Proc. Natl. Acad. Sci. USA
Vol. 90, pp. 138–142, January 1993
Biochemistry

The type 1 human immunodeficiency virus Tat binding protein is a transcriptional activator belonging to an additional family of evolutionarily conserved genes

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Communicated by Herbert Weissbach, October 2, 1992

ABSTRACT **The type 1 human immunodeficiency virus Tat protein is a powerful transcriptional activator when bound to an RNA structure (TAR) present at the extreme 5′ terminus of viral mRNA. Since transcriptional activation requires binding of Tat to RNA, it has been suggested that Tat enhances initiation or elongation through a direct interaction with cellular transcription factors. Here we show through protein fusion experiments that the previously identified cellular Tat binding protein, TBP-1, although unable to bind DNA, is a strong transcriptional activator when brought into proximity of several promoter elements. Transcriptional activity depends upon the integrity of at least two highly conserved domains: one resembling a nucleotide-binding motif and the other motif common to proteins with helicase activity. Our studies further reveal that TBP-1 represents one member of a large, highly conserved gene family that encodes proteins demonstrating strong amino acid conservation across species. Finally, we identified a second family member that, although 77% similar to TBP-1, does not activate transcription from the promoters examined. This finding, together with the observation that TBP-1 does not activate each promoter examined, suggests that this gene family may encode promoter-specific transcriptional activators.**

The type 1 human immunodeficiency virus (HIV) Tat protein (1, 2) represents one of two viral regulatory proteins essential for virus replication (3). Although the mechanism of Tat function has been controversial, the establishment of a cell-free transactivation system clearly demonstrates that Tat is a potent transcriptional activator (4–7). The *in vitro* results further suggest that Tat functions at the level of elongation (4, 5).

The mechanism of Tat function appears to differ from that of other transcriptional activators. For example, Tat elicits its effect after interaction (6, 8) with an RNA structure [referred to as TAR (9)] present at the extreme 5′ terminus of each viral transcript. The TAR sequence has been shown to form a stem–loop structure containing a bulge in the stem. Sequences within the loop and bulge are essential for Tat function *in vivo* (8, 10–12), although *in vitro* analysis suggests that the bulge sequences and not the loop are required for interaction with Tat (6, 8). These findings imply that cellular factors that associate with loop sequences may be essential for the transactivation response. Indeed several studies have identified specific cellular factors that associate with the loop sequence (13–16).

The ability to elicit transactivation without direct interaction with DNA suggests that Tat might associate with cellular factors, which in turn interact with DNA or promoter-bound transcription factors. Using purified Tat protein to screen a λgt11 cDNA library, we previously described the identification of a cDNA that encodes a protein designated Tat binding protein 1 (TBP-1), which associates with Tat (17). More recent reports suggest that TBP-1 represents one member of a large highly conserved gene family. One human homolog, designated MSS-1, shares 42% sequence identity with TBP-1 and has been shown to enhance Tat-mediated transactivation (18). MSS-1 was originally identified by transcomplementation of a yeast *Sgv-1* mutation, which encodes a CDC28/CDC2 related kinase that appears to regulate G1 cyclins (19). A second homolog termed SUG-1, or TBP-γ, identified in the yeast *Saccharomyces cerevisiae* also shares considerable homology with TBP-1 (20, 21). A mutant form of SUG-1, which is an essential gene in yeast, was isolated as a supressor in an assay to determine whether the yeast transcriptional activator GAL4 can function when its C-terminal activation domain is deleted. The diverse manner in which each of these genes was identified does not provide a clear picture relating to their function.

To gain further insight toward the function of TBP-1 and the related family members, a series of protein fusion experiments were performed. A fusion protein of TBP-1 with the DNA binding domain of the yeast transcriptional activator GAL4 demonstrated strong transcriptional activity on several promoters bearing the GAL4 DNA-binding site. Transcriptional activity was abolished following mutation of a putative nucleotide-binding site and a helicase motif. Using a genetic assay in yeast, we observed that TBP-1 can heterodimerize with at least one related human homolog. Surprisingly, the dimerization domain lies outside of the area sharing strong sequence conservation. The ability of TBP-1 to activate only a subset of the promoters examined and the inability of a highly conserved human homolog to activate any of the promoters tested suggest that this gene family may encode a class of promoter-specific transcriptional activators.

