcription and nucleotide excision repair of DNA (9). TFIIH is the only GTF which has enzymatic activities: it has two helicase subunits and a cyclin-dependent protein kinase subunit which phosphorylates the pol II large-subunit C-terminal domain (CTD) (12, 52, 58, 60). Both of these enzymatic activities are implicated in steps in transcription which occur shortly after initiation of the RNA chain. First, a helicase is required for efficient formation of open complexes and for promoter clearance on linear templates in vitro (20). Second, when paused polymerases resume elongation on several *Drosophila* genes in vivo, the CTD becomes phosphorylated, suggesting a possible role for TFIIH kinase in regulating elongation (50, 70). Furthermore, inhibitors of the TFIIH kinase inhibit elongation under activated (74), but not basal (57), transcription conditions.

In vivo, rate-limiting steps after initiation have been well documented for a number of genes. For example, polymerases stall 20 to 40 bases downstream of the start sites in the *Drosophila hsp70* and human c-*myc* genes (38, 51, 64) and terminate prematurely in the human immunodeficiency virus (HIV) provirus (31, 34). Promoter-proximal pausing appears to be a general phenomenon, not restricted to a small subset of promoters (37). It is not clear how promoter-proximal pausing and premature termination of transcription in vivo relate to promoter clearance in vitro because no specific assay for promoter clearance has been developed in vivo. In this paper, we therefore use the term elongation in its broadest sense to refer to all nucleotide addition steps after initiation.

Certain activators, including HIV Tat and the Gal4-VP16 fusion protein, stimulate elongation by pol II (1, 21). Because rate-limiting steps after initiation appear to be widespread in vivo, the abilities of activators to affect elongation are of great potential importance to the control of gene expression. Stimulation of elongation, like that of initiation, may involve contacts between activation domains and GTFs. Squelching by excess VP16 in *trans* inhibited elongation, suggesting that this activation domain can sequester a factor(s) required for efficient elongation (73).

It is not known if Tat and VP16 stimulate elongation by similar mechanisms. Tat is able to stimulate elongation, in some cases without any apparent effect on initiation (31, 33, 40, 47), whereas VP16 also strongly stimulates initiation. Tat is a unique activator because it is recruited to the transcription complex by binding to nascent RNA rather than to promoter DNA. Although Tat can activate transcription when tethered to DNA (2, 29, 62), it is not known whether, under these circumstances, it affects elongation or initiation.

The binding of two or more activators to a single template often causes more-than-additive stimulation of transcription, a phenomenon called synergy. Multiple copies of a single activator, as well as two different activators, can synergize (4, 43). For example, Tat synergizes with Sp1, but not with VP16 (29, 62). In many cases, it is not known why some combinations synergize while others do not. Three mechanisms of transcriptional synergism have been identified: (i) cooperative DNA binding (18), (ii) formation of multiple contacts between activators and GTFs (4, 6, 42), and (iii) derepression of chromatin

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templates by displacement of nucleosomes (5). It has also been suggested that synergy could result from the stimulation of different rate-limiting steps in transcription (25).

In this study, we have surveyed a number of cellular and viral activation domains for their abilities to stimulate postinitiation steps in transcription. We show that a subset of cellular activation domains have the ability to stimulate elongation, while others stimulate only initiation. Only those activators which do not stimulate elongation synergize with HIV Tat. We show that a mutant of the VP16 activation domain uncouples the stimulation of elongation from that of initiation. Furthermore, we have found a correlation between those activators that stimulate elongation and those that bind to TFIIH.

## MATERIALS AND METHODS

**Plasmids.** Expression vectors for Gal4 fusion proteins were derived from pSG424 (55). Gal4(1-94) was expressed from pSG1-94, made by deleting sequences downstream of the *Hpa*I site in pSGVPΔ490 (54). Gal4(1-147) was expressed from pSG1-147 (73). Gal4-VP16(410-490) was expressed from pSGVPΔ 490. Gal4-SW6 was expressed from pSGSW6 in which the *Xho*I-*Sty*I fragment of SW6 (68) was inserted into pSGVPΔ490. Gal4-Tat(1-48) was expressed from pHKGTat1-48, made by inserting the *Xho*I-*Eco*NI fragment of Gal4-Tat (62) into pHKG4 (22). Gal4-Sp1B(263-499) was expressed from pHKGSp1 (516Δ112), a gift of B. Khoo and S. Jackson. Gal4-E2F1(380-437) was expressed from pHKGE2F(380-437) (22). Gal4-CTF(399-499) was expressed from pHKGCTF(399-499), a gift of B. Khoo and S. Jackson. Gal4-p53(1-92) was expressed from pGalMp53(1-92) (46).

pSp65-VA1 (48) contains the adenovirus type 2 VA1 gene. $pGal_5$-HIV2 CAT has been described previously (73). $pGal_1$-HIV2 CAT was made by inserting the *Xba*I-*Nco*I HIV2-CAT fragment from $pGal_5$-HIV2 CAT into Gal1 E1b CAT (22). $pGal_5$-c-myc P2 luciferase was constructed by inserting the *Pvu*II-*Sac*I fragment of $pGal_5$-P2 CAT (73) into the *Sma*I-*Sac*I sites of pGL2 (Promega). $pGal_5$-$Sp_4$-HIV2 CAT was constructed from $pGal_5$-HIV2 CAT (73) by insertion of an oligonucleotide [$(GGGGCGGGAC)_4$] into the *Xba*I site. $pGal_5$-$Sp_4$-E1b CAT was constructed by inserting the same oligonucleotide into the *Xba*I site of $pGal_5$-E1b CAT (Gal4/E1bTATA) (41).

pGX-3X-Tat was constructed by inserting a Tat gene that was PCR amplified from ptat72 (14) into the *Bam*HI site of pGEX-3X. pGX-3X-$Tat_{1-48}$ was constructed by digesting pGX-3X-Tat with *Eag*I and *Eco*RI, filling in, and recircularizing.

The GST-Sp1A and GST-Sp1B expression plasmids were described previously (11). GST-CTF was expressed from pGEX20T-CTF, which contains residues 399 to 499 of CTF. GST-VP16 was expressed from pGEX2T-VP16, made by inserting the *Eco*RI fragment from pSGVPΔ490 (54) encoding residues 410 to 490 of VP16. GST-SW6 was constructed by insertion of the *Sph*I-*Sty*I fragment of pSW6 (68) into the pGEX2T-VP16 plasmid.

**Transfection, chloramphenicol acetyltransferase (CAT) assay, nuclear run-on reaction, and RNase protection.** 293 cells were transiently transfected with calcium phosphate precipitates. HeLa cells were transfected with DEAE-dextran and then treated with dimethyl sulfoxide and chloroquine. All cells were harvested after 40 h. For Fig. 2, 293 cells on 15-cm-diameter plates were transfected with 24 μg of $pGal_5$-c-myc P2 luciferase, 200 ng of RSV-CAT, and 4 μg of various Gal4 expression plasmids. For Fig. 3, 4, and 5, 293 cells were transfected with 10 μg of $pGal_5$-HIV2 CAT, 1 μg of pSp65-VA1, 200 ng of RSV-luciferase, and 2 μg of various Gal4 expression plasmids. For Fig. 3, 4, and 5, 293 cells were transfected with 10 μg of $pGal_5$-HIV2 CAT, 1 μg of pSp65-VA1, 200 ng of RSV-luciferase, and 2 μg of various Gal4 expression plasmids. For Fig. 6 and 7, HeLa cells were transfected with 8 μg of $pGal_5$-HIV2 CAT, 1.5 μg of Gal4 expression plasmid, 1 μg of RSV-luciferase, 1.5 μg of pSV-HIV1-tat (10) or empty PECE vector, and 1 μg of pSp65-VA1 (Fig. 7). For Fig. 8 and 9, 293 cells were transfected with 5 μg of reporter, 100 ng of RSV-luciferase, 1 μg of various Gal4 expression plasmids, and 0.5 μg of pSp65-VA1 (Fig. 9).

RNA isolation (which includes a DNase step) and RNase protection assays were as described previously (26, 73). All RNase protection experiments shown were repeated at least twice in independent transfections. For quantitation of RNase protection, autoradiographs were scanned by densitometry and the areas under the curves were integrated. Correction factors for the difference in radioactive U content between the read-through (RT) band and the shorter terminated (TM) bands were 1.67 and 5 for the longer and shorter TM bands, respectively.

Nuclear run-on reactions were carried out in a final concentration of 105 mM potassium glutamate as previously described (73) except that the extension reaction was reduced from 10 to 1 min. M13 probes for HIV type 2 (HIV-2) TAR and mouse c-*myc* sequences have been described previously (73). Luciferase probes 4 and 5 in Fig. 2 are M13 clones of the *Eco*RI-*Taq*I and *Eco*RV-*Bst*YI fragments. The upstream probe G is the *Pst*I-*Bam*HI fragment containing five Gal4 binding sites from Gal4/E1bTATA (41) cloned into M13mp18. The CAT probes 2 and 3 in Fig. 3 contain the *Hin*dIII-*Pvu*II and *Eco*RI-*Sca*I fragments, respectively, in M13.

