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In Vitro Interaction of the Human Immunodeficiency Virus Type 1 Tat Transactivator and the General Transcription Factor TFIIB with the Cellular Protein TAP

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We have reported the molecular cloning, expression, and characterization of a human cellular protein, TAP, which possesses a strong transcriptional activation domain and binds the human immunodeficiency virus type 1 Tat transactivator in vitro and in vivo (L. Yu, Z. Zhang, P. M. Loewenstein, K. Desai, Q. Tang, D. Mao, J. S. Symington, and M. Green, J. Virol. 69:3007–3016, 1995). Here we show that TAP binds the general transcription factor TFIIB. Furthermore, we delineate the binding domains of TAP, Tat, and TFIIB, as well as measure the strengths and specificity of these protein-protein interactions. TAP binds strongly to Tat, with a K_d of (~2 to 5) × 10^{-7} M. The Tat activation region contains a 17-amino-acid conserved core domain which is the single contact site for TAP. Single-amino-acid substitutions within the Tat core domain inactivate transactivation in vivo and in vitro and greatly reduce binding of Tat to TAP in vitro. TAP binds strongly to TFIIB, with about the same K_d as for Tat. The interaction between TAP and TFIIB requires a sequence near the carboxy terminus of TFIIB which is also required for binding the strong acidic activator VP16. The contact sites for Tat and TFIIB map within the TAP C-terminal region, which contains the TAP activation domain. These combined results are consistent with the hypothesis that TAP is a cellular coactivator that bridges the Tat transactivator to the general transcription machinery via TFIIB.

The human immunodeficiency virus type 1 Tat transactivator is essential for viral gene expression and virus replication (for a review, see reference 25). How Tat functions is therefore of great interest. Studies of Tat's mechanism of action have been controversial and difficult to interpret (for a review, see reference 3). Using Tat peptide-affinity chromatography, we have identified a human cellular protein, TAP, that strongly binds Tat and have cloned and expressed its cDNA (4, 26). Transfection experiments with a series of TAP deletion mutants identified a strong transcriptional activation domain within the TAP C-terminal region that is masked by flanking sequence elements (26). Here we describe detailed protein-protein interaction studies with purified components and demonstrate that TAP can interact specifically with the Tat core activation domain and with the general transcription factor TFIIB. The binding sites for Tat and TFIIB are located within the TAP C-terminal region, which contains the TAP activation function. In a separate study, we provide evidence that Tat transactivation in vitro involves the function of a coactivator (22). Taken together, these findings support a model in which TAP may be an intermediary in Tat transactivation.

MATERIALS AND METHODS

Construction of TAP plasmids and TAP deletion mutants. A full-length TAP template for in vitro translation, pcTAP1–279, was constructed by subcloning the *Hin*dIII-*Sma*I fragment from pBTAP2 (26) into the *Hin*dIII-*Eco*RV sites of pcDNAI (Invitrogen). TAP71–279 was PCR synthesized with pBTAP2 as template, with ATGGAATTCATGCTGCACACCGAC as an upstream sense primer, and with ATCTCTAGAAAGCACATGTCTAGC containing an *Xba*I site as a downstream antisense primer. pcTAP71–279 was generated by inserting the PCR product, digested with *Xba*I, between the *Eco*RV and *Xba*I sites in the pcDNAI vector. The expression plasmids for glutathione *S*-transferase (GST)-TAP fusion proteins were constructed by inserting the open reading frame of the different TAP deletions, prepared by PCR synthesis, between the *Bam*HI and *Eco*RI sites of the pGEX-2TK vector (8). The following primers were used:

ATGGGATCCCTGCCTCTGCTGCGCTGC (primer 1)
ATGGGATCCATGCTGCACACCGACGGAGAC (primer 2)
ATGCCATGGGATCCCAGAAGGTTGAAGAAC (primer 3)
ATGGGATCCCAGTCCACTGGCGAG (primer 4)
ATGGGATCCGGGGTGGACAACACT (primer 5)
TCAGAATTCCTGGCTCTTGACAAA (primer 6)

The fragments were PCR synthesized as follows: TAP1–279, primers 1 (upstream) and 6 (downstream); TAP71–279, primers 2 (upstream) and 6 (downstream); TAP150–279, primers 3 (upstream) and 6 (downstream); TAP208–279, primers 4 (upstream) and 6 (downstream); and TAP244–279, primers 5 (upstream) and 6 (downstream).