MATERIALS AND METHODS

Plasmid Constructions. The plasmids pGAL-TBP-1 and pGAL-TBP-7 were prepared as follows: the full-length genes encoding the TBP-1 and TBP-7 cDNAs were amplified by PCR and ligated into the *Eco*RI–*Xba* I site of the pSG424 mammalian expression vector (22) to create a fusion to the DNA-binding domain of the yeast transcriptional activator protein GAL4 (amino acids 1–147).

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Abbreviations: HIV, human immunodeficiency virus; HA, hemagglutinin; CAT, chloramphenicol acetyltransferase; LTR, long terminal repeat; GAL_{UAS}, GAL4 DNA-binding site; TBP, Tat binding protein; TK, Herpes virus thymidine kinase; MMTV, mouse mammary tumor virus; VP16, herpes simplex viral protein 16.

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The GAL-TBPΔGKT, GAL-TBP/K(235)-H, and GAL-TBP/D(285)-A mutants were prepared in two-step PCR reactions. Epitope-tagged TBP-1 (HATBP-1), TBP-7 (HATBP-7), and TBP1ΔGKT (HATBP-1ΔGKT) genes were constructed by PCR, using a 5′ primer encoding the amino acid sequence MYPYDVPDYA corresponding to the influenza virus hemagglutinin (HA) protein (23) and cloned into the *Hin*dIII–*Xba* I site of the eukaryotic expression vector soC-MVIN (24).

Yeast Plasmids. pGAL-TBP-1, pGAL-TBP-7, and pGAL4-(1–147) were subcloned out of the pSG424-based vectors and inserted into the *Hin*dIII–*Xba* I sites preceding the *ADH1* promoter of pVL100 (25). Deletions of pGAL-TBP-1 were created by PCR to generate the set of pYGAL-TBP plasmids. For herpes simplex viral protein 16 (VP16) fusions, the C-terminal 78 amino acids of VP16 were isolated by PCR from pMSVP16 (26) and subcloned into the *Hin*dIII–*Pvu* II sites preceding the *ADH1* promoter present in pVU100 (25) to create pYVP16. TBP-1 was then inserted in frame into the *Pst* I–*Xba* I site of pYVP16 to generate pYVP16-TBP-1.

Transfections and CAT Assays. Each of the reporter plasmids used in this study contains the bacterial chloramphenicol acetyltransferase (CAT) gene fused to different promoters containing between two and six copies of the GAL4 DNA-binding site (GAL_{UAS}). GAL4-tk-CAT contains five copies of the GAL4 binding site cloned upstream of the Herpes virus thymidine kinase (TK) promoter in the eukaryotic expression vector pBL2CAT (27). Plasmid pGMCSΔGRE/UAS contains the CAT gene downstream of a mouse mammary tumor virus (MMTV) long terminal repeat (LTR) promoter that has five copies of the GAL4 binding site (28). pGAL4-HIV(−40)CAT contains two copies of GAL binding site at nucleotide −40 in the HIV LTR. The G6 LTR plasmids contain six copies of the GAL_{UAS} sequence present at the nucleotide position indicated within the HIV promoter (29).

Cell Culture and Transfection. COS-7 cells were maintained in Iscove's modified Dulbecco's medium supplemented with glucose (4.5 mg/ml), 10% fetal calf serum, and gentamicin (50 μg/ml; GIBCO). For transient transfection assays, 3×10^5 cells were transfected by the DEAE-dextran procedure (30). Cells were transfected with 0.5–1.0 μg of reporter plasmid and 1–2 μg of the pSVGAL-TBP expression vectors. Cells were harvested 48 h after transfection, and CAT assays were performed as described (31). For quantitation of CAT activity, a fluorescence diffusion method was used (32).

Immunofluorescence. COS-7 cells were transfected with plasmid pHATBP-1, pHATBP-7, or pHATBP-1ΔGKT; 2 days posttransfection they were fixed and permeabilized (33). Permabilized cells were incubated with monoclonal antibody raised against the influenza HA epitope, incubated with 10 mM glycine in phosphate-buffered saline at 4°C, washed four times with 10 mM glycine in phosphate-buffered saline, and then incubated with a 1:500 dilution of rhodamine isothiocyanate-conjugated goat anti-mouse IgG.