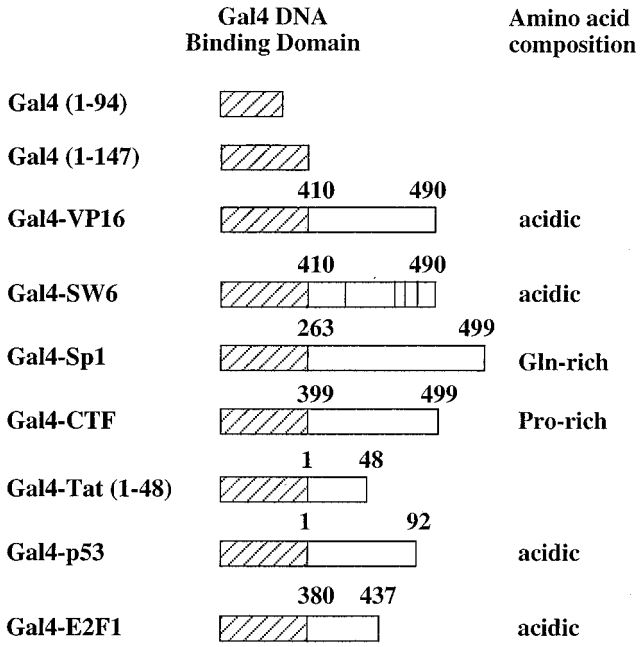


FIG. 1. Gal4 fusion proteins used as activators in this study.

CAT assays utilized the $^{14}C$-labeled acetyl coenzyme A substrate, and quantification was by scintillation counting. Each sample was assayed in duplicate.

**Protein-protein interaction assays.** For Fig. 10A, 800-μl aliquots of HeLa whole-cell extracts (61) were chromatographed on affinity columns (40 μl) containing 4 mg of glutathione *S*-transferase (GST) or GST fusion protein per ml, except for GST-Sp1A and GST-Sp1B, which were immobilized at 6 mg/ml. Bound proteins were eluted with 160 μl of 1 M NaCl in ACB buffer (72) and subsequently with 160 μl of 1% sodium dodecyl sulfate (SDS) (72).

For Fig. 10B, 10-mg samples of HeLa nuclear extracts in buffer D (59) with 50 mM KCl were loaded onto 0.6-ml columns containing immobilized GST proteins at 1 mg/ml. Proteins were eluted with buffer D supplemented with 0.6 M KCl.

Eluted samples (20 μl) were immunoblotted with anti-p62 monoclonal antibody 3c9 (13) or anti-MO15 antibody 2F8 (52) and visualized by enhanced chemiluminescence (ECL; Amersham).

## RESULTS

**Activators differ in their abilities to stimulate elongation: nuclear run-on analysis.** We measured the abilities of different activation domains to stimulate transcriptional elongation by using the nuclear run-on assay. Fusions of various activation domains with the DNA binding domain of *Saccharomyces cerevisiae* Gal4 (amino acids 1 to 147) (Fig. 1) were coexpressed in transiently transfected 293 cells with the $pGal_5$-c-myc P2 luciferase reporter plasmid (see Fig. 2). After 40 h, nuclei were harvested and allowed to continue transcription in the presence of [$\alpha$-$^{32}P$]UTP. RNA was isolated and hybridized to a series of five single-stranded M13 probes spanning the length of the reporter plasmid. The intensity of the signal on each probe gives a measure of the relative polymerase density on that region of the gene at the time the nuclei were isolated. This assay therefore gives a "snapshot" view of the distribution of polymerases along the gene averaged over the population of cells in the culture. If polymerases are distributed evenly along the length of the gene, we infer that elongation is efficient. If, on the other hand, a higher polymerase density is observed at the 5′ end than at the 3′ end, we infer that elongation is inefficient and that polymerases are stalling or terminating prematurely. Our run-on conditions (105 mM $K^+$ glutamate)

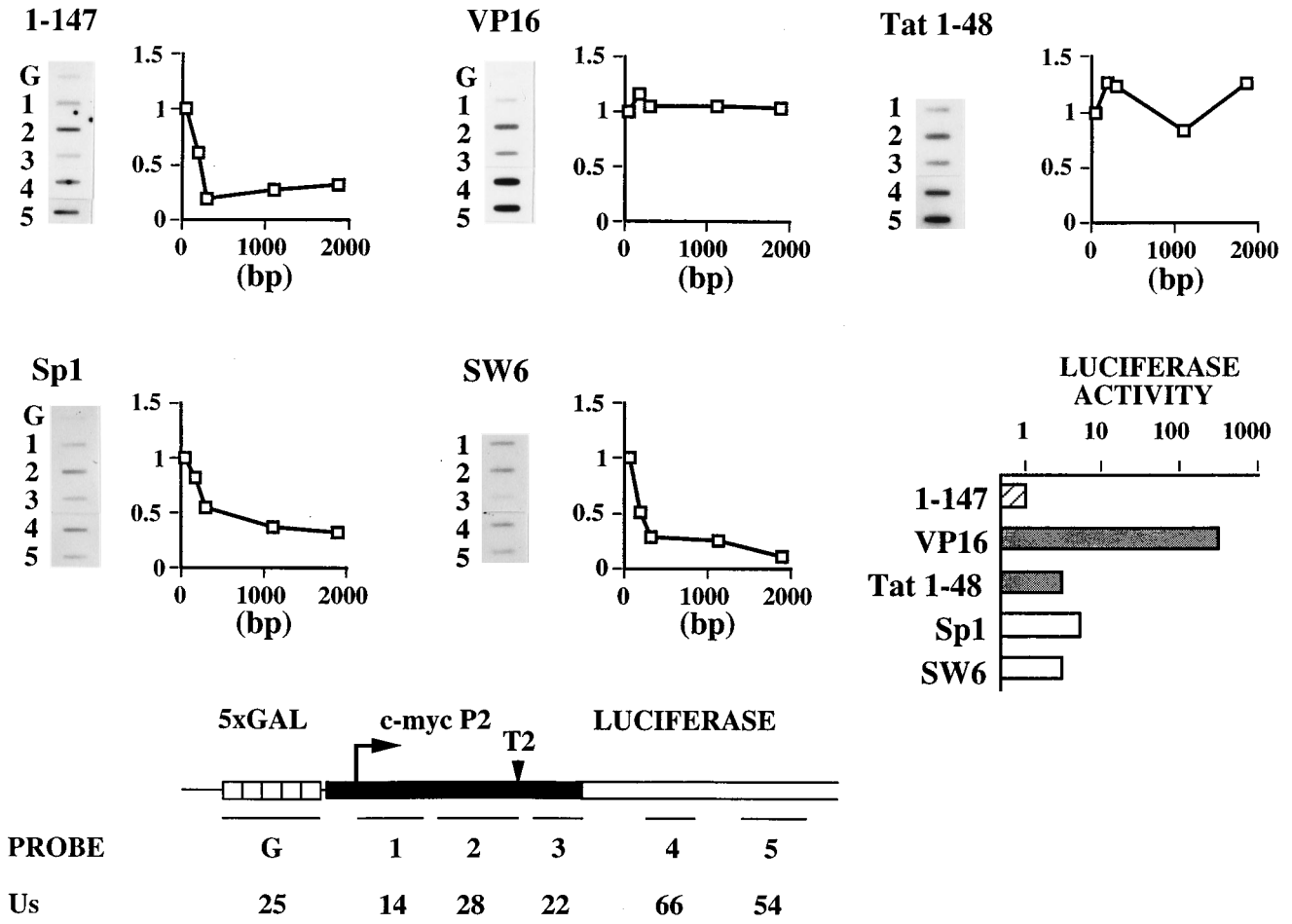


FIG. 2. Activators differ in their abilities to stimulate elongation. Nuclear run-on analysis of $pGal_5$-c-myc P2 luciferase transiently transfected into 293 cells with Gal4(1-147), -VP16, -Tat(1-48), -Sp1, or -SW6 (VP16 mutant) is shown. Autoradiograms were exposed for 7 days, 6 h, 7 days, 24 h, and 48 h, respectively. Polymerase densities were calculated from PhosphorImager data corrected for U content (see map). After correction for transfection efficiency, the relative 5′ (probe 1) polymerase densities were approximately 1, 10, 1, 2, and 2 for Gal4(1-147), -VP16, -Tat(1-48), -Sp1, and -SW6, respectively. In the graphs, polymerase density was normalized to 1.0 for probe 1 and plotted versus distance from the start site to the center of each probe. The upstream probe G is shown for Gal4(1-147), -VP16, and -Sp1. Luciferase activity is expressed relative to that for Gal4(1-147) and normalized to CAT activity from for cotransfected RSV-CAT. 5×GAL, five Gal4 binding sites.

permit the release of stalled polymerases. Because the 5′ run-on signal reflects both stalled and actively elongating polymerases, it is not an accurate measure of initiation rate but only of the density of preinitiated polymerases (see Discussion). To control for upstream initiation and transcription which reads all the way around the plasmid, in some experiments we included a probe, G, which is located upstream of the c-*myc* start site. In all cases the amount of read-around transcription detected by this probe was low relative to that which is correctly initiated (see Fig. 2 to 4).

Our results revealed two classes of activation domains: those that stimulate transcription by polymerases which elongate efficiently and those that stimulate transcription by polymerases which elongate poorly. So that elongation efficiencies can easily be compared, the autoradiographs in Fig. 2 were exposed to give approximately equal signals for the 5′ end (probe 1), and polymerase densities were plotted relative to the density at the 5′ end. After correction for transfection efficiency, the relative 5′ polymerase densities were approximately 1, 10, 1, 2, and 2 for Gal4-(1-147), -VP16, -Tat(1-48), -Sp1, and -SW6, respectively. In agreement with previous results (73), transcription in the absence of an activation domain [Gal4(1-147)] showed a decline in polymerase density between the 5′ and 3′ ends of the gene, implying that, under these conditions, transcripts were elongated poorly (Fig. 2). In contrast, transcription activated by Gal4-VP16 had equivalently high polymerase densities at the 5′ and 3′ ends of the gene, suggesting that this activator stimulates elongation, as previously shown (73).

Gal4-Tat(1-48) contains the activation domain of Tat but not its arginine-rich RNA binding domain (amino acids 49 to 74); it does not bind TAR RNA and cannot activate transcription from the HIV-2 long terminal repeat (2a). Gal4-Tat(1-48), like Gal4-VP16, stimulated elongation efficiency relative to Gal4(1-147), although it is a 100-fold-weaker activator than Gal4-VP16 as measured by luciferase expression (Fig. 2). We conclude that stimulation of elongation by a transactivator is not necessarily correlated with a high level of initiation.

Gal4-Sp1, like Gal4-Tat(1-48), is a weak activator (8), but in contrast to Gal4-Tat(1-48), it gave rise to a pattern of transcription with a higher polymerase density at the 5′ end of the gene than at the 3′ end (Fig. 2). This observation shows that Gal4-Sp1 activates transcription complexes with low processivity which tend to stall and/or fall off shortly after initiation, in agreement with two recent reports (37, 75).

We also examined transcription stimulated by a mutant VP16 activation domain by using Gal4-SW6 (68), which has

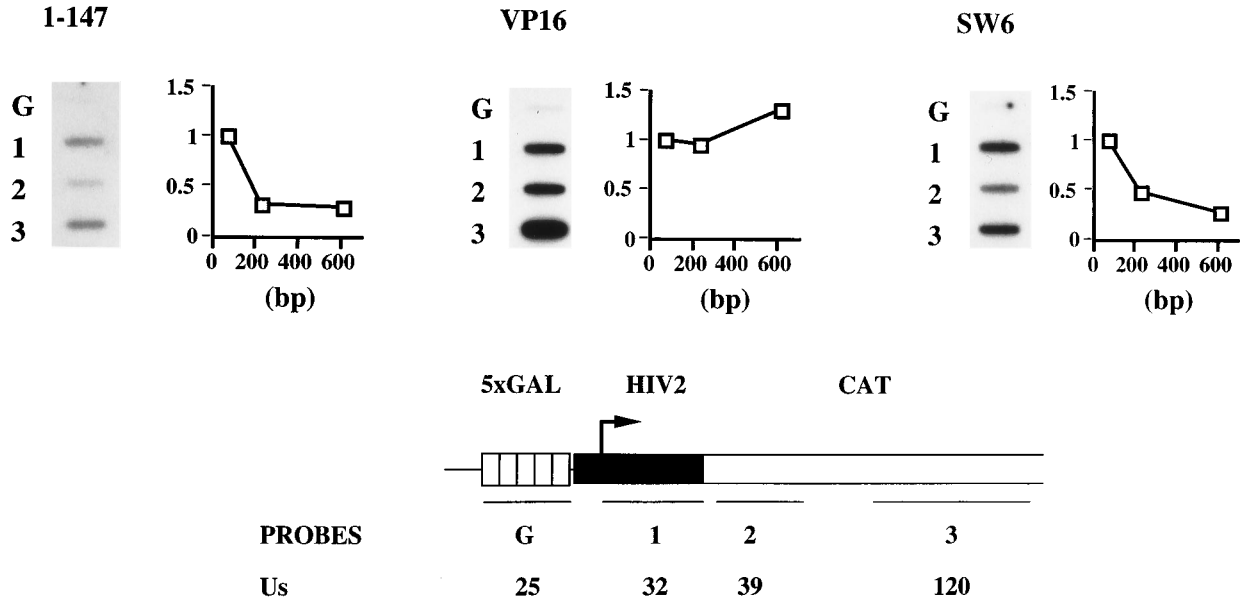


FIG. 3. Gal4-VP16 stimulates more efficient elongation than Gal4-SW6. Nuclear runon analysis (as in Fig. 2) of $pGal_5$-HIV2 CAT in transfected 293 cells activated by Gal4(1-147), -VP16, or -SW6 is shown. Autoradiograms were exposed for 10, 2, and 10 days, respectively. 5×GAL, five Gal4 binding sites.

point mutations of Phe residues at positions 442, 473, 475, and 479. By the criterion of luciferase gene expression, this mutant activator was about 100-fold weaker than wild-type VP16 and approximately equivalent in strength to Gal4-Sp1 and Gal4-Tat(1-48) (Fig. 2). In contrast to wild-type VP16, Gal4-SW6 resembled Gal4-Sp1 in stimulating transcription, which elongated poorly as manifested by the sharp drop in polymerase density between the 5′ and 3′ ends of the gene.

To test whether the nuclear run-on results with the $pGal_5$-myc luciferase template applied to a different promoter, we transfected $pGal_5$-HIV2 CAT into 293 cells with an expression vector for Gal4(1-147), Gal4-VP16, or Gal4-SW6. Nuclear run-on analysis (Fig. 3) showed that Gal4-VP16 stimulated transcription that elongated with high efficiency, whereas transcription activated by Gal4(1-147) or Gal4-SW6 elongated poorly. Gal4-VP16 and Gal4-SW6 stimulated the 5′ polymerase density relative to Gal4(1-147) by five- and twofold, respectively, in this experiment after normalization to a cotransfected VA gene (data not shown). In all cases, read-around transcription detected by the upstream probe G was low relative to transcription downstream of the HIV-2 start site. We conclude that the differences in elongation efficiency between different activators apply to two unrelated reporter genes. In summary, the nuclear run-on analysis showed that some activators stimulate elongation—the steps in transcription which occur after initiation—while others do not and that these differences are not related to the amounts of transcription that they stimulate.

**RNase protection assay for the efficiency of transcriptional elongation.** To test whether the nuclear run-on results were supported by an independent method, we used an RNase protection assay to analyze $pGal_5$-HIV2 CAT transcripts activated by Gal4-VP16 and -SW6. This assay (outlined in Fig. 4C) is based on the observation that prematurely terminated HIV RNAs are stable (31, 34). The TM RNase protection bands represent a diffuse set of RNAs with 3′ ends in the HIV-2 TAR sequence, which acts as a terminator and/or stabilizer of terminated transcripts (66). The RT RNase protection product represents transcripts which initiate correctly and extend beyond position +165. The weak bands marked RA in Fig. 4A correspond to RNAs which either read around the plasmid or initiate upstream of position +1. (Two read-around bands were observed because of cleavage at a 2-base mismatch with the probe just downstream of the TATA box.) We measured the amounts of RT and TM products by densitometry and expressed the efficiency of elongation as the ratio of RT to total transcription (RT plus TM) after compensation for the lower number of radioactive U residues protected by the shorter TM transcripts. This value is useful for comparing efficiencies of elongation but overestimates the absolute efficiency because termination sometimes occurs downstream of position +165, particularly with activators like Sp1 and SW6 (Fig. 2 and 3).

For Fig. 4, transcription of $pGal_5$-HIV2 CAT was activated by Gal4-VP16 and Gal4-SW6, which were expressed at equal levels as demonstrated by Western blot (immunoblot) and gel mobility shift analyses (data not shown). Transcripts were analyzed with two RNase protection probes which differ at their 3′ ends as shown in Fig. 4C. The shift in the positions of all of the TM bands with probe 2 relative to those with probe 1 shows that these RNase protection products are not the products of random degradation during RNA isolation but rather correspond to genuine 3′ ends. Lanes 3 and 4 in Fig. 4A were exposed threefold longer than lanes 1 and 2. Gal4-VP16-stimulated transcription had an RT/(RT + TM) ratio of 62%, versus 9% for Gal4-SW6. These results are in agreement with those of the nuclear run-on experiment (Fig. 3) and confirm that the wild-type VP16 activation domain stimulates more efficient transcriptional elongation than the SW6 mutant.

We investigated the effect of the number of activator binding sites on elongation efficiency. HIV-2 CAT reporters with either one or five Gal4 binding sites were transfected into 293 cells with the Gal4-VP16 expression vector. Transcripts were analyzed by RNase protection with probe 1 (Fig. 4A, lanes 6 and 7) and quantified (Fig. 4B, right panel). The elongation efficiency [RT/(RT + TM)] was 4.5-fold greater (58 versus 13%) with five binding sites than with only one. In subsequent experiments we used the five-site reporter, which was previously

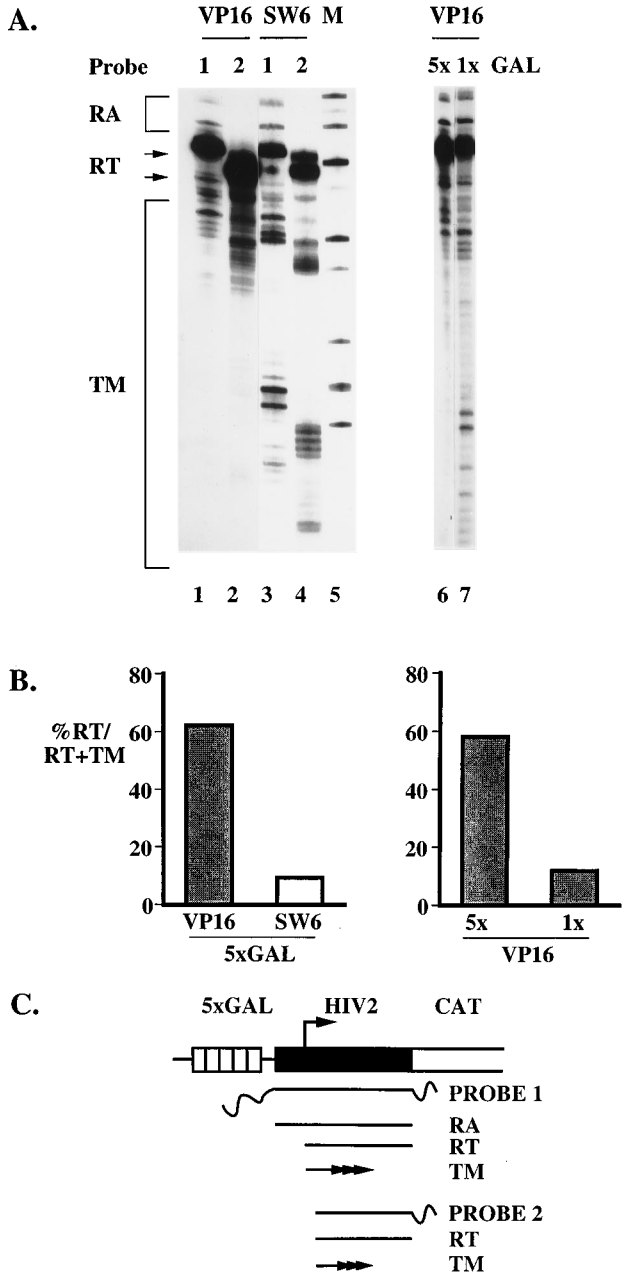


FIG. 4. RNase protection assay for transcriptional elongation through HIV-2 TAR. (A) Lanes 1 to 5, RNase protection of $pGal_5$-HIV2 CAT transcripts activated by Gal4-VP16 or Gal4-SW6. RNA from transfected 293 cells was analyzed with probes 1 and 2 (see panel C). Size markers (M) are 180, 160, 148, 123, 110, 90, 76, and 67 bases. Read-around (RA), RT, and TM transcripts are marked. Lanes 3 and 4 were exposed three times longer than lanes 1 and 2. Lanes 6 and 7, RNase protection of $pGal_5$- and $pGal_1$-HIV2 CAT transcripts with probe 1. Lane 7 was exposed three times longer than lane 6. (B) Percent RT/(RT + TM) values determined by densitometry of the gels in panel A after correction for the U content of the RT and TM bands. (C) RNase protection strategy. Probes 1 and 2 were made from the pVZ HIV-2 template (73) cleaved with *Eco*RI and *Aci*I, respectively. Probe 1 extends to position −32 before its sequence diverges; probe 2 extends to position +12. 5×GAL and 1×GAL, five Gal4 binding sites and one Gal4 binding site, respectively.

shown to be near saturating for the effect of a particular activator (4).

**Two classes of activators differ in their abilities to stimulate transcriptional elongation.** The RNase protection assay was used to analyze a panel of activation domains (Fig. 1) for stimulation of overall RNA synthesis and elongation efficiency [RT/(RT + TM)]. Transcription with the Gal4(1-147) or Gal4(1-94) DNA binding domains alone (Fig. 5A, lanes 6, 7, 9, and 10 [lanes 9 and 10 show a four-times-longer exposure]) elongated poorly, as indicated by the relatively low percent RT/(RT + TM) values (Fig. 5B). Expression of these DNA binding domains did not stimulate transcription (relative to a cotransfected VA control) above that observed for empty vector (2a). Gal4-SW6, Gal4-Sp1, and Gal4-CTF all clearly stimulated RNA synthesis relative to Gal4(1-147) (Fig. 5A; compare lanes 2 to 4 with lanes 6 and 7), but the efficiency of elongation remained low. The results suggest that these activators stimulate initiation of transcription but not elongation. We refer to this class of activators as type I.

In contrast to type I activators, Gal4-VP16, Gal4-E2F1, and

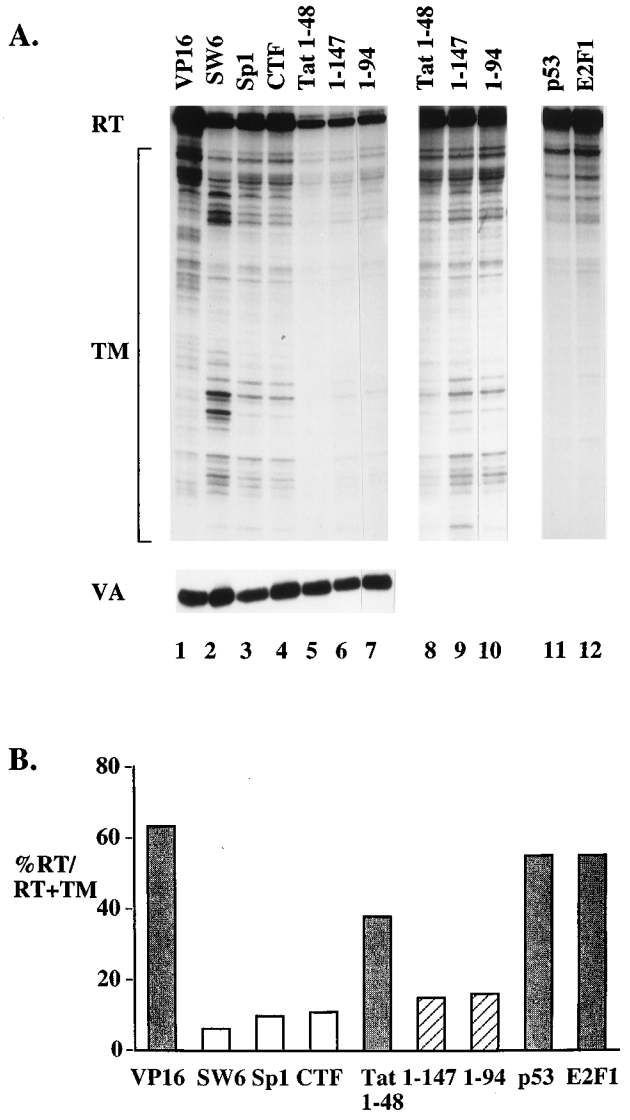


FIG. 5. Production of RT and TM transcripts by seven chimeric Gal4 activators. (A) RNase protection of $pGal_5$-HIV2 CAT (probe 1 [Fig. 4C]) and VA1 (VA) transcripts. Lanes 8 to 10 are identical to lanes 5 to 7, respectively, except that they were exposed four times longer. Lanes 11 and 12 are from an independent experiment. RT and TM transcripts are marked. (B) The RNase protection products in the gel in panel A were quantified by densitometry, and percent RT/(RT + TM) values were calculated as for Fig. 4B.

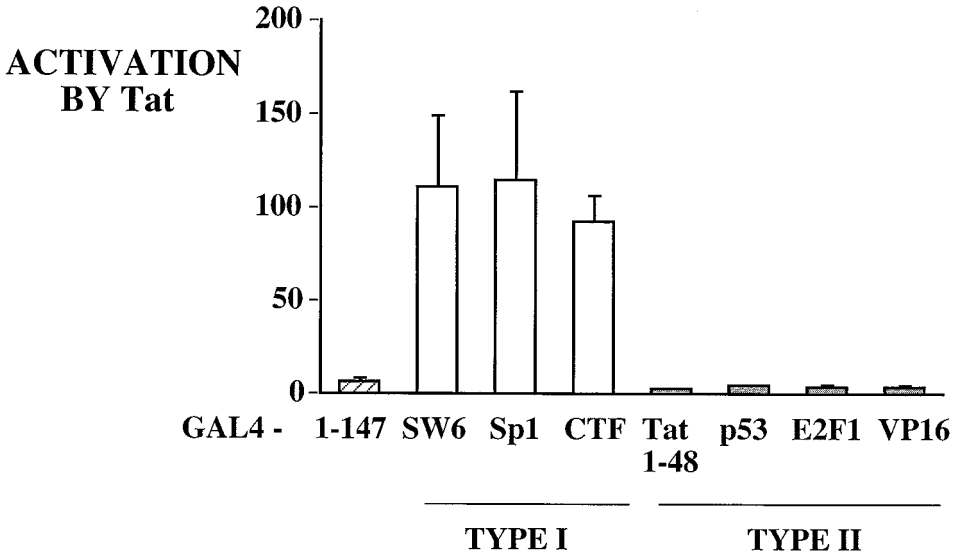


FIG. 6. Synergy between HIV-1 Tat and type I but not type II activators. HeLa cells were transfected with $pGal_5$-HIV2 CAT, RSV-luciferase, and various Gal4 expression vectors plus pSV-HIV1-tat or empty PECE vector. CAT expression was determined relative to luciferase activity, and the fold induction by Tat was determined. Average values and standard deviations for at least three experiments for each activator are plotted.

Gal4-p53 gave rise to abundant transcripts with a high RT/TM ratio (Fig. 5) characteristic of efficient elongation. Gal4-Tat(1-48)-activated transcripts also elongated relatively efficiently, but the amount of RNA synthesis was low relative to that with VP16, E2F1, and p53 (Fig. 5A, lane 5, and a longer exposure in lane 8). A strong effect of this activator on elongation was more obvious when Sp1 was also present (see Fig. 9). In addition to affecting the RT/TM ratio, these activators shifted the positions of the 3′ ends of TM products towards the 3′ end of TAR (Fig. 5A; compare lanes 1, 8, 11, and 12 with lanes 2 to 4). We call the class of activators which stimulate efficient elongation type II.

In summary, the RNase protection data reinforce and extend the conclusions of the run-on experiments of Fig. 2 and 3. Together these experiments distinguish two functional classes of activator: type II activators stimulate elongation; type I activators do not.

**Only type I activators synergize with Tat.** Southgate and Green (62) showed that promoter-bound activators differ in their responses to HIV Tat bound to nascent TAR RNA. They found that Gal4-Sp1 synergized with Tat, whereas Gal4-VP16 did not. We have extended these results by using the Tat cooperation assay to investigate our panel of activators (Fig. 1). HeLa cells were transfected with the $pGal_5$-HIV2 CAT reporter, expression vectors for Gal4 fusion proteins, and either a Tat expression plasmid or an empty vector. CAT activity in the presence of Tat was divided by activity in the absence of Tat after normalization for transfection efficiency with an RSV-luciferase reference gene. The results are plotted in Fig. 6. The type I activators Gal4-Sp1, -SW6, and -CTF synergized strongly (92- to 114-fold) with Tat. In contrast, Tat had only a two- to fivefold effect in the presence of the type II activators Gal4-Tat(1-48), -VP16, -p53, and -E2F1. In the absence of Tat, type I activators stimulated 200- to 400-fold less CAT expression than the strong type II activators, Gal4-p53, -E2F1, and -VP16, whereas in the presence of Tat, they were only about 5-fold weaker. In each case, the combined effect of Tat plus the type I activator was far greater than the product of stimulation by either factor alone.

Tat cooperation with the type I and II activators Gal4-SW6 and -VP16, respectively, was also examined by RNase protection (Fig. 7). Tat had a significant effect on the efficiency of elongation in the presence of Gal4-SW6 (Fig. 7; compare lanes 1 and 2) but little effect in the presence of Gal4-VP16 (Fig. 7, lanes 3 and 4). Lanes 1 and 2 in Fig. 7 were exposed six times longer than lanes 3 and 4.

In summary, the Tat cooperation assay precisely distinguishes between type I and type II activators. This assay therefore provides independent confirmation of the conclusion based on studies of elongation efficiency (Fig. 2 to 5) that there are two functionally distinct classes of activators.

**Type II activators differ in their abilities to cooperate with Sp1.** We also used an Sp1 cooperation assay in our attempts to distinguish the functional properties of the activation domains shown in Fig. 1. Southgate and Green (62) previously used this assay to show that Gal4-Tat(1-48), but not Gal4-VP16, synergizes with Sp1. CAT expression in transfected 293 cells was measured for two pairs of reporter genes which differ only by four tandem Sp1 sites inserted next to the TATA box (Fig. 8). The CAT activity in the presence of Sp1 sites was divided by the activity in the absence of Sp1 sites, and the fold activation was plotted. The two pairs of reporters were $pGal_5$-HIV2 CAT with $pGal_5$-$Sp_4$-HIV2 CAT (Fig. 8A) and $pGal_5$-E1b CAT with $pGal_5$-$Sp_4$-E1b CAT (Fig. 8B). The latter pair of plasmids does not contain any obvious terminator element analogous to the HIV-2 TAR sequence. The two pairs of reporter genes behaved identically in response to different Gal4 activators. A 1.5- to 3-fold effect of Sp1 sites on stimulation of CAT expression was observed with type I Gal4 activators. On the other hand, there were significant differences among type II activators in their abilities to cooperate with Sp1. Gal4-Tat(1-48) plus Sp1 activated 15- to 40-fold better than Gal4-Tat(1-48) alone. This cooperation of Sp1 with Gal4-Tat(1-48) was slightly more than multiplicative. On the other hand, Sp1 enhanced transcription only 1.5- to 3-fold in the context of bound Gal4-p53, -E2F1, or -VP16. The Sp1 cooperation assay therefore distinguishes two subclasses of type II activator: type IIA, represented by Gal4-Tat(1-48), synergizes with Sp1; type IIB, represented by Gal4-p53, -E2F1, and VP16, does not synergize.

RNase protection (Fig. 9) showed that Gal4-Tat(1-48), like

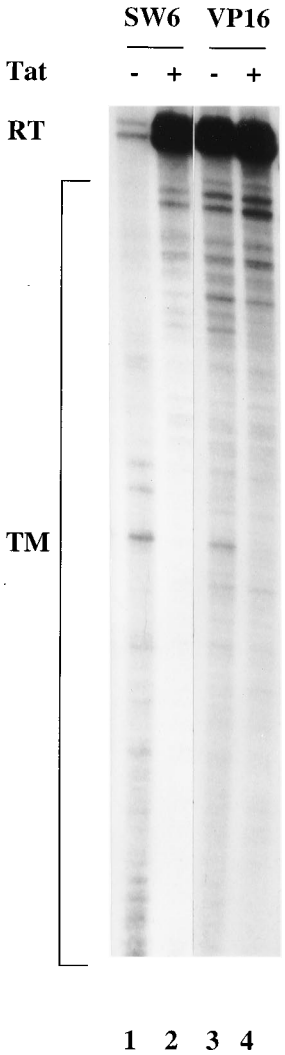


FIG. 7. Tat stimulates elongation with Gal4-SW6 but not Gal4-VP16. RNase protection of $pGal_5$-HIV2 CAT transcripts from HeLa cells transfected as for Fig. 6 with the Gal4-VP16 or -SW6 expression vector without or with Tat is shown. RNase protection of the cotransfected pSp65-VA1 transcripts demonstrated equal transfection efficiencies (not shown).

Tat itself (Fig. 7), greatly enhanced elongation of transcripts from the $pGal_5$-$Sp_4$-HIV2 CAT gene compared with Gal4(1-147), Gal4-SW6, or Gal4-Sp1. Lanes 1 to 3 in Fig. 9A were exposed 3.5 times longer than lanes 4 and 5. Transcription activated by Gal4-Tat(1-48) elongated as efficiently as that stimulated by Gal4-VP16 (Fig. 9B). The data in Fig. 9 therefore confirm the strong effect of Gal4-Tat(1-48) on elongation which was observed in the absence of a cooperating factor (Fig. 2 and 5) when the overall amount of transcription was much less.

**Activators that stimulate elongation bind to TFIIH.** The functional difference between type I and type II activators might be explained by the nature of the GTFs that they contact in the preinitiation complex. For example, the Tat, VP16, E2F1, and p53 activation domains may stimulate elongation by targeting a common component of the transcription machinery which is not contacted by Sp1, CTF, or SW6. With the exception of SW6, all of these activators have been reported to interact with TFIID subunits (7, 11, 17, 19, 22, 32, 67, 71). In addition, VP16 and CTF also bind TFIIB (35, 44). There are no obvious correlations between the reported contacts with TFIID or TFIIB and the classification of an activator as type I or type II. On the other hand, we have noted that the type II acidic activators VP16, p53, and E2F1 all interact with TFIIH, whereas a VP16 mutant similar to SW6 cannot (50a, 72). To test whether this correlation extended to the other activation domains shown in Fig. 1, we examined whether TFIIH could interact with Tat, Sp1, CTF, and SW6. HeLa cell extracts were chromatographed on columns containing immobilized activation domains fused to GST, and proteins retained on the columns were eluted and analyzed by Western blotting with antibodies against subunits of TFIIH.

Like VP16, both full-length HIV-1 Tat (positions 1 to 72) and its 48-amino-acid N-terminal activation domain bound the TFIIH in HeLa extracts as judged by Western blotting with antibody against the p62 subunit of TFIIH (Fig. 10A, lanes 5 and 6). This interaction between Tat and TFIIH is likely to be direct because the immobilized Tat activation domain also bound to the $^{35}$S-labelled p62 subunit of TFIIH when it was made by translation in a rabbit reticulocyte lysate (70a). Western blotting did not reveal any binding of TFIIB, TFIIE, or TFIIF to Tat in these experiments, although Tat did bind TFIID as detected by anti-TBP antibody, in agreement with published results (32) (data not shown). In contrast to Tat, GST-Sp1A, -Sp1B, -CTF, and -SW6 did not bind any more TFIIH than the GST control resin (Fig. 10A, lanes 3, 8, and 9; Fig. 10B, lane 6). In addition, the experiment of Fig. 10B shows that the form of TFIIH retained by the Tat and VP16 activation domains contains the $p40^{MO15}$ protein kinase subunit. These results therefore extend our observations that type II activators bind TFIIH and show, in addition, that type I activators do not bind TFIIH.

## DISCUSSION

**Three classes of activators differ in their abilities to affect initiation and postinitiation steps in transcription.** In this paper, we have distinguished between activation domains on the basis of whether they stimulate the postinitiation (elongation) stage in pol II transcription. Three independent lines of evidence were used to make this distinction: (i) polymerase distribution along the gene determined by nuclear run-on analysis, (ii) production of truncated transcripts detected by RNase protection, and (iii) synergy with other activators assayed by reporter gene activity.

The type I activators Sp1, CTF, and the VP16 mutant SW6 gave rise to a skewed polymerase distribution with a sharp drop in density immediately downstream from the 5′ end (Fig. 2 and 3 and data not shown). These activators gave rise to abundant truncated RNAs (Fig. 5), indicating that they stimulated initiation of transcription. Type I activators synergized strongly with HIV Tat but not with Sp1 (Fig. 6 and 8). In contrast, type II activators gave rise to a uniform distribution of polymerases along the gene (Fig. 2 and 3) and produced relatively few truncated transcripts (Fig. 5). There is, however, functional heterogeneity among type II activators. Type IIA has only one identified representative, Gal4-Tat(1-48), a weak activator that synergized well with the type I activator Sp1 (Fig. 8). We think it likely that there are as-yet-unidentified cellular type IIA DNA binding factors similar to Gal4-Tat(1-48). One possible candidate is PBP, an ATF/CREB family member which activates promoter clearance but not preinitiation complex assembly or open complex formation (49). Type IIB activators, represented by Gal4-p53, -E2F1, and -VP16, are strong activators that do not synergize with Sp1 (Fig. 8). To explain these dif-

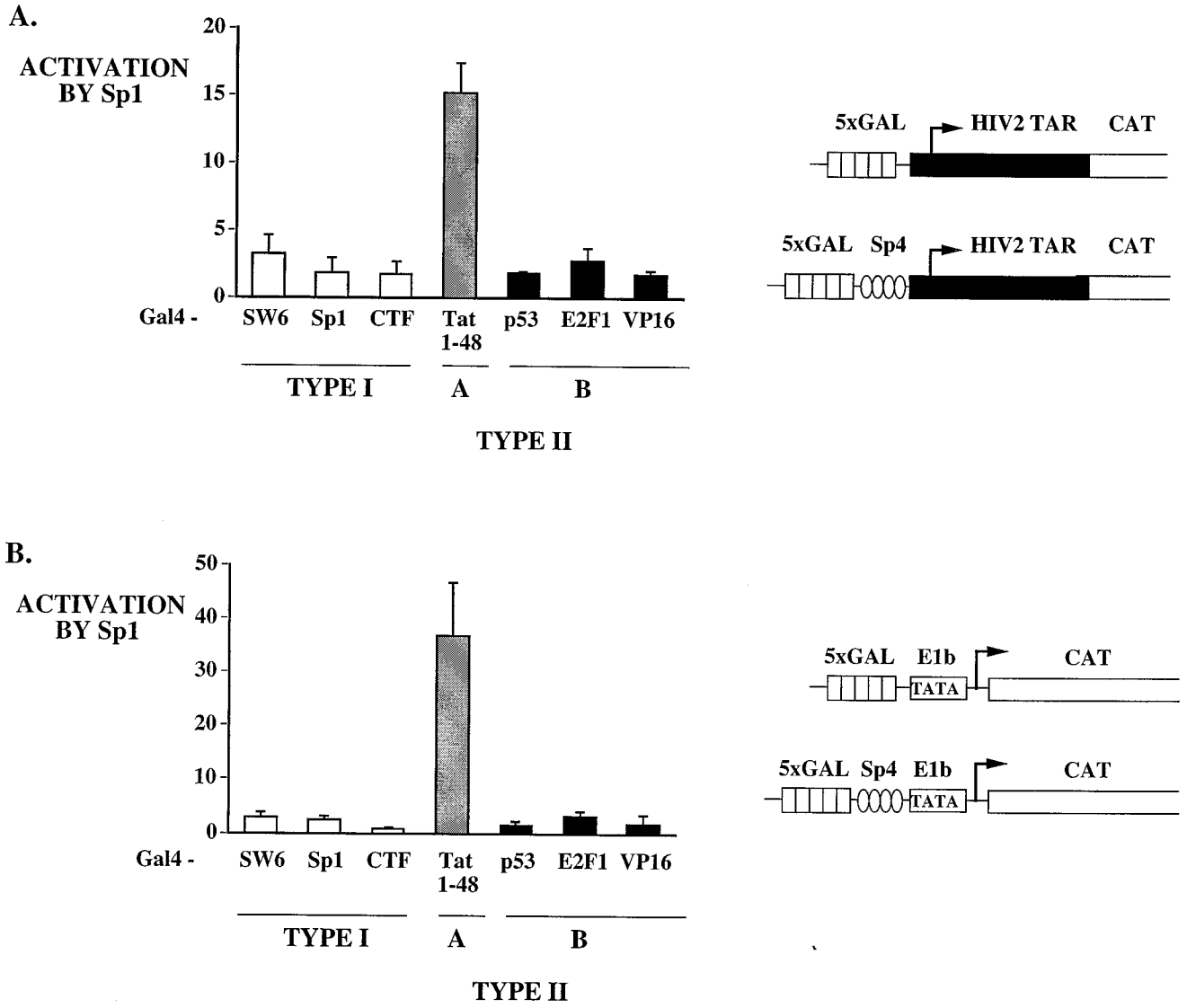


FIG. 8. Sp1 synergizes with Gal4-Tat(1-48) but not with other activators. (A) 293 cells were transfected with expression vectors for various Gal4 activators and either $pGal_5$-HIV2 CAT or $pGal_5$-$Sp_4$-HIV2 CAT. CAT activity was normalized to the activity of cotransfected RSV-luciferase, and the fold induction by Sp1 was plotted. The data shown are average values and standard deviations based on results from three independent experiments. (B) Experiments were conducted as for panel A except that the reporter was $pGal_5$-E1b CAT or $pGal_5$-$Sp_4$-E1b CAT. Fold induction by Sp1 is plotted. The data are from three independent experiments. 5×GAL, five Gal4 binding sites; Sp4, four Sp1 binding sites.

ferent functional properties, we suggest that type I activators stimulate initiation but not elongation, type IIA activators stimulate predominantly elongation, and type IIB activators strongly stimulate both initiation and elongation. This model for the three functional classes of activators is summarized in Fig. 11. Further analysis may reveal more functional subclasses. For example, different type I activators could stimulate different steps in initiation by contacting different sets of GTFs.

We observed production of truncated RNAs (Fig. 5) and promoter-proximal pausing by pol II (Fig. 2) (38, 64) when examining nonactivated transcription or transcription activated by type I but not type II activators. In contrast, a recent nuclear run-on analysis with various Gal4-fusion proteins led to the conclusion that all activation domains stimulated transcription which pauses in the promoter-proximal region, although pausing was reduced in the presence of VP16 (37). In order to reveal functional differences between activators when reporter genes with multiple binding sites are used, it is probably necessary for the activators to be saturating so as to maximize synergy between multiple copies of the same activator. When the occupancy of the binding sites is low, most activators may predominantly stimulate initiation. Our experiments may have demonstrated more clear-cut differences between activation domains than observed previously (37) because our reporter genes (which had five, rather than three, Gal4 binding sites) were more fully saturated with each activator. In support of this explanation, transcription activated by Gal4-VP16 from a single-site reporter is less processive than that from a five-site reporter (Fig. 4A). This effect of binding site number on the elongation efficiency of Gal4-VP16-activated transcription presumably contributes in part to the synergistic activation of gene expression by multiple copies of this activator (4).

**Mutation of VP16 switches it from type II to type I.** Our experiments suggest that type IIB activators like VP16 have a

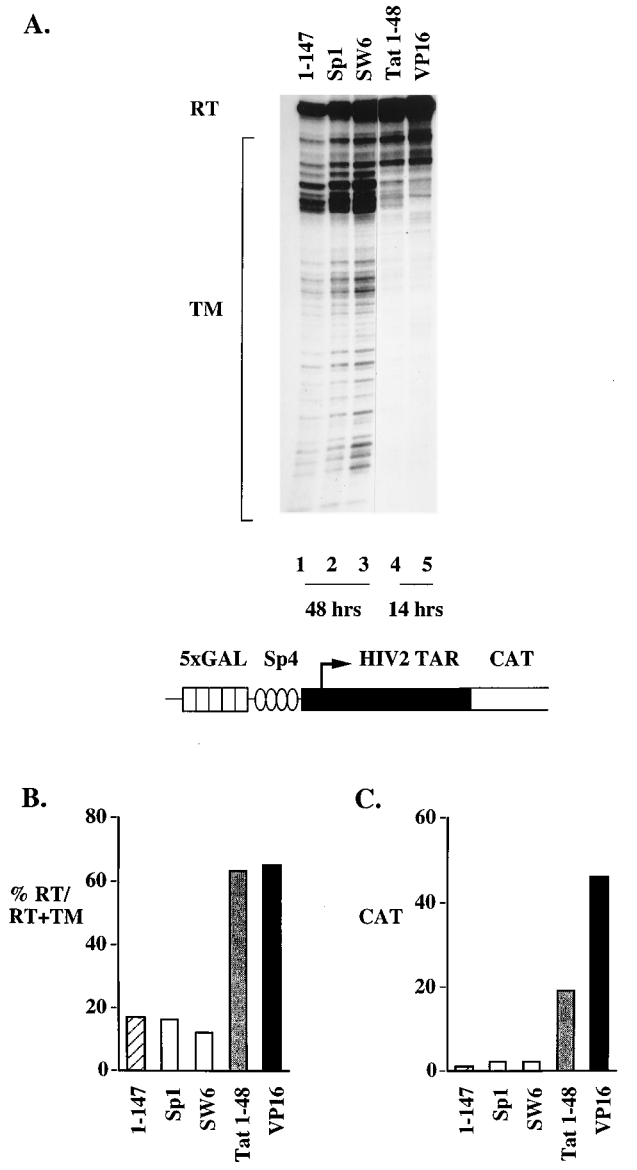


FIG. 9. Gal4-Tat(1-48) synergizes with Sp1 by istimulating transcriptional elongation. (A) 293 cells were transfected with $pGal_5$-$Sp_4$-HIV2 CAT, RSV-luciferase, pSp65-VA1, and Gal4 expression plasmids. HIV-2 transcripts were analyzed by RNase protection as for Fig. 5A. Lanes 1 to 3 were exposed 3.5 times longer than lanes 4 and 5, as indicated. RNase protection demonstrated equal expression of VA1 transcripts (not shown). 5×GAL, five Gal4 binding sites; Sp4, four Sp1 binding sites. (B) Percent RT/(RT + TM) determined by densitometry of the autoradiogram in panel A. (C) Relative CAT activity normalized to RSV-luciferase activity for each Gal4 activator.

dual function in that they stimulate both initiation of transcription and at least one postinitiation event. The SW6 mutant of VP16 has four substitutions of Phe residues at positions 442, 473, 475, and 479. Gal4-SW6 is a much weaker activator than Gal4-VP16 as assayed by luciferase and CAT reporter gene expression (Fig. 2) (68), but it does not behave like a weak type II activator such as Gal4-Tat(1-48). Instead, it behaves like a type I activator by the criteria of the nuclear run-on assay (Fig. 2 and 3), the RNase protection assay (Fig. 4 and 5), and synergy with other activators (Fig. 6 to 8). In fact, SW6 was functionally indistinguishable from the CTF and Sp1 activation domains. The four mutations in SW6 therefore result in a qualitative, as well as a quantitative, change in transcription, effectively converting the VP16 activation domain from type IIB to type I. The initiation and elongation functions of a type IIB activator are therefore at least partially separable by genetic means.

The behavior of SW6 underscores the fact that there is no simple relationship between amino acid composition and function. The three type IIB activators VP16, p53, and E2F1 all have acidic activation domains, but Gal4-E1a(121-222), which also belongs to this class (2a, 74), is not highly acidic. SW6 is as acidic as VP16 yet behaves like the type I proline- and glutamine-rich activation domains of CTF and Sp1.

**Implications for the function of HIV Tat.** The first activator shown to stimulate pol II elongation was HIV Tat, but its mechanism of action remains a matter for speculation (28). The binding of Tat to nascent RNA, rather than DNA, suggested that it may act directly on the elongating ternary complex as an antitermination factor similar to the phage lambda N protein (21). This idea was challenged by the observation that Tat could also stimulate transcription after binding to DNA when it was fused to the DNA binding domain of c-Jun or Gal4 (2, 29, 62). It was not determined whether activation by Jun-Tat or Gal4-Tat in these experiments occurred by stimulation of elongation or initiation. It was therefore possible that

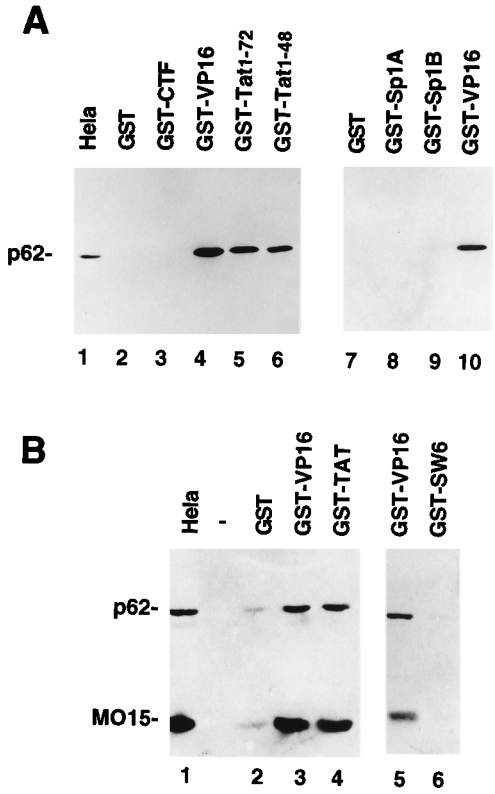


FIG. 10. Type II but not type I activation domains bind to TFIIH. (A) HeLa whole-cell extracts (800 μl) were chromatographed on microcolumns containing various immobilized GST fusion proteins. Bound proteins were eluted in 160 μl of buffer containing 1 M NaCl, and 20 μl was analyzed by immunoblotting with monoclonal antibody 3c9 against the p62 subunit of TFIIH. HeLa cell extract (10 μl) was run as a marker in lane 1. No significant amount of p62 was detected when the GST, GST-Sp1A, GST-Sp1B, and GST-CTF columns were subsequently eluted with SDS (data not shown). (B) HeLa cell nuclear extracts were chromatographed on 0.6-ml columns containing various immobilized GST fusion proteins, and the bound proteins were eluted with 0.6 M KCl and analyzed by immunoblotting with monoclonal anti-p62 and anti $p40^{MO15}$ antibodies.

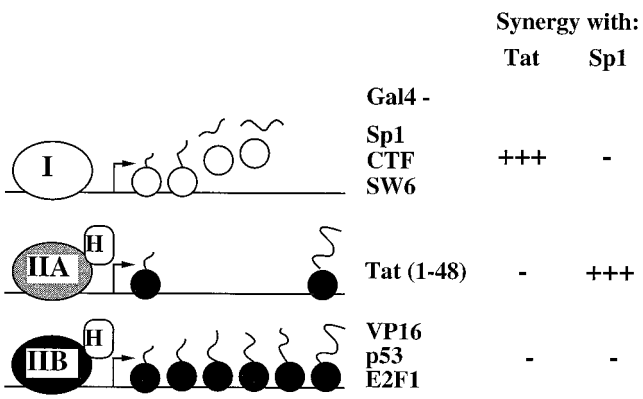


FIG. 11. Model for three functional classes of activation domains. Type I does not bind TFIIH and activates initiation by nonprocessive transcription complexes (open circles). Types IIA and IIB bind TFIIH (H) and cause elongation to be processive (filled circles). Tat synergizes well with all type I activators but not with type IIA or IIB. Sp1, a type I activator, synergizes with Gal4-Tat(1-48) (type IIA) but not with type IIB or other type I activators.

different mechanisms operate when Tat is recruited to the promoter by DNA binding or to the ternary complex by RNA binding. Only Tat activation via TAR RNA, for example, is likely to involve cellular TAR binding factors (28).

Our results (Fig. 2, 5, and 9) show that Gal4-Tat(1-48) bound to DNA operates in a way similar to Tat bound to TAR RNA, that is, by stimulating transcriptional elongation by pol II. The effect of Tat on elongation is therefore not peculiar to its unusual mode of recruitment to the transcription complex. The strong effect of Gal4-Tat(1-48) on elongation also shows that stimulation of this step is not a trivial consequence of a high initiation rate, since it is a very weak activator. Indeed, we did not find evidence for significant stimulation of initiation by Gal4-Tat(1-48) relative to the Gal4(1-147) DNA binding domain (Fig. 5A). There could be differences between the behaviors of Gal4-Tat(1-48) and Tat itself, however, since the latter does stimulate initiation in some cases (39).

Gal4-Tat(1-48) strongly activated transcription from $pGal_5$-$Sp_4$-E1b CAT (Fig. 8B) (30, 62), which does not contain a known terminator. Our other results make it likely that Gal4-Tat(1-48) activated this reporter gene by stimulating elongation. Postinitiation effects of activators therefore do not appear to be limited to a subset of genes containing specific terminators, consistent with the report that promoter-proximal pausing is, in fact, a very general phenomenon (37).

**Synergy between activators that stimulate initiation and elongation.** Sp1 was previously shown to synergize with Tat and Gal4-Tat, while VP16 did not, but the reasons for this difference remained unclear (29, 30, 62). In one case, in which Tat cooperation with VP16 was reported (16), the reporter plasmids contained multiple Sp1 sites which almost certainly contributed to the effect observed. A second reported instance of synergy between Gal4-VP16 and Sp1 was exceptional in that Gal4-VP16 was bound downstream of the gene and activated transcription only threefold (69). We have observed synergy between Sp1 and Gal4-VP16 bound to a single Gal4 site but not with five Gal4 binding sites (2a).

Our experiments suggest an explanation for why some combinations of activator synergize and others do not. When self-cooperation between identical activators was maximized by saturating the promoter, the only synergistic combinations we observed were those involving type I and type IIA activators, which affect initiation and elongation, respectively. Only Gal4-Tat(1-48) (type IIA), and none of the type I or type IIB activators, synergized with Sp1 (type I) (Fig. 8). Conversely, only the type I activators, Gal4-Sp1, -CTF, and -SW6, synergized with Tat, while the type IIA and IIB activators, Gal4-Tat(1-48), -VP16 -p53, and -E2F1, did not (Fig. 6). Synergy with Tat was not inversely related to the activator strength as previously proposed (62). Although Gal4-Tat(1-48) is a weak activator, it does not synergize with Tat, while other weak activators (Gal4-CTF, -Sp1, and -SW6) do synergize with Tat. In summary, the only combinations of activators which synergized under our conditions were those which stimulated two apparently distinct rate-limiting steps in transcription: initiation and elongation. This result is consistent with a model for synergy proposed by Herschlag and Johnson (25).

**Contributions of elongation and initiation to gene activation.** Neither the nuclear run-on assay nor the RNase protection assay directly measures initiation rates; therefore, we cannot accurately estimate the contribution of increased initiation rate to the stimulation of gene expression. However, there is no doubt that it plays an important role. The nuclear run-on assay measures the density of preinitiated polymerases at the 5′ end of the gene but does not distinguish between stalled and actively elongating polymerases. Stalled polymerases resume transcription in the run-on assay and produce high 5′ run-on signals even if little or no active initiation is occurring. Run-on reactions therefore underestimate the stimulation of initiation by activators. For this reason, the 5- to 10-fold enhancement of 5′ polymerase density by Gal4-VP16 relative to Gal4(1-147) (Fig. 2 and 3) is a minimum estimate of the effect on initiation. Gal4-VP16 and other type II activators stimulated elongation efficiency by about 10-fold relative to Gal4(1-147) and type I activators (Fig. 2 and 3). Together the effects on initiation and elongation account for most of the 200- to 300-fold stimulation of CAT activity by Gal4-VP16 relative to Gal4(1-147).

In contrast to type IIB activators, the type I activators, Gal4-Sp1, -CTF, and -SW6, stimulate only initiation, and the type IIA activator, Gal4-Tat(1-48), stimulates only elongation. Type I and type IIA activators stimulate similar levels of reporter gene expression (Fig. 2) by two different mechanisms. Only in combination with one another do they generate high levels of gene expression comparable to that generated by type IIB activators (Fig. 9C).

By stimulating elongation and suppressing promoter-proximal pausing, type II activators may indirectly permit higher initiation rates by removing stalled polymerases which would otherwise obstruct the start site. The *Drosophila* heat shock factor, HSF, is believed to function in this way (45). This type of effect could make an important contribution to the synergy between type I and type IIA activators (Fig. 9) and may also contribute to the high initiation rates associated with type IIB activators such as VP16, E2F1, and p53. We suggest that the reason that type IIB activators are stronger than other types is precisely because they stimulate both initiation and elongation. Further support for this idea could be provided by isolation of a second type of mutant VP16 which, unlike SW6, behaves as a type IIA activator that stimulates elongation but not initiation.

**A role for TFIIH in activation of transcription after initiation?** Several previous reports have identified functional differences between certain type I and type II activators. Gal4-Sp1 and Gal4-CTF activate transcription significantly better from promoter-proximal positions than from distal positions, whereas Gal4-VP16 activates equally well from both positions (56). Gal4-VP16 and -Sp1 also behave differently in their abilities to activate various mRNA and small nuclear RNA promoters (8). Additionally, transcription that is activated by Gal4-

VP16 and Gal4-Tat increases with overexpression of TBP, whereas Gal4-CTF and -Sp1 are largely unresponsive (53). An additional functional distinction was found in an elegant study showing that activation by Gal4-VP16 is inhibited by deletion of the pol II CTD, whereas Sp1-activated transcription is unaffected (15).

This last observation is particularly intriguing given the correlation we observed between binding of TFIIH and stimulation of elongation. Phosphorylation of the pol II CTD by TFIIH could be involved in the stimulation of elongation by type II activators. All of the type II activators that we have identified bind TFIIH, whereas type I activators do not (Fig. 10). This correlation suggests that TFIIH may be involved in activator-mediated stimulation of postinitiation events in transcription. In several *Drosophila* genes, CTD phosphorylation is correlated with stimulation of elongation by stalled polymerases near the start sites (50, 70). The binding of an activation domain to TFIIH could modulate the activity of the MO15 cyclin-dependent kinase associated with this GTF.

The binding of TFIIH to VP16 is severely reduced by the mutations in SW6 (72) (Fig. 10B), which also abolish the ability of this activator to stimulate elongation. The altered properties of the SW6 mutant are consistent with the model that binding to TFIIH is required for stimulation of elongation, but they do not prove the case, since interactions with other factors, such as TBP, are also affected (reference 27 and data not shown). Reduced binding of SW6 to TBP may explain the reduced level of initiation relative to that with wild-type VP16 apparent in some of our experiments (Fig. 2 and 4). An elongation function of TFIIH is also consistent with our observation that inhibitors of TFIIH kinase, such as dichlororibofuranosylbenzimidazole (DRB), also inhibit pol II elongation (74). Herrmann and Rice (23) showed that Tat interacts with a CTD kinase other than TFIIH (although of similar molecular weight) (24) which is also inhibited by DRB. It is therefore possible that activators interact with multiple CTD kinases in order to modulate transcription.

On the basis of this evidence, we propose, as a working model, that type II activators stimulate elongation, at least in part, by recruiting and/or stimulating the TFIIH protein kinase, which converts the polymerase from a nonprocessive to a processive form (Fig. 11). In contrast, type I activators such as Sp1, CTF, and the SW6 mutant of VP16 lack this capability and stimulate only initiation by virtue of their interactions with factors such as TFIID and TFIIB (11, 17, 35, 71).

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