Construction of Tat expression plasmids and synthesis of Tat peptides. The following primers were used for the PCR synthesis of Tat expression plasmids:

ATGGGATCCATGCCAGTAGATCCT (primer 7)
CTAGAATTCCGATTCCTTCGGGCCTGT (primer 8)
GGGCCATGGAGCCAGTAGATCCT (primer 9)
CGAAGATCTTTCCTTCGGGCCTGT (primer 10)

pGEX-2TK-Tat was constructed by inserting PCR-synthesized Tat between the *Bam*HI and *Eco*RI sites of pGEX-2TK; the Tat insert was made with pcTat (17) as a template with primers 7 (upstream) and 8 (downstream). pQTat and pQTat41Ala, which express Tat and Tat41Ala with six histidine residues at the C terminus, were constructed by inserting PCR-synthesized Tat and Tat41Ala into the pQE60 vector (Qiagen) between the *Nco*I and *Bgl*II sites; the Tat and Tat41Ala inserts were synthesized with pcTat and pLJTat41Ala (11, 16) as templates with primers 9 (upstream) and 10 (downstream). The construction of pGEX-2T-Tat1–48 and pGEX-2T-Tat1–48, 41Ala has been described previously (22). pGEX-2T-Tat1–48, 38Ala was constructed in the same manner by C.-Z. Song, with GAL4(1–47) HIV-1 Tat38Ala as a template (23). Tat peptides were synthesized with an ABI 430A peptide synthesizer with FASTMOC chemistry (Applied Biosystems) and purified by reverse-phase high-performance liquid chromatography.

Expression of recombinant proteins. The various GST-2TK fusion proteins containing TAP, Tat, and their mutant derivatives, as well as GST-2T-TFIIB derivatives from Roberts et al. (21), were prepared and bound to glutathione-agarose as described by Kaelin et al. (8). Aliquots of beads were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) to determine the concentration and purity of protein. Beads were diluted with glutathione-agarose to give 1 μg of protein per 100 μl of 10% slurry for studies utilizing ligand bound to agarose beads. To produce Tat and Tat 41Ala, *Escherichia coli* M15 cells transformed by pQTat or pQTat41Ala were grown to an A_{600} of 0.6, induced with 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside),

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harvested after 3 h of further growth, and purified by Ni-nitrilotriacetic acid affinity chromatography in 6 M guanidine-HCl (Qiagen). Tat and Tat41Ala were renatured by stepwise removal of guanidine-HCl and concentrated with a Centricon 3 microconcentrator (Amicon).

Preparation of Affigel 10-immobilized peptides and proteins. Affigel 10 (Bio-Rad) was used as described by the manufacturer. Briefly, protein or peptide was dissolved in 250 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid [pH 7.5]) at 2.5 mg/ml. Affigel 10 was washed rapidly three times in cold water, added to the protein solution (1 ml of packed resin per ml of protein solution), and incubated at 4°C with rocking for 4 h. Ethanolamine (pH 8.0) was added to 100 mM, and incubation continued for 1 h. Beads were washed extensively in phosphate-buffered saline (PBS) and diluted with blocked Affigel 10 to give a concentration of 1 μg of ligand per 100 μl of a 1:10 slurry. Peptides not soluble in HEPES buffer were dissolved in dimethyl sulfoxide and coupled to Affigel 10 as described above, except that the resin was not washed with water. Bound protein was measured by the decrease in A_{280} after acidification of an aliquot of the protein solution before and after binding to Affigel. Reproducibly, over 95% of the protein or peptide was bound to Affigel.