Western Blot Analysis. For Western blot analysis, 2×10^6 COS-7 cells were transfected with the GAL-TBP expression vectors. Proteins were resolved on 10% polyacrylamide gels and blotted onto nitrocellulose membrane filters. Blots were quenched for 2 h at room temperature with block solution (2% gelatin/50 mM Tris, pH 7.5/0.5 M NaCl), incubated at 4°C with an anti-GAL4 rabbit polyclonal antibody (1:1000) in binding buffer (50 mM Tris, pH 7.5/500 mM NaCl/0.5% gelatin/0.05% Tween 20), and washed four times with washing solution (50 mM Tris, pH 7.5/150 mM NaCl/0.05% Tween 20). Filters were incubated with ^{125}I-labeled protein A (Amersham) in binding buffer, washed, and autoradiographed with Kodak XAR-5 film.

Dimerization Assays. Growth and manipulation of yeast strains were done according to standard procedures (34). YJ0-Z (*GAL4* Δ, *GAL80* Δ, *ura3-52, leu2-3, 112, ade1, his3, MEL-1, GAL-1:lacZ*) contains an integrated copy of the *Escherichia coli* β-galactosidase (*lacZ*) gene under the control of the GAL-1 promoter (35). YJ0-Z was transformed with the indicated expression vectors, and transformants were grown on 3% glycerol/2% lactic acid. β-Galactosidase activity was determined as described (36), and units of activity were calculated by the method of Miller (37).

RESULTS

Isolation of the Full-Length TBP-1 cDNA and a Highly Related Gene. Using biotinylated Tat protein to screen a λgt11 cDNA library, we previously described the isolation of the TBP-1 cDNA (17). The presence of an open reading frame preceding the putative initiator methionine suggested that the full-length clone may not have been obtained. To examine this possibility, the TBP-1 cDNA was used as a probe to rescreen a λgt11 library. Of several clones identified, some were identical to the original TBP-1 cDNA. However, at least two cDNAs that contained additional sequence preceding the AUG codon originally identified were obtained (Fig. 1). The new TBP cDNA contains two additional codons encoding methionine residues preceding the codon encoding the methionine first identified. Translation of this cDNA in an *in vitro* translation lysate suggests that the 5′ AUG is efficiently utilized by the translational machinery (data not shown). Thus, this cDNA likely represents the full-length TBP-1 cDNA. The full-length sequence differs from the cDNA originally described by the presence of an additional 35 N-terminal amino acids.

During rescreening of the library, a gene that demonstrates a striking similarity with TBP-1 was also identified (Fig. 1). The encoded protein, designated TBP-7, is 57% identical and 77% similar to TBP-1 over a 210-amino-acid stretch. As with the other related family members MSS-1 (18) and SUG-1 (20), the TBP-7 sequence homology does not extend through the amino terminus.

Consistent with the premise that TBP-1 functions with Tat to activate gene expression, its subcellular localization was found to be nuclear (17). The functional relatedness between TBP-7 and TBP-1 was also examined by identification of its subcellular localization. As observed with TBP-1, the TBP-7 protein showed a predominant nuclear localization (Fig. 2).

Transcriptional Activity of a GAL–TBP-1 Fusion Protein. To explore the hypothesis that TBP-1 functions with Tat to facilitate transcriptional activation, its ability to interact with DNA was examined. In numerous attempts, using highly purified recombinant TBP-1, we were unable to demonstrate sequence-specific interaction between TBP-1 and DNA. It is possible that TBP-1 does not associate with DNA if it functions through an association with other promoter-bound transcription factors. The requirement for Tat in transactivation might imply that its function is simply to bring TBP-1 to the promoter. To examine the ability of TBP-1 to transactivate in the absence of Tat, a series of protein fusions designed to bring TBP-1 in proximity to the promoter were made. Initially, the entire TBP-1 gene was fused to the DNA-binding domain of the yeast transcriptional activator GAL4 (22). Cotransfection of pGAL-TBP-1 with either the GAL_{UAS}-driven TK or HIV promoter elicited a strong transcriptional activation (Fig. 3*A*). Under quantitative conditions, GAL–TBP-1 was found to activate the GAL4-tk-CAT and HIV LTR 4.8- and 8.6-fold, respectively. Moreover, the transcriptional activity was only slightly below that induced by a chimeric GAL4 derivative [GAL4–p65 (24)] containing the activation domain of NF-κB p65, a bonafide transcriptional activator (38). GAL–TBP-1 was unable to stimulate the GAL_{UAS}–MMTV promoter. The inability to activate this promoter may reflect a function of TBP-1 because activation was achieved in the presence of the

FIG. 1. Amino acid sequence of the TBP-1 and TBP-7 cDNAs and homology with related proteins SUG-1 and MSS-1. The beginning of the amino acid sequence of the longest open reading frame in the TBP-1 and TBP-7 cDNAs is compared to the published amino acid sequence of SUG-1 (20) and MSS-1 (18). The numbers that appear below the sequence correspond to the amino acid positions in TBP-1. Uppercase letters correspond to identity among each of the proteins. The putative nucleotide-binding domain and helicase motifs are single and double underlined, respectively. The shading indicates the conserved amino acids.

GAL–p65 protein. Alternatively, since activation of the MMTV LTR by GAL4–p65 protein is very weak, GAL–TBP-1 may be able to activate this promoter but the activity is below the limit of detection.

The positional requirement of TBP-1 function on a minimal HIV promoter element, which had previously been shown to be sufficient for full activation (39), was examined. To address this question, a series of reporter plasmids were used that contain six copies of the GAL_{UAS} introduced at various points upstream to the minimal HIV promoter (29). The ability of the GAL–TBP-1 proteins to transactivate was independent of the position of the GAL-binding site at positions −83 and −69, but was somewhat reduced at positions −119 and −31. Thus, no significant positional dependency for TBP-1 function was observed (Fig. 3*B*).

Sequence comparison of TBP-1 with the other related family members reveals the presence of a highly conserved potential nucleotide binding motif (GPPGTGKT) and a second region that is similar to a motif found in many helicases (Fig. 1) (40). The importance of the putative nucleotide-binding domain for the transcriptional activity of TBP-1 was examined by creating the mutations TBPΔGKT and TBP/K(235)-H, which deletes residues Gly-Lys-Thr and changes the lysine at position 235 to histidine, respectively. These plasmids were unable to activate either the GAL_{UAS}-driven HIV LTR or TK promoter in cotransfection assays (Fig. 4). A third mutation, TBP/D(285)-A, within the putative helicase motif, which converts the aspartic acid at position 285 to alanine, was unable to transcriptionally activate the promoters examined (Fig. 4). The inability of these plasmids to activate the promoters does not reflect lack of protein expression as the expression of each mutant protein was comparable to that of wild-type GAL–TBP-1 (Fig. 5). The diffuse band observed migrating above the GAL–TBP-1 protein on the Western blot most likely represents a post-translationally modified form of the protein. The strong amino acid conservation between TBP-7 and TBP-1 suggested that TBP-7 might also possess transcriptional activity. However, a full-length GAL–TBP-7 fusion protein, although expressed (Fig. 5), failed to activate the promoters examined (data not shown).

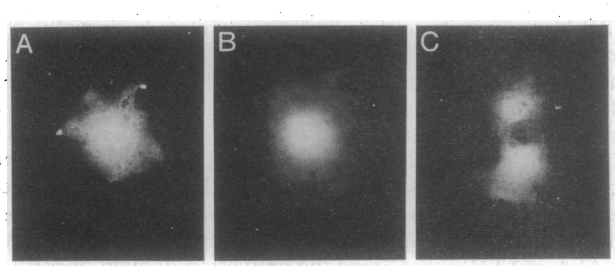

FIG. 2. Subcellular localization of TBP-1, TBP-7, and TBPΔGKT. COS-7 cells were transfected with plasmids pHATBP-1 (*A*), pHATBP-7 (*B*), and pHATBP-1ΔGKT (*C*) and fixed for indirect immunofluorescence 48 h posttransfection.

Dimerization Properties of TBP-1 and Related Family Members. Many of the well-characterized transcriptional activators function after dimerization or heterodimerization with a related family member. To determine if TBP-1 has the ability to dimerize, a genetic system developed to study protein–protein interactions in *Saccharomyces cerevisiae* was employed (41). Briefly, a yeast strain bearing an integrated copy of a GAL_{UAS}–*lacZ* reporter was transformed with a GAL-TBP-1 expression vector (pYGAL-TBP-1) either alone or together with a second plasmid that encodes a fusion protein of TBP-1 with the acidic transcriptional activation domain of the Herpes simplex virus VP16 protein (pYVP16-TBP-1), a strong transcriptional activator in yeast (42). Neither pYGAL-TBP-1 nor pYVP16-TBP-1 was able to induce *lacZ* expression on its own (Fig. 6*A*). However, the combination of both plasmids induced strong transcriptional activity. This reflects an association between the TBP-1 domains bringing the VP16 activation domain into the proximity of the GAL_{UAS}

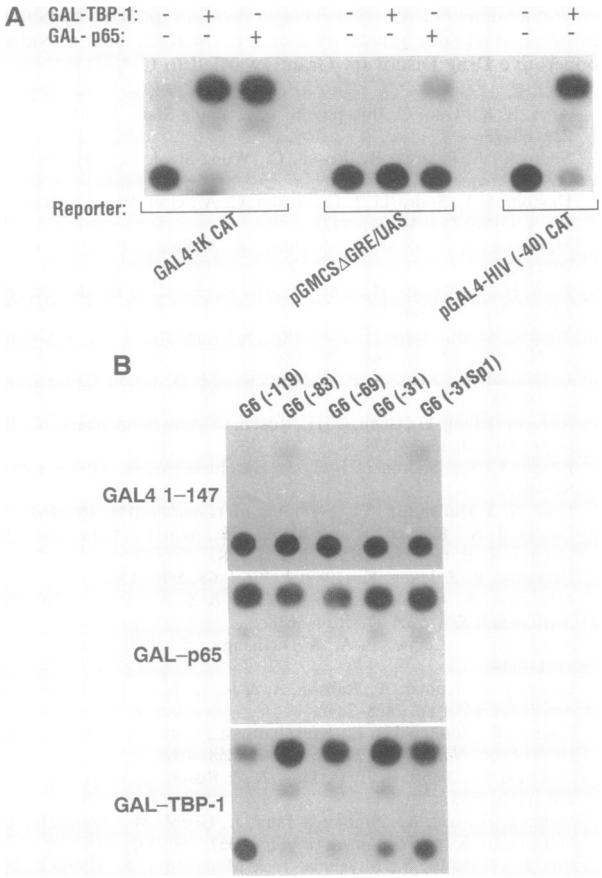

FIG. 3. Transcriptional activity of the GAL-TBP-1 fusion protein. (*A*) CAT assays shown were prepared 48 h posttransfection from cells transfected with the indicated reporter constructs and with GAL4(1–147) or in the presence of the pGAL-TBP-1 or GAL-p65 expression vector. (*B*) Positional requirement for TBP function. The CAT assays shown were performed using cell lysates prepared from cells transfected with the G6 series reporter constructs in the presence of either an expression vector encoding the GAL4(1–147), GAL-p65 fusion protein, or the GAL-TBP-1 fusion protein. The G6 reporter constructs contain six copies of a GAL_{UAS} present at the nucleotide position indicated in an HIV LTR lacking the TAR sequence (29).

and the formation of an active transcription complex. The observation that GAL-TBP-1 does not activate in yeast is not surprising; several mammalian transcriptional activators do not function in this system. The inability to stimulate expression in yeast may suggest that additional cellular factors are required for TBP-1 function.

Deletion analysis of TBP-1 demonstrated that neither deletion of the conserved C-terminal end [pYGAL-TBP(1–396)]

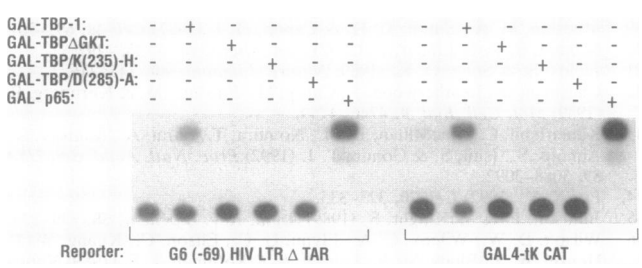

FIG. 4. Mutational analysis of GAL-TBP-1 function. CAT assays shown were prepared from cell lysates obtained from cells transfected with either the G6 (−69) or GAL4-tk-CAT reporter in the presence of the expression vectors encoding the GAL-TBP-1 fusion proteins indicated.

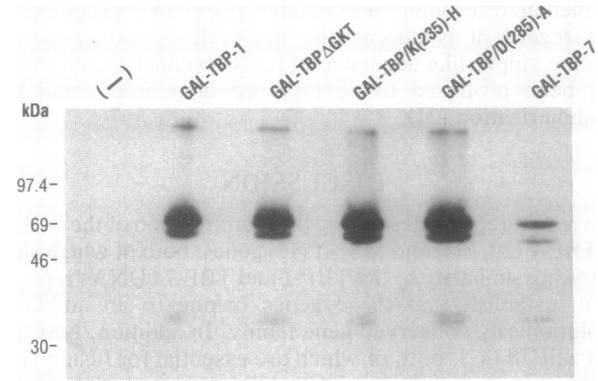

FIG. 5. Expression of GAL-TBP-1, GAL-TBP-7, and GAL-TBP-1 mutants in COS-7 cells. Western blot analysis was performed on total cell homogenates that were prepared from COS-7 cells transfected with the indicated GAL-TBP expression vectors. The samples were separated by polyacrylamide gel electrophoresis, transferred onto a nitrocellulose filter, and overlaid with anti-GAL4 antibody, incubated with ^{125}I-labeled protein A, and autoradiographed.

nor deletion within the putative nucleotide-binding domain [pYVP16-TBP-1(ΔGKT)] affected dimerization (Fig. 6*A*). However, deletions at the N terminus did affect dimerization, and indeed the N-terminal 100 amino acids were shown to be sufficient for dimerization [pYGAL-TBP(1–100); Fig. 6*A*]. Thus, the N-terminal 100 amino acids confer the dimerization potential of TBP-1. Finally, the ability of pYVP16 TBP-1 to activate *lacZ* expression in the presence of pYGAL-TBP-7, albeit less than pYGAL-TBP-1, indicates that TBP-1 has a potential to heterodimerize with at least one related family member.

Inspection of the N-terminal 100 amino acids, of both TBP-1 and TBP-7, reveals the presence of a conserved leucine zipper-like structure (Fig. 6*B*), a motif known to mediate protein–protein interactions (45). In accord with previously characterized leucine zipper motifs, the TBP-1 and TBP-7 motifs are composed of a heptad repeat of five hydrophobic amino acids (predominantly leucine) interdigitated with a high density of oppositely charged amino acids. Thus, dimerization between TBP-1 and related members may

A

DNA Binding Domain Fusion	Activation Domain Fusion	β-galactosidase Activity
pYGAL4 1-147	-	<0.5
-	pYVP16-TBP-1	<0.5
pYGAL-TBP-1	-	<0.5
pYGAL-TBP-1	pYVP16-TBP-1	22.6
pYGAL-TBP-1	pYVP16-TBP-1(ΔGKT)	13.3
pYGAL-TBP (1-396)	pYVP16-TBP-1	27.1
pYGAL-TBP (50-446)	pYVP16-TBP-1	<0.5
pYGAL-TBP (100-446)	pYVP16-TBP-1	<0.5
pYGAL-TBP (1-100)	pYVP16-TBP-1	19.3
pYGAL-TBP-7	-	<0.5
pYGAL-TBP-7	pYVP16-TBP-1	3.6

B

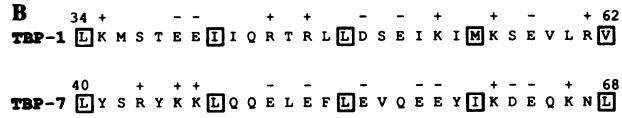

FIG. 6. Dimerization properties of TBP-1. (*A*) The yeast strain YJ0-Z was transformed by the indicated plasmids, and the β-galactosidase activity was determined. The numbers in parentheses denote amino acids present in the GAL-TBP-1 deletion constructs. Activity is expressed in Miller units, and each value presented is the mean activity of four independent transformants. (*B*) The sequence of the putative leucine zipper structures between amino acids 34 and 62 of TBP-1 and amino acids 40 and 68 of TBP-7 is shown. The hydrophobic amino acids forming the heptad repeat of the zipper are boxed, and the charged amino acids are depicted by "+" and "−" signs. While leucine is by far the most common amino acid at every seventh position of the leucine zipper, methionine, isoleucine, and valine appear to be suitable alternatives (43, 44).

be mediated through these leucine zipper-like structures. In this regard, it is noteworthy that TBP-y also contains a leucine zipper-like sequence in its N-terminal region, which has been proposed to be involved in a homo- and heterodimerization (21).

DISCUSSION

The recent reports describing the identification of the SUG-1, or TBP-y (20, 21), and MSS-1 (18) genes, both of which show a striking similarity to the TBP-1 and TBP-7 cDNAs reported here, establish that these genes belong to an additional evolutionarily conserved gene family. In addition, NSF (46) and SEC18 (47), both of which are essential for fusion steps of the secretory pathway, VCP (48), a protein with unknown function, and PAS1 (49), a yeast protein required for peroxisome biogenesis, all show strong sequence similarity.

The diverse circumstances under which each of these genes was identified does not provide insight toward their mechanism of action. The group of proteins appears to fall into two classes: nuclear proteins (TBP-1, TBP-7, SUG-1, and MSS-1) and nonnuclear proteins (NSF, Sec18, VCP, and PAS1). We postulate that the nuclear family is involved in transcription. First, we demonstrate that at least one member of the nuclear family (TBP-1) is a strong transcriptional activator. This activity is dependent on conserved putative nucleotide-binding motif and helicase domains. In addition, MSS-1 and SUG-1 are also thought to be involved in a transcriptional pathway (18, 20). No role or function for TBP-7 was elucidated. However, the observation that the GAL–TBP-1 fusion protein, although a potent transcriptional activator of the HIV and TK promoters, is unable to activate the MMTV promoter, may imply that the nuclear family members, including TBP-7, will show different promoter specificities. The ability of TBP-1 and TBP-7 to form heterodimers suggests that a homodimer unable to activate a specific promoter may indeed function when complexed with a different family member. This possibility, which needs to be addressed further, would potentially expand the function of this gene family.

The demonstration of a transcriptional activation function for TBP-1, which was originally identified through its association with Tat protein (17), may provide further insight toward the mechanism of HIV-1 Tat function. As Tat elicits strong transcriptional activation after its association with RNA, it has been suggested that Tat serves to bring cellular factors, some of which may be transcription factors themselves, into the proximity of the promoter. The recent demonstration that Tat can also function when bound to upstream promoter DNA (29) would be consistent with this hypothesis. If TBP-1 functions with Tat to facilitate transactivation, the importance of the putative nucleotide-binding and helicase motifs for transcriptional activity may provide clues relating to the function of TBP-1. For example, it has been suggested that Tat acts as a processivity factor on RNA polymerase II in a manner analogous to that of transcription factor IIF (5). If TBP-1 provides this function, it might be envisioned that the nucleotide-binding and helicase motifs are important for unwinding of the DNA or RNA·DNA complex to facilitate elongation. Purification of TBP-1 and analysis of its biochemical properties should provide further insight toward its mechanism of action that will likely shed light on its potential role in Tat-mediated transcriptional activation.

We thank Stephen Johnston for providing the yeast strain YJ0-Z, Thomas Shenk for the GAL4-tk-CAT plasmid, Grahm Mao for help with the cDNA screening, and Tina Rose for preparation of the manuscript. C.D.S. is a scholar of the American Foundation for AIDS Research and S.M.R. is a recipient of a fellowship from the Leukemia Society of America. This work was supported by National Institutes of Health grants awarded to M.R.G. and a National Cooperative Drug Discovery Grant awarded to C.A.R.

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