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Mutations in the Carboxy-Terminal Domain of TBP Affect the Synthesis of Human Immunodeficiency Virus Type 1 Full-Length and Short Transcripts Similarly

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The human immunodeficiency virus type 1 promoter generates two types of RNA molecules, full-length transcripts and short transcripts. Synthesis of the short transcripts depends on the inducer of short transcripts (IST), an element located downstream of the start site. In the presence of the viral activator Tat, the synthesis of full-length transcripts is up-regulated while that of short transcripts is down-regulated. Full-length and short transcripts are probably generated by different types of transcription complexes. The first is IST independent, capable of efficient elongation, and up-regulated by Tat. The second is IST dependent, incapable of efficient elongation, and down-regulated by Tat. We have used an in vivo assay to assess the role of TBP in human immunodeficiency virus type 1 transcription and to test the effect of mutations in TBP on synthesis of full-length and short transcripts. We find that TBP bound to the TATA box is required for the synthesis of short and full-length transcripts as well as for Tat activation and that both yeast TBP and the carboxy-terminal domain of human TBP can replace full-length human TBP for these processes. Mutations in TBP affect the synthesis of short and full-length transcripts as well as Tat activation similarly, and these effects correlate with the previously described effects of these mutations on binding of TBP to the TBP-associated factor $TAF_{II}250$ in vitro. Together, these results suggest that if short and full-length transcripts are generated by variant transcription complexes, these complexes use TBP similarly, probably as part of the TFIID complex.

The human immunodeficiency virus type 1 (HIV-1) promoter is unusual in its ability to generate two types of RNA molecules; full-length transcripts that extend through the entire transcription unit, and short transcripts that end around position +60 downstream of the transcription start site (7, 16, 32, 36, 40, 41). Synthesis of the short transcripts depends on a bipartite DNA element, the inducer of short transcripts (IST), located downstream of the transcription start site (36, 41). Mutations in IST reduce or suppress the synthesis of short transcripts without affecting that of full-length transcripts. Thus, IST activates transcription from the HIV-1 promoter, but the resulting RNA molecules are short. IST acts in concert with upstream HIV-1 promoter elements and is not functional on its own. Since it activates transcription, IST does not correspond to a transcription terminator. Rather, it is a promoter element that appears to direct the assembly of transcription complexes that are incapable of efficient elongation (36, 41).

The HIV-1 promoter is regulated in large part by the viral activator Tat. Tat is unique among activators in that it acts through an RNA target known as the *trans*-activation-responsive region (TAR), encoded between positions +18 and +44 downstream of the HIV-1 cap site (8, 9, 14, 38, 40; see reference 15 for a review). In transfection experiments, the presence of Tat results in a large increase in the amount of full-length transcripts and a decrease in the amount of short transcripts. This observation originally prompted the suggestion that Tat acts by converting the short transcripts into full-length transcripts (16). However, when IST is mutated, the synthesis of short transcripts is dramatically reduced, and yet activation by Tat is unaffected (41). Thus, although activation by Tat most probably affects transcription elongation (7, 18, 25–27, 29, 30, 54), it does not depend on IST function and the formation of short transcripts. Rather, it appears that Tat stimulates the assembly of transcription complexes that are capable of efficient elongation to the detriment of IST-dependent, elongation-deficient transcription complexes (reviewed in reference 4). The mechanisms of Tat function are not known, but Tat interacts directly with TBP in vitro and the interaction is dependent on the Tat activation domain, suggesting that the direct Tat-TBP interaction may be functionally relevant (17).

Both the IST and TAR elements work in concert with HIV-1 promoter elements located upstream of the transcriptional start site, one of which is a TATA box. In RNA polymerase II mRNA promoters, the TATA box is thought to be recognized in vivo by the TATA box-binding protein (TBP)-containing complex TFIID (47). Indeed, although TBP on its own can bind to the TATA box and sustain basal levels of transcription in vitro, the TBP-associated factors (TAFs) present in the TFIID complex are required to mediate activation of basal transcription by a number of *trans* activators, including Sp1 (6, 45, 53; see reference 48 for a review). The HIV-1 promoter contains three functional Sp1 binding sites just upstream of the TATA box and is therefore likely to recruit TFIID. However, since the HIV-1 promoter appears to direct the formation of two types of transcription complexes that differ in their elongation properties (28, 30, 41; reviewed in references 4 and 15), these transcription complexes may be assembled by variant TBP-containing complexes.

In a previous study, Tansey et al. (47) used an in vivo assay to test the effect of mutations in TBP on activation by different

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types of activation domains. They showed that whereas several single clusters of alanine substitutions in TBP had little effect on transcription activation in vivo, different combinations of mutation clusters reduced activation by various activation domains. These effects did not correlate with the abilities of the mutant TBPs to direct basal transcription in vitro, to bind to the TATA box, or to interact directly with activation domains (46, 47). They did correlate, however, with the abilities of the mutant TBPs to interact in vitro with the largest subunit of TFIID, $TAF_{II}250$, which is essential for the assembly of the TFIID complex (3, 39, 51).

Here, we have used the same assay to study the role of TBP in assembly of the two putative transcription complexes assembled on the HIV-1 promoter. We have determined whether the synthesis of short transcripts and the synthesis of full-length transcripts in the absence and presence of Tat differ in their sensitivities to mutations in TBP. We find that synthesis of both full-length and short transcripts as well as Tat activation require TBP bound to the TATA box and that yeast TBP (yTBP) and the conserved carboxy-terminal of human TBP (hTBP) can replace full-length hTBP for these processes. Furthermore, the synthesis of full-length transcripts, the synthesis of short transcripts, activation by Tat, and activation by DNA-targeted activators are all affected similarly by various mutations in the carboxy-terminal domain of TBP. These effects do not correlate with the abilities of the mutant TBP molecules to interact with Tat in vitro but do correlate with their previously reported abilities to interact with $TAF_{II}250$ in vitro. Together, these data suggest that a direct Tat-TBP interaction is not critical for Tat function in vivo and that the transcription complexes that synthesize full-length and short transcripts in the absence and presence of Tat use TBP similarly, probably as part of TFIID.

MATERIALS AND METHODS

Plasmid constructions. pHIV-1/R has been described previously (36). Point mutations in the HIV-1 TATA box to generate the construct pHIV-1/R-AS, with a TGTAAA altered TATA box, or the construct pHIV-1/R-FAS, with an altered TATA box surrounded by c-*fos* sequences, were introduced by site-directed mutagenesis as described previously (23, 55) with the oligonucleotides 5′ CCCT CAGATGCTGCTGTAAAGCAGCTGC 3′ and 5′ GAGCCCTCAGATCGCT TCTGTAAAGGCGCCAGCTTTCTCGAG 3′, respectively. In preliminary transfection experiments, the pHIV-1/R-AS and pHIV-1/R-FAS constructs behaved indistinguishably. To introduce GAL4 binding sites to generate the construct p(4xG17)HIV-1/R-FAS, pHIV-1/R-FAS was digested with *Eco*RI, which in this construct cuts 152 bp upstream of the HIV-1 transcriptional start site; the ends were dephosphorylated with calf intestine phosphatase and then filled in with Klenow enzyme. An *Eco*RI-*Hin*cII fragment containing four GAL4 binding sites was excised from plasmid pUC1194xG17 (a kind gift of M. Tanaka), which contains four 17-mer GAL4 binding sites (50) cloned into the *Sma*I site of the pUC119 polylinker, the ends were filled in with Klenow enzyme, and the fragment was ligated to the *Eco*RI-digested vector. The four GAL4 binding sites in the resulting constructs are in the same orientation as the HIV-1 transcription unit. p(4xG17)pHIV-1/R was constructed by insertion of an *Asp* 718 fragment containing the four GAL4 binding sites into an *Asp* 718 site located 140 bp upstream of the HIV-1 transcription start site. The GAL4 sites are in the same orientation as the HIV-1 transcription unit. To generate the p(4xG17)HIV-1/R+34Δ4 and p(4xG17)HIV-1/R+34Δ4-FAS constructs, the p(4xG17)HIV-1/R and p(4xG17)HIV-1/R+34Δ4 constructs were partially digested with *Sac*I, which cleaves three times in the construct, the 3′ overhangs were removed with Klenow enzyme, and linear molecules resulting from a single cleavage were isolated by gel electrophoresis and religated. The correct constructs, which were checked by sequencing, have lost the *Sac*I site at position +34 downstream of the HIV-1 transcription start site as a result of a 4-bp deletion. All of the altered-specificity TBP expression plasmids as well as pCG-GAL(1–94)VP16(413–490), which expresses the GAL4(1–94)-VP16 (G-VP16) fusion protein, and pCG-GAL(1–94)CTF^{P}(399–499), which expresses the G-proline-rich CTF activation domain ($G\text{-}CTF^{P}$) protein, have been described previously (5, 47). pCGN-Tat was constructed by subcloning Tat-encoding sequences from pCG-Tat (36) into the mammalian expression vector pCGN (44), which adds a 15-amino-acid hemagglutinin (HA) epitope tag to the amino terminus of the expressed protein. To construct pCGN-GAL(1–94)Tat, which expresses the G-Tat protein, a *Sty*I-*Xba*I fragment from pUC119sma::GAL(1–94) (a kind gift from M. Tanaka) containing GAL(1–94)-encoding sequences was ligated into pCGN-Tat cleaved with *Xba*I, which cleaves just 5′ of the sequences encoding the amino terminus of Tat.

Transfections. HeLa cells were transfected by electroporation. Cells were grown in Dulbecco minimal essential medium supplemented with 10% fetal calf serum to a density of 80 to 90%. For each sample, the cells from one 10-cm-diameter dish were trypsinized and resuspended in 250 μl of medium. A mixture of plasmids containing 7 μg of the HIV-1 reporter plasmid, 1 μg of pα1x72, a plasmid carrying the human α-globin gene, 0.5 μg of pCGN or pCGNTat, various amounts of a TBP-expressing plasmid as indicated in the figure legends, and salmon sperm DNA carrier to a total amount of 20 μg in 30 μl of TE (10 mM Tris-HCl [pH 7.5], 1 mM EDTA) was added to the cells. The mixture was incubated at room temperature for 5 min, transferred to a Gene Pulser cuvette (0.4-cm-wide electrode gap; Bio-Rad), and electroporated with a pulse of 260 V and 960 μF in the Gene Pulser (Bio-Rad). The cells were then transferred to 10-cm-diameter dishes containing 10 ml of medium and collected 24 h later. RNase T_1 protection analyses were performed on RNA extracted from half of each sample, while Western blot (immunoblot) analyses were performed on protein extracts collected from the other half of each sample with an antibody (12CA5) directed against the HA epitope tag to determine TBP and Tat expression levels. All transfection experiments were repeated at least once, and most of them were repeated two or more times, with similar results.

RNase T_1 mapping. RNA was collected, and RNase T_1 protections were performed as described previously (11). Two probes were used: α98, which detects correctly initiated RNA derived from the α-globin internal reference gene, and T3/534, which detects the two classes of transcripts derived from the HIV-1 reporter constructs (36). To generate the T3/534 riboprobe, each HIV-1 construct was cleaved with *Eco*RI and used as a template for bacteriophage T3 RNA polymerase. Correctly initiated transcripts were quantitated with a Bio-Imaging Analysis System (Fuji) with normalization to the α-globin signal from the internal reference.

GST pull-down assays. Glutathione *S*-transferase (GST)–Tat (obtained from S. Brand, originally from A. Rice) and GST were expressed and purified from *Escherichia coli* as described by Herrmann and Rice (13). GST fusion proteins were bound to glutathione-agarose beads. The amounts of protein bound to the beads were quantitated by fractionation on a sodium dodecyl sulfate (SDS)-polyacrylamide gel along with known amounts of bovine serum albumin. The different TBP_{AS} (TBP with a triple amino acid substitution mutants subcloned in pCite or pTβ (a kind gift of Siegfried Ruppert) were expressed as L-[^{35}S]methionine-labeled proteins by translation in vitro with a TnT kit from Promega. The amounts of the different TBP_{AS} mutants were quantitated with a phosphor imager (Fuji) after fractionation on an SDS-polyacrylamide gel. The volumes for each TBP_{AS} mutant protein were then adjusted to contain equal counts per microliter.

Beads containing 3 μg of GST fusion protein were incubated with equivalent amounts of ^{35}S-labeled TBP_{AS} mutants in a final volume of 24 μl for 40 min at 4°C with constant rotation. Beads were then gently spun down, and the supernatant was removed. The beads were then washed four times with 800 μl of HEMGN (25 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid [HEPES; pH 7.9], 12.5 mM $MgCl_2$, 0.1 mM EDTA, 10% glycerol, 0.1% Nonidet P-40, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride) containing 0.15 M KCl and twice with 800 μl of HEMGN containing 0.3M KCl as described previously (46). The beads were then boiled in Laemmli buffer (24), and the supernatants were fractionated by polyacrylamide gel electrophoresis (PAGE) on SDS–12.5% polyacrylamide gels. The gels were fixed and dried, and the amounts of ^{35}S-labeled TBP_{AS} mutant proteins bound to the beads were quantitated by phosphor imager (Fuji) analysis. The amounts of $hTBP_{AS}$ retained on GST beads were less than 1.5% of the amounts bound to the GST-Tat beads. The results are expressed as percentages of the amount of $hTBP_{AS}$ bound to the GST-Tat beads, which was set at 100% after subtraction of the background signal obtained with the GST beads. Approximately 10% of the $hTBP_{AS}$ input bound to the GST-Tat agarose beads.

RESULTS

To assess the effects of different mutations in TBP on transcription from the HIV-1 promoter, we used an assay based on a triple amino acid substitution of TBP, as described by Strubin and Struhl (42). The triple mutation relaxes the DNA binding specificity of the protein, allowing it to bind to an altered TATA box, TGTAAA, as well as to the canonical TATAAA box. Endogenous TBP cannot recognize the altered TGTAAA box; thus, the effects of further mutations in TBP_{AS} on transcription from a promoter containing the altered TATA box can be assessed in vivo without interference from endogenous TBP (10, 19, 42, 47).

We introduced the altered TATA box into the previously

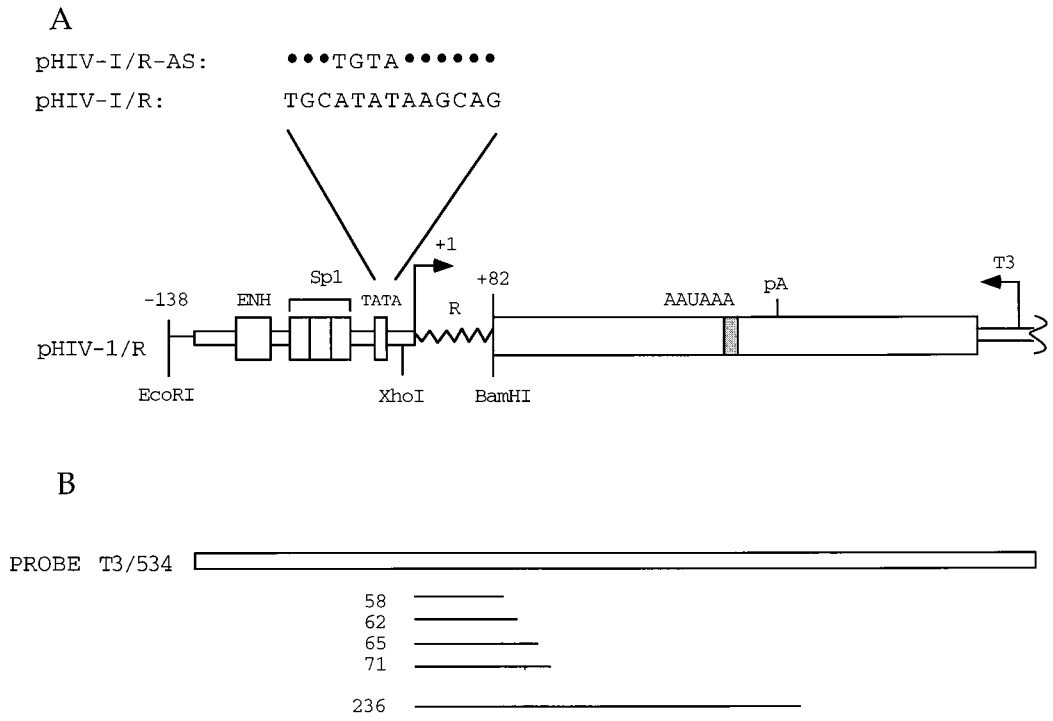

FIG. 1. Reporter constructs. (A) pHIV-1/R (36) contains HIV-1 sequences extending from positions −138 to +82 that include (i) the HIV-1 promoter with the enhancer (ENH), the three Sp1 binding sites, and the TATA box and (ii) R sequences from positions +1 to +82 (wavy line), which contain the IST and TAR elements. Downstream is an adenovirus type 2 fragment (large open rectangle) containing the L3 polyadenylation site (pA), followed by a fragment carrying the bacteriophage T3 promoter. The mutations introduced in the TATA box region in pHIV-1/R-AS are indicated. (B) Complementary RNA probe T3/534 synthesized from the indicated bacteriophage T3 promoter is protected over 58 to 71 nucleotides by short transcripts and over 236 nucleotides by full-length transcripts.

described construct pHIV-1/R (36), which is diagrammed in Fig. 1A. This construct contains HIV-1 sequences from positions −138 to +82 relative to the cap site (position +1) and thus comprises the HIV-1 promoter region as well as sequences from the HIV-1 long terminal repeat R region spanning the IST element and encoding the TAR RNA element (positions +18 to +44). Downstream of the HIV-1 R region is a fragment derived from adenovirus type 2 that contains the L3 polyadenylation signal, followed by an antisense bacteriophage T3 promoter which serves to generate perfectly complementary RNA probes that map simultaneously the 5′ and 3′ ends of short and long transcripts. We replaced the wild-type HIV-1 TATA box (ATATAA) with the altered TATA box TGTAAA to generate the construct pHIV-1/R-AS (Fig. 1A).

The wild-type HIV-1 reporter and its altered TATA box derivative were transfected into HeLa cells together with (i) an expression vector (pCGNTat) that produces a Tat protein tagged at its amino terminus with the HA epitope (34) or a control vector (pCGN) lacking Tat coding sequences, (ii) an expression vector (e.g., pCGNhTBP$_{AS}$) that produces an HA-tagged altered-specificity TBP or various derivatives thereof (47), and (iii) an internal control plasmid (pα1x72) carrying the human α-globin gene, whose expression is not affected by Tat. In all experiments, we assayed both Tat and TBP expression by performing immunoblots with monoclonal antibody 12CA5, which recognizes the HA tag (34). We also extracted RNA to quantitate the accumulation of short and long HIV-1 transcripts as well as α-globin transcripts by RNase T_1 protection analysis. As indicated in Fig. 1B, the HIV-1 probe (designated T3/534) is protected over 58 to 71 nucleotides by short transcripts and over 236 nucleotides by full-length transcripts polyadenylated at the L3 site.

Synthesis of short and full-length transcripts in the absence of Tat as well as activation by Tat depend on TBP bound to the TATA box. To determine whether human TBP$_{AS}$ can selectively activate transcription from the HIV-1 promoter containing an altered TATA box, we compared the patterns of transcripts generated by the wild-type HIV-1 reporter (pHIV-1/R) with that generated by the altered TATA box reporter (pHIV-1/R-AS) in the presence or absence of Tat and various TBP expression constructs. The resulting RNase T_1 analysis is shown in Fig. 2A. As observed previously (36, 41), transfection of the parent construct pHIV-1/R into HeLa cells gave rise to a large amount of short transcripts (lane 1, bands labeled ST) and a small amount of full-length transcripts (lane 1, band labeled FL). Cotransfection of the Tat expression vector led to a dramatic 50-fold increase in full-length transcripts and an 85% reduction in short transcripts (compare lanes 1 and 2). We have shown before that both the short and long transcripts are correctly initiated and that the long transcripts are polyadenylated at the L3 polyadenylation site (36).

Changing the HIV-1 TATA box to the sequence TGTAAA (construct pHIV-1/R-AS) dramatically reduced both synthesis of full-length and short transcripts in the absence of Tat (compare lane 3 with lane 1) and Tat activation of full-length transcripts (compare lane 6 with lane 2), indicating that the HIV-1 TATA box is required for all these processes. We then tested whether transcription from the HIV-1 promoter with the altered TATA box could be rescued by the altered-specificity hTBP. Cotransfection of increasing amounts of the hTBP$_{AS}$ expression plasmid resulted in increasing amounts of full-length and short transcripts in the absence of Tat, up to the levels observed with the parent construct (compare lanes 4 and 5 with lane 1). Moreover, activation by Tat could be restored to

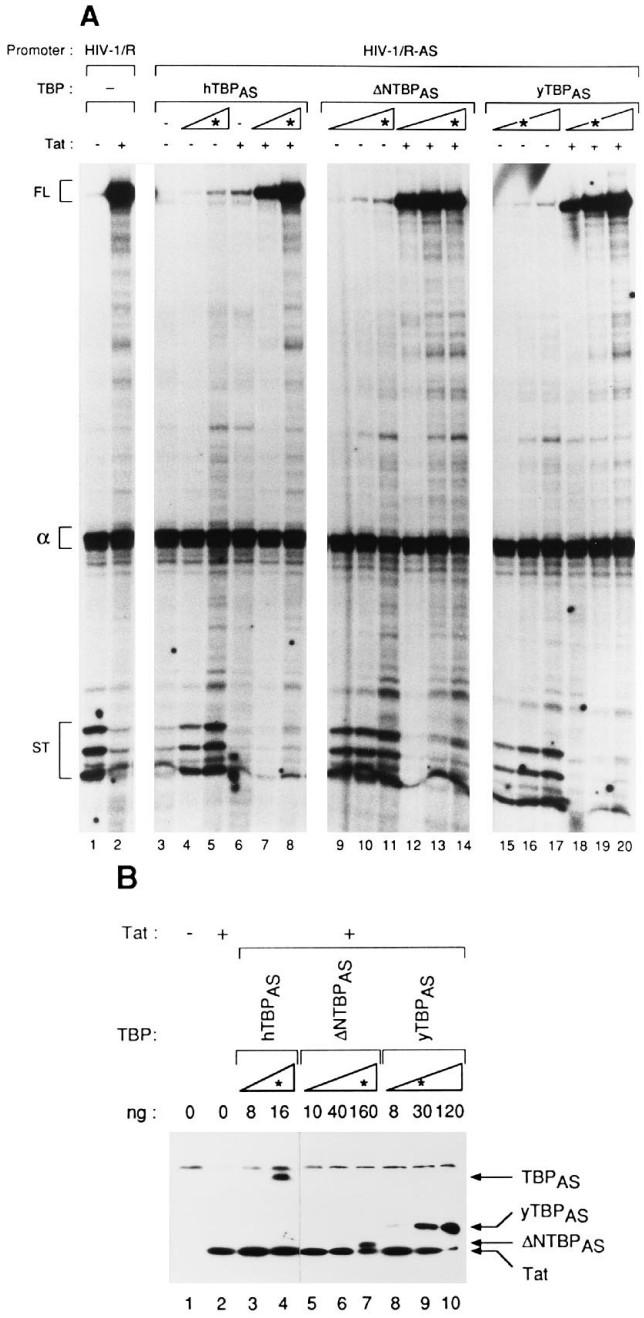

FIG. 2. TBP bound to the TATA box is required for HIV-1 long terminal repeat transcription. (A) RNase T_1 analysis of RNA isolated from HeLa cells transfected with a plasmid (pα1x72) carrying the α-globin internal reference gene, pCGN (− lanes) or pCGNTat (+ lanes), pHIV-1/R (lanes 1 and 2) or pHIV-1/R-AS (lanes 3 to 20), and the TBP_{AS} expression plasmids indicated above the lanes. The probes used were α98, a probe that protects correctly initiated α-globin RNA over 98 nucleotides, and T3/534. α, correctly initiated α-globin RNA; ST, correctly initiated short transcripts; FL, RNA correctly initiated and polyadenylated at the L3 site. The samples transfected with amounts of TBP expression plasmids yielding equivalent amounts of expressed protein (see panel B) are indicated by asterisks. (B) An aliquot of the transfected cells used for the RNase T_1 analysis shown in panel A was used for enhanced chemiluminescence analysis with antibody 12CA5. The amounts of the different TBP expression vectors used in the transfections are indicated above the lanes. The positions of TBP_{AS}, $yTBP_{AS}$, $\Delta NTBP_{AS}$, and Tat are indicated. The lanes showing equivalent amounts of expressed protein are marked by asterisks.

near wild-type levels (compare lanes 7 and 8 with lane 2). As controls, cotransfection of hTBP (without the altered-specificity mutation) restored less than 10% of HIV-1 transcription in the absence and presence of Tat, and $hTBP_{AS}$ did not rescue transcription from an HIV-1 promoter containing another mutation in the TATA box (CTGTAAC replacing the wild-type sequence ATATAAG) (data not shown). Together, these results demonstrate that in vivo, synthesis of full-length and short transcripts in the absence of Tat as well as activation by Tat require TBP bound to the HIV-1 TATA box.

The conserved carboxy-terminal domain of hTBP and full-length yTBP can support the synthesis of short and full-length transcripts in the absence and presence of Tat. TBP consists of two domains: an N-terminal domain that has diverged widely during evolution, and a phylogenetically conserved, protease-resistant, C-terminal domain that can bind to TATA boxes and perform virtually all of the TBP functions tested so far (reviewed in reference 12; see also reference 47). The yeast and human C-terminal domains are 81% identical and have been shown to be interchangeable for response to a number of different activators, including acidic, glutamine-rich, and proline-rich activators, both in vitro and in vivo (19, 20, 42, 47, 52). However, yTBP cannot substitute for hTBP to mediate retinoic acid-dependent activation in EC cells (19). Thus, we wanted to determine whether HIV-1 transcription could be mediated by a truncated human TBP lacking the conserved amino-terminal domain and by yTBP.

We cotransfected plasmids expressing an $hTBP_{AS}$ missing the nonconserved N-terminal domain ($\Delta NTBP_{AS}$) or yeast altered-specificity TBP ($yTBP_{AS}$), along with the altered TATA box HIV-1 reporter plasmid and the Tat-expressing or the control vector. Figure 2A shows that increasing amounts of both the $\Delta NTBP_{AS}$ and $yTBP_{AS}$ expression vectors could rescue the ability of the altered TATA box HIV-1 reporter to transcribe short and full-length transcripts in the absence and presence of Tat. Equivalent amounts of HIV-1 transcription could be restored with 160 ng of the $\Delta NTBP_{AS}$, 30 ng of the $yTBP_{AS}$, and 16 ng of the $hTBP_{AS}$ expression vectors (lanes labeled with asterisks in Fig. 2A). In Fig. 2B, an immunoblot analysis with monoclonal antibody 12CA5 directed against the HA tag shows that 160 ng of the $\Delta NTBP_{AS}$, 30 ng of the $yTBP_{AS}$, and 16 ng of the $hTBP_{AS}$ expression vectors yielded approximately equivalent amounts of protein. Thus, comparable levels of the $hTBP_{AS}$, $\Delta NTBP_{AS}$, and $yTBP_{AS}$ proteins are able to support comparable synthesis of full-length and short transcripts in the absence of Tat and comparable levels of Tat activation. This result suggests that the TBP residues important for all transcription from the HIV-1 long terminal repeat reside in the C-terminal domain of TBP and are conserved between yeast and human species.

Synthesis of full-length transcripts and synthesis of short transcripts display similar sensitivities to mutations in the C-terminal domain of TBP in vivo. We next wanted to determine whether TBP exerts different functions in the synthesis of full-length and short transcripts from the HIV-1 promoter. For this purpose, we tested the effects of several alanine substitutions in the C-terminal domain of hTBP, which had been tested before for their effects on activation by a variety of activation domains (46a, 47). These substitutions target conserved, charged residues located on the solvent-exposed surface of the DNA-bound protein (21, 22, 33). Table 1 indicates the amino acids changed to alanine in each TBP mutant, and Fig. 3 shows their locations on the crystal structure of *Arabidopsis thaliana* TBP (21). The mutations target the four α helices (H1, H1′, H2, and H2′) and two prominent loops connecting strands S3 and S4 (S3/S4) and S3′ and S4′ (S3′/S4′). We also tested

TABLE 1. Altered-specificity TBP molecules

Name	Mutation
$yTBP_{AS}$	None
$hTBP_{AS}$	None
ΔN	Δ2-159
H1 DA	D-179→A
H1 KA	K-181→A
H1′	R-269→A
	E-271→A
H2	R-231→A
	R-235→A
	R-239→A
H2′E1A	E-323→A
H2′E2A	E-326→A
S3/S4	E-206→A
	R-208→A
S3′/S4′	K-297→A
	R-299→A

combinations of two or more of the mutations listed in Table 1; for example, the TBP mutant H2+S3/S4 contains both the H2 and S3/S4 sets of alanine substitutions. All of the TBP mutants tested in this study have been shown previously to be efficiently expressed in HeLa cells (46a, 47).

Figure 4A shows the results for three of these mutant TBPs. Immunoblot analysis revealed that transfection of 16 ng of $hTBP_{AS}$, 12 ng of S3/S4, 20 ng of H2+S3/S4, and 16 ng of S3/S4+S3′/S4′ expression vectors resulted in similar amounts of expressed protein (data not shown); the pairs of lanes corresponding to these amounts are marked with asterisks in Fig. 4A. Because the levels of full-length transcripts synthesized in the absence of Tat are very low and therefore difficult to quantitate, we measured the levels of Tat-activated full-length transcripts, as well as the levels of short transcripts in the absence of Tat, and the results are shown in a graph form in Fig. 4B. Also shown are the results obtained for all of the other TBP mutations tested except the two single point mutations in helices H1 and H2′ (Table 1), which behaved indistinguishably from $hTBP_{AS}$ (data not shown). These latter mutations also have no effect on activation by several types of DNA-targeted activation domains (46a).

None of the TBPs mutated in a single region of the protein significantly impaired the synthesis of short transcripts in the absence of Tat or Tat's ability to induce full-length transcripts (Fig. 4B, mutants H1′, H2, S3/S4, and S3′/S4′). In fact, the S3/S4 mutation resulted in higher than wild-type synthesis of short transcripts in the absence of Tat (Fig. 4A; compare lanes 9 and 3) and Tat activation (Fig. 4A; compare lanes 13 and 14 with lanes 7 and 8; Fig. 4B). Thus, the ability of TBP to mediate IST function and activation by Tat is surprisingly resistant to single sets of alanine substitutions, as observed before for activation by other activators (47). However, several mutant TBPs containing alanine substitutions in two or more regions of the protein mediated reduced synthesis of short transcripts in the absence of Tat and a reduced response to Tat. For example, with the H2+S3/S4 TBP mutant, the levels of short transcripts were reduced by slightly more than 80%, and the levels of Tat-activated full-length transcripts were reduced by more than 60%, relative to those obtained with $hTBP_{AS}$ (Fig. 4A, lanes 7, 8, 19, and 20; Fig. 4B). Notably, the H1′+H2+S3/S4+S3′/S4′ TBP mutant did not restore HIV-1 transcription above the levels observed in the absence of any cotransfected $hTBP_{AS}$ (Fig. 4B).

As is evident from the graph in Fig. 4B, all of the TBP mutations had very similar effects on formation of short transcripts in the absence of Tat and full-length transcripts in the presence of Tat. This finding suggests that these mutations target interactions of TBP with compounds of the basal transcription machinery that are required for the synthesis of both short transcripts and full-length transcripts, rather than factors specifically required for one or the other of these two processes. The similar sensitivities of full-length and short transcript synthesis to mutations across a wide region of the surface of TBP suggests that TBP is used similarly for production of both full-length and short transcripts.

The abilities of mutant TBP_{AS} molecules to support Tat activation in vivo do not correlate with their abilities to bind to GST-Tat in vitro. Tat has been shown to interact with a GST-TBP fusion protein in vitro (17). This interaction is competed for by a peptide containing the conserved core domain of Tat but not by a peptide containing a mutation that impairs Tat activation in vivo, suggesting that a direct Tat-TBP interaction is required for Tat activation in vivo. To explore this question further, we tested the abilities of the different TBP mutants used in the in vivo Tat activation assay described above to interact with Tat in vitro in a GST pull-down assay.

In vitro-translated radiolabeled wild-type and mutant TBP_{AS} molecules were mixed with GST-Tat fusion protein bound to glutathione-agarose beads. The beads were washed extensively, and the amounts of radiolabeled TBP that remained bound to GST-Tat were determined by SDS-PAGE followed by quantitation with a phosphor imager (see Materials and Methods for details). We first determined that $\Delta NTBP_{AS}$ and $yTBP_{AS}$ bound to GST-Tat beads similarly to full length $hTBP_{AS}$ (data not shown) and then tested several mutant TBP_{AS} molecules. Figure 4B shows the results, expressed as percentages of the amount of wild-type $hTBP_{AS}$ retained on the GST-Tat beads. Many of the mutations had significant effects on binding of TBP to GST-Tat. Strikingly, however, two TBP mutants that were severely reduced in their abilities to interact with Tat in vitro (H2 and S3′/S4′) still supported wild-type (or, in the case of S3′/S4′, higher than wild-type) levels of Tat activation in vivo. Further, the mutant H2+S3/S4 bound GST-Tat more efficiently than, for example, S3′/S4′ but sustained Tat activation in vivo much less efficiently. Thus, the abilities of mutant TBP molecules to sustain Tat activation in vivo do not correlate with their abilities to interact with Tat in vitro, suggesting that a TBP-Tat interaction is not essential for Tat activation in our in vivo assay.

Tat responds to C-terminal TBP mutants like G-CTF^P. A number of activation domains targeted to the DNA by fusion

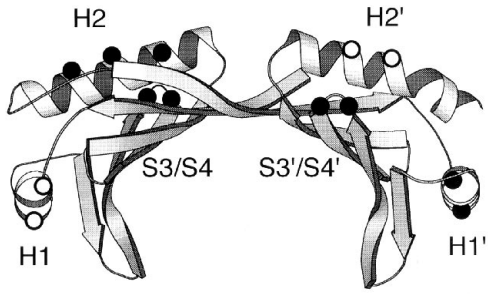

FIG. 3. Locations of the alanine substitutions on the *A. thaliana* TBP structure (33). The positions of clustered alanine substitutions in the H1′ and H2 helices as well as in the loops between the S3 and S4 strands (S3/S4) and S3′ and S4′ strands (S3′/S4′) are indicated by black dots. The single alanine substitutions in the H1 (D-179→A and K-181→A) and H2′ (E-323→A and E-326→A) helices are indicated by white dots.

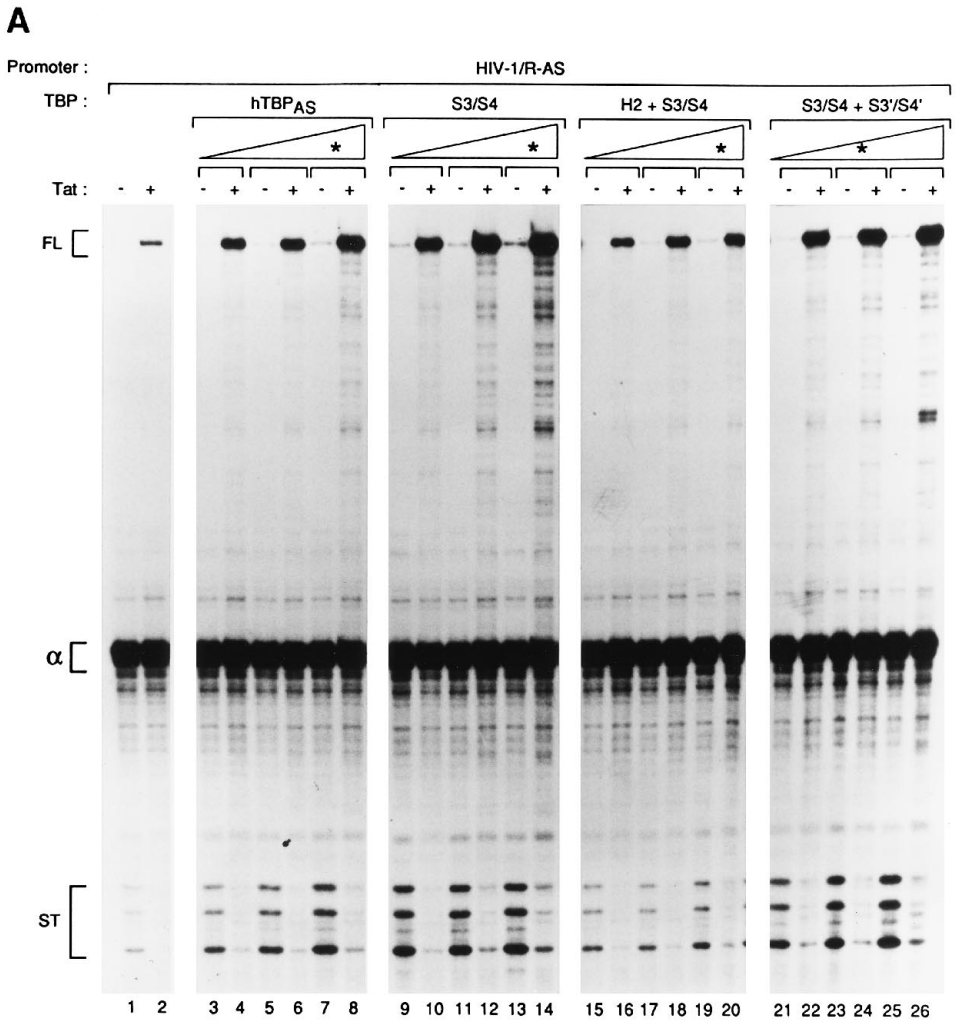

FIG. 4. Mutations in the C-terminal domain of TBP affect synthesis of short and full-length transcripts similarly. (A) RNase T_1 analysis of RNA isolated from HeLa cells transfected with pα1x72, pCGN (−lanes) or pCGNTat (+ lanes), pHIV-1/R-AS, and increasing amounts of the TBP expression plasmids hTBP$_{AS}$ (lanes 3 to 8), S3/S4 (lanes 9 to 14), H2+S3/S4 (lanes 15 to 20), and S3/S4+S3′/S4′ (lanes 21 to 26). The lanes transfected with amounts of TBP expression plasmid yielding equivalent amounts of expressed protein as determined by Western blotting with monoclonal antibody 12CA5 (data not shown) are indicated by an asterisk. Transcripts are designated as in Fig. 2A. (B) Quantitation of RNase T_1 and in vitro GST pull-down results for the different TBP$_{AS}$ mutations tested. The mutants' abilities to support Tat *trans* activation of full-length (FL) transcripts (left-hand bars) and short (S) transcript formation in the absence of Tat (middle bars) and, for some of them, to bind to GST-Tat in vitro (right-hand bars) are graphed as percentages of wild-type TBP$_{AS}$ activity. Tat *trans* activation of full-length transcripts and short transcript formation in the absence of Tat were determined by quantitation of the RNase T_1 analyses with a phosphor imager and normalization to the α-globin internal control. Amounts of TBP proteins expressed were normalized by Western blotting as described in the legend to Fig. 2. The relative abilities to bind to GST-Tat are expressed as percentages of the amount of hTBP$_{AS}$ bound to the GST-Tat beads (see Materials and Methods for details).

to the heterologous GAL4 DNA-binding domain (residues 1 to 94) (2), including G-VP16 (5), which contains a single copy of the VP16 activation domain (49), and G-CTF^P (47), which contains a single copy of the proline-rich CTF activation domain (31), have been previously tested for their sensitivities to mutations in TBP (47). The responses of such activators were found to be surprisingly resilient to sets of alanine substitutions in a single region of TBP but were affected by combinations of alanine substitutions in two or more regions of the protein. Different types of activation domains generally responded similarly to the different mutations in TBP, although the VP16 activation domain was more affected by the H1′+S3/S4+S3′/S4′ mutation. Importantly, except for the VP16 activation domain, the effects correlated with the abilities of the different TBP mutants to bind to the largest subunit of TBP, TAF$_{II}$250, in vitro (47).

To determine whether activation by Tat through TAR is sensitive to the same mutations in the C-terminal domain of TBP as activation by DNA-targeted factors, including Tat targeted to the DNA, we inserted four GAL4 binding sites upstream of the HIV-1 enhancer in the construct containing the altered TATA box to generate the construct p(4xG17)HIV-1/R-FAS (Fig. 5A). We then compared the abilities of some mutant hTBP$_{AS}$ derivatives to restore activation of full-length transcripts by Tat and by DNA-targeted activators.

We first compared RNA-targeted Tat with G-VP16 and G-CTF^P (Fig. 5B and C). As observed previously (47), the response of G-VP16 was more strongly affected than that of G-CTF^P by the H1′+S3/S4+S3′/S4′ mutation (Fig. 5B, lane 3; Fig. 5C). Strikingly, RNA-targeted Tat responded to this mutation like DNA-targeted G-CTP^P; in fact, the responses of Tat and G-CTF^P to the different TBP mutations were almost identical. Second, we compared RNA-targeted Tat with G-Tat, in which the entire Tat protein (residues 1 to 86) is targeted to

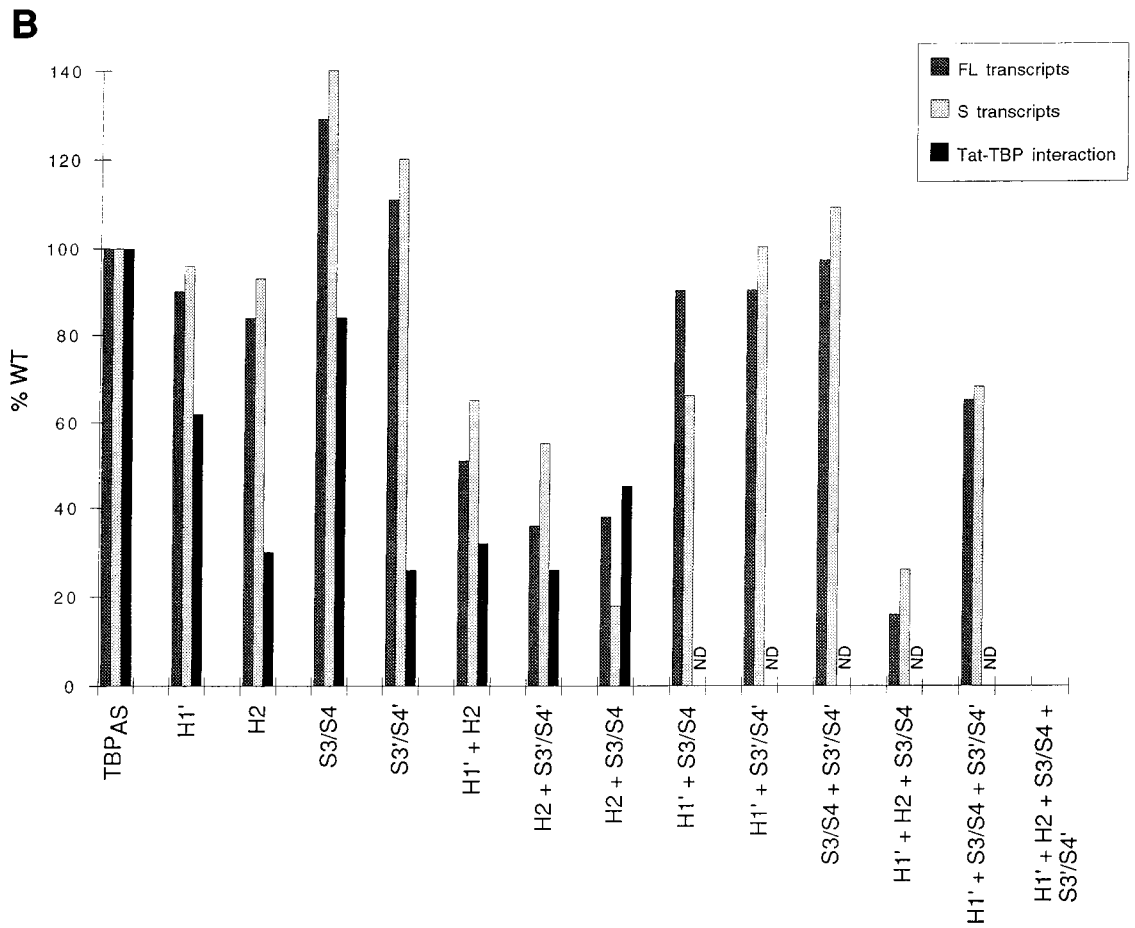

FIG. 4—*Continued.*

the DNA by fusion to the GAL4(1–94) DNA binding domain. To ensure that the G-Tat fusion protein could activate only through its DNA target, the GAL4 binding sites, we tested G-Tat with a derivative of the HIV-1 reporter construct containing the four GAL4 binding sites in which TAR had been debilitated by a four-nucleotide deletion (mutation +34Δ4) [construct p(4xG17)HIV-1/R+34Δ4-FAS (Fig. 5A)]. Figure 5D shows that the two activators responded virtually identically to the various mutations in TBP. Thus, mutations in the C-terminal domain of TBP affect activation by Tat and activation by the DNA-targeted activator G-Tat and G-CTF^P similarly. Since the effects of these TBP mutations on activation by G-CTF^P have been shown before to correlate with effects on binding to $TAF_{II}250$, this result suggests that the HIV-1 TATA box recruits TFIID in vivo and that regardless of whether it is targeted to the DNA or the RNA, the Tat activation domain functions through TFIID to activate transcription.

DISCUSSION

As a first step toward characterization of the transcription complexes that assemble on the HIV-1 promoter in vivo, we have examined the TBP requirements for the synthesis of short and full-length transcripts in the absence and presence of Tat. We find that all these processes are similarly affected by various mutations in the C-terminal domain of TBP, suggesting that they use TBP similarly.

TBP bound to the TATA box is required for the synthesis of short and full-length transcripts in the absence and presence of Tat. By using an RNase protection assay, which, unlike a chloramphenicol acetyltransferase assay, ensures that the observed transcription signals result from correct initiation at the HIV-1 promoter, we show that mutation of the HIV-1 TATA box to the sequence TGTAAA strongly reduces the synthesis of short and long transcripts in the absence of Tat. This mutation also severely impairs activation by Tat. Further, expression of an altered specificity TBP molecule capable of binding to the altered TATA box restores original levels of short and long transcripts in the absence of Tat as well as activation by Tat. Together, these results indicate that the TATA box is critically important for all transcription from the HIV-1 promoter, consistent with the results of Rittner et al., (37), who find that the TATA box is required for HIV-1 transcription in vitro and functions by recruiting TBP. This result is in contrast, however, with those of previous experiments in which mutations in the HIV-1 TATA box affected Tat activation but not basal synthesis of long transcripts in the absence of Tat (1, 28, 35). Because basal levels of long transcripts are low, we cannot exclude the possibility that mutations in the TATA box affect activation by Tat more severely than synthesis of long transcripts in the absence of Tat. However, the observation that expression of the altered-specificity TBP restores basal synthesis of long transcripts to wild-type levels clearly implicates TBP in the process.

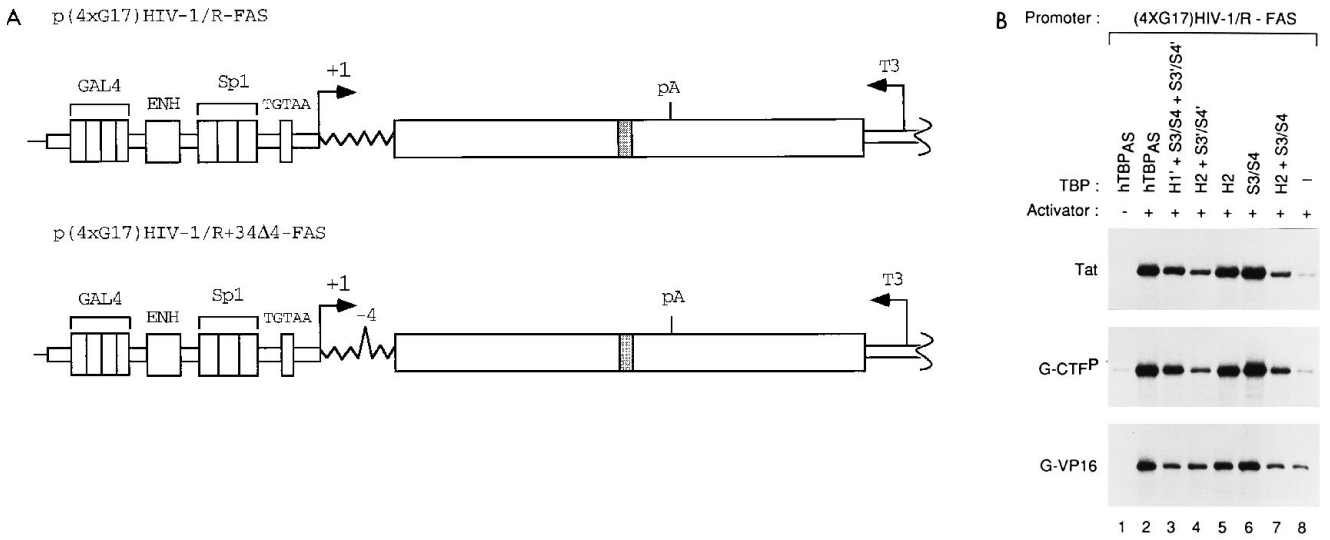

FIG. 5. Tat responds to TBP_{AS} mutants like the DNA-targeted activators G-Tat and G-CTF^P but unlike G-VP16. (A) p(4xG17)HIV-1/R-FAS contains four GAL4 DNA binding sites at position −152 and an altered TATA box. p(4xG17)HIV-1/R+34Δ4-FAS has in addition a four-nucleotide deletion at position +34 in TAR that prevents Tat activation in vivo (36). For abbreviations, see the legend to Fig. 1. (B) RNase T_1 analysis of RNA isolated from HeLa cells transfected with pα1x72, p(4xG17)HIV-1/R-FAS, a Tat (top), G-CTF^P (middle), or G-VP16 (bottom) expression vector, and the TBP_{AS} expression plasmids indicated. Only the full-length transcripts are shown. For comparison's sake, the middle panel is from a longer exposure than the top and bottom panels. (C) Quantitation of the data in panel B expressed as a percentage of activation by $hTBP_{AS}$. The amount of activation by the activator alone (without cotransfection of TBP_{AS} expression plasmid [lane 8]) was subtracted from each band after normalization to the α-globin internal control. (D) Tat *trans* activation of full-length transcripts as determined by RNase T_1 analysis of RNA isolated from HeLa cells transfected with pα1x72, either the Tat expression plasmid and p(4xG17)HIV-1/R-FAS or the G-Tat expression plasmid and p(4xG17)HIV-1/R+34Δ4-FAS, and the indicated TBP_{AS} expression plasmids. The quantitation was performed as described for panel C.

We find that the carboxy-terminal DNA binding domains of both hTBP and yTBP, whose amino-terminal domain is very different from that of hTBP, can mediate the synthesis of full-length and short transcripts as well as activation by Tat. In this respect, Tat behaves like a number of activation domains, including the VP16 acidic, CTF proline-rich, and Sp1 glutamine-rich activation domains, which do not require the nonconserved amino terminal domain of TBP and can use yTBP in human cells (47). This property of Tat is consistent with the observation that the Tat activation domain can function in yeast cells when directed to the DNA (43) and suggests that difficulties in reproducing Tat activation through a TAR RNA element in yeast cells are due to species-specific differences in factors other than TBP.

The synthesis of short transcripts is similar to that of full-length transcripts in its sensitivity to mutations in TBP. We tested a number of mutations in the carboxy-terminal domain of TBP that were tested before for their effects on activation of the c-*fos* promoter by activation domains derived from Sp1, Oct-2, CTF, p53, and VP16 (46, 47). These activation domains respond similarly to the different TBP mutations except for the VP16 activation domain, which is more sensitive to particular mutations in TBP. The effects of mutations in TBP on responses by these different activation domains correlate very well with the abilities of these mutant TBPs to bind to $TAF_{II}250$ in vitro (47).

We find that when targeted to the HIV-1 promoter, the different mutant TBPs affect similarly synthesis of short transcripts, activation by Tat, and activation by the DNA-targeted activators G-CTF^P and G-Tat. As observed previously for activation by DNA-targeted activators, all transcription from the HIV-1 promoter was surprisingly resistant to single sets of alanine substitutions in TBP but could be impaired by combinations of mutations that also impair binding to $TAF_{II}250$ (47). This finding emphasizes that DNA-targeted and RNA-targeted activators can use TBP similarly. Strikingly, although the abilities of mutant TBPs to sustain Tat activation in vivo correlate well with their previously described abilities to bind to $TAF_{II}250$ in vitro, they do not correlate with their abilities to bind to a GST-Tat fusion protein in vitro. This finding suggests that the TBP-Tat interaction disrupted by the TBP mutations tested here is not required for in vivo function. Similarly, mutations in TBP that debilitate interaction with the VP16 and p53 activation domains have no effect on activation by these domains in vivo (46). Thus, many of the numerous interactions that can occur between TBP and activators in vitro may not be directly relevant in vivo.

The mutations that we have tested do not uncover a TBP domain uniquely required for the synthesis of short transcripts, for activation by Tat, or for activation by DNA-targeted activators. Rather, they seem to disrupt an interaction with a member of the basal transcription machinery required for all types of transcription from the HIV-1 promoter, presumably $TAF_{II}250$ and hence TFIID. These results suggest that although activation by Tat in vitro contrasts with activation by DNA-targeted activators in that it can be reproduced with just recombinant TBP (54), activation by Tat in vivo probably targets a TFIID-containing transcription complex. Further, they suggest that if the HIV-1 promoter generates short and full-length transcripts by assembling different transcription complexes, these transcription complexes both use TBP similarly, probably as part of TFIID.

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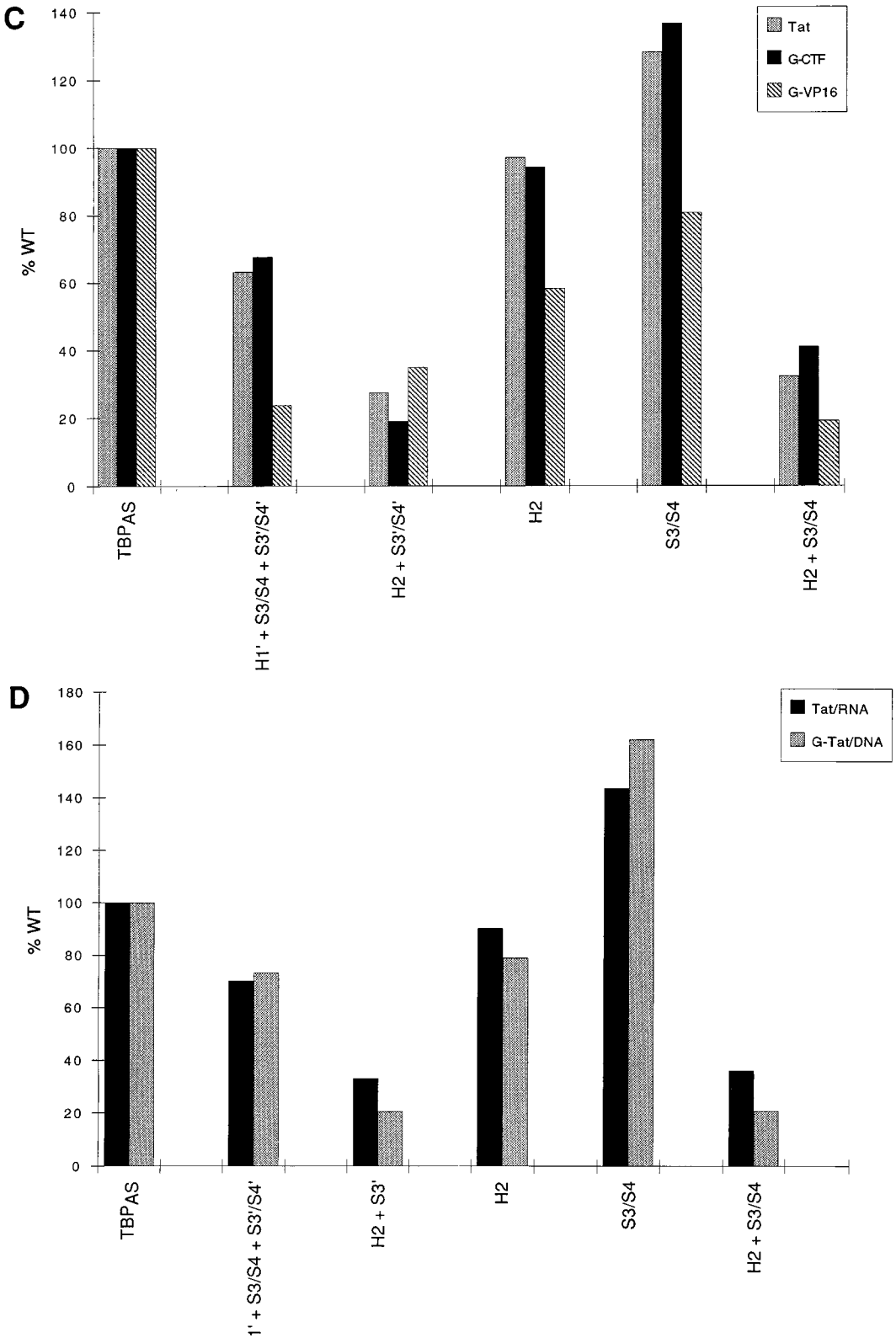

FIG. 5—*Continued.*

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