Preparation of labeled proteins. To prepare ^{35}S-labeled TAP1–279 and TAP71–279, pcTAP1–279 and pcTAP71–279 were linearized with *Xba*I and used as a template in a coupled transcription-translation system (Promega TNT) with T7 RNA polymerase-rabbit reticulocye lysate and labeled with [^{35}S]cysteine as recommended by the manufacturer. To prepare ^{35}S-labeled GAL4-VP16, GAL4(1–94)-VP16 cloned into pGEM-3 was linearized with *Eco*RI and used as a template in Promega TNT system with SP6 polymerase-wheat germ extract and labeled with [^{35}S]methionine (21). GST-2TK-TAP fusion proteins and GST-2TK-Tat were labeled with ^{32}P by the kinase reaction and purified as described previously (8). ^{32}P-Tat was cleaved from GST-2TK-Tat by incubation with human thrombin (6a). Recombinant Tat was labeled with ^{125}I by incubation of 50 μg of protein in 200 μl of PBS with 0.5 mCi of Na-^{125}I (NEN) and five IODO-BEADS (Pierce) for 20 min. The solution was removed from the IODO-BEADS, cytochrome *c* (400 μg) was added, and labeled Tat was purified by exclusion chromatography on a 6-ml Sephadex G-10 column equilibrated in PBS–0.01% Tween 80.

Protein-protein binding assays. Ligands immobilized on Affigel 10 or GST fusion proteins bound to glutathione-agarose were incubated with labeled TAP or GAL4-VP16 translates, ^{32}P-labeled GST-2TK-TAP or ^{32}P-labeled GST-2TK-Tat fusion proteins, or ^{125}I-labeled recombinant Tat in binding buffer by either of two protocols. Incubation of bound ligand with labeled protein was performed in 100 μl of TBST (50 mM Tris-HCl, [pH 7.6], 150 mM NaCl, 0.2% Tween 20, 200 μg of bovine serum albumin [BSA] per ml); in most experiments, TBST containing 500 mM NaCl was used. After incubation for 1 h at 4°C with rocking in a 1.5-ml microcentrifuge tube, beads were washed four times with the same TBST binding buffer lacking BSA by vortexing for 10 s and were centrifuged at 360 × *g* for 2 min. Bound protein was eluted by incubating beads in 30 μl of SDS-PAGE sample buffer at 90°C for 10 min and electrophoresed on an SDS–12.5% polyacrylamide gel. The dried gel was exposed for 1 to 2 days to a storage phosphor screen, and bound protein was visualized and quantitated with a Molecular Dynamics 400B PhosphorImager. In most experiments with TFIIB, an alternate protocol was used in which the immobilized ligand was preincubated for 1 h at 4°C in 300 μl of Nonidet P-40 binding buffer containing 40 mM HEPES (pH 7.5), 150 mM KCl, 5 mM $MgCl_2$, 0.5 mM EDTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 0.2% Nonidet P-40, and 0.5 mg of a sonicated and clarified *E. coli* extract per ml, essentially as described by Roberts et al. (21). Labeled protein was added, and incubation continued for 1 h at 4°C. Beads were washed with the same Nonidet P-40 binding buffer lacking bacterial extract and analyzed as described above.

For solution binding, GST fusion proteins were stripped batchwise with 50 mM glutathione (pH 7.0) from 200 to 300 μl of packed glutathione-agarose containing the GST fusion protein. Proteins were concentrated and buffer was exchanged with PBS by centrifugation in a Centricon-30 (Amicon). The GST fusion protein and the labeled translate were incubated in 30 μl of binding buffer as described above. After 1 h at 4°C, 70 μl of a 1:10 dilution of glutathione-agarose in binding buffer was added and incubation continued with rocking for 15 min at 4°C. Beads were washed four times in binding buffer, and bound protein was analyzed as described above.

The binding of T7 epitope-tagged TFIIB (a gift from S. G. E. Roberts and M. R. Green) to GST-TAP derivatives was performed in Nonidet P-40 binding buffer as described above. Beads were washed and bound protein was eluted with SDS-sample buffer and resolved by SDS-PAGE. Proteins were transferred to nitrocellulose (S&S; BA83) with an LKB Novablot apparatus. Immunoblot analysis was performed by ECL chemiluminescence as recommended by Amersham with T7 Tag monoclonal antibody (Novagen) as the primary antibody at 1:3,000 and donkey anti-mouse immunoglobulin G conjugated with horseradish peroxidase (Amersham) as the secondary antibody at 1:1,000.

RESULTS

A 17-amino-acid sequence within the Tat activation region is the target for TAP. TAP was cloned on the basis of its ability to bind strongly at high ionic strength to the full-length Tat protein of 86 amino acids (4). It is important to identify the Tat sequences responsible for binding in order to develop an understanding of the possible role of TAP in Tat transactivation. Tat has been functionally divided into two major regions (Fig. 1A). The first is the transcriptional activation region (amino acids 1 to 48) (9, 23), which includes a conserved core domain (1, 6). The second is the basic region (amino acids 49 to 57), which is required for binding of Tat to TAR. One would expect a cellular protein involved in Tat transactivation to bind the Tat activation region. To delineate Tat sequences that interact with TAP, recombinant Tat and a series of chemically synthesized Tat peptides, Tat1–48, Tat14–48, Tat22–48, Tat32–48, and Tat34–48, which span the Tat activation domain; and Tat49–57, which encodes the basic domain, were covalently immobilized in large excess on Affigel 10. Affigel 10 is widely used to measure protein-protein interactions (for review, see reference 5). Although each protein molecule can be linked through the primary amine at the N terminus or at internal lysine residues, the large amount of peptide used ensures that the N-terminal-linked ligand is present in vast excess over that needed for interaction with its target in solution.

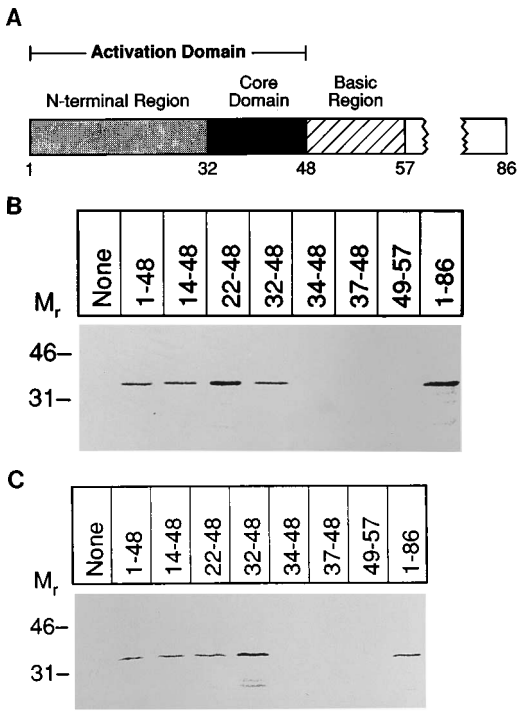

FIG. 1. Mapping of the human immunodeficiency virus type 1 Tat sequences that interact with the cellular protein TAP identifies a 17-amino-acid contact site within the Tat activation domain. (A) Schematic diagram of Tat domains. (B and C) Protein-protein interactions were carried out between Affigel 10-immobilized recombinant Tat1–86 or Tat deletion peptides with in vitro ^{35}S-labeled TAP1–279 translate (B) or TAP71–279 translate (C). TAP translates containing about 12,000 trichloroacetic acid-precipitable counts were incubated with 1 μg of bound ligand in TBST binding buffer containing 500 mM NaCl. Shown is PhosphorImager analysis of labeled TAP bound to immobilized Tat derivatives and resolved by SDS-PAGE. The positions of ^{14}C-labeled molecular size protein markers (in kilodaltons) (Amersham) run on a contiguous lane are shown on the left.

The immobilized Tat ligands were incubated with a ^{35}S-labeled translate of full-length TAP (TAP1–279) in high-stringency buffer (500 mM NaCl). As shown in Fig. 1B, TAP binds strongly to Tat1–86, Tat1–48, Tat14–48, Tat22–48, and Tat32–48 but not to Tat34–48 or Tat49–57. These results define Tat32–48 as a minimal binding domain. Significantly,

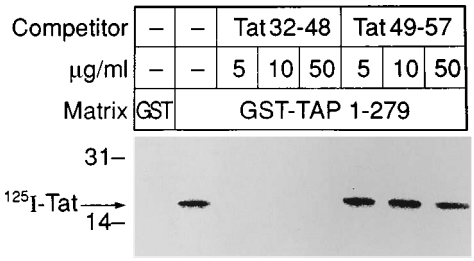

FIG. 2. Tat32–48 contains the single binding site for TAP. ^{125}I-labeled recombinant Tat (7 ng, 58,000 cpm) was incubated with agarose-bound GST-2TK-TAP1–279 or GST-2TK in the presence of competitor peptide Tat32–48 or basic region peptide Tat49–57. Bound protein was eluted, resolved by SDS-PAGE, and subjected to PhosphorImager analysis. M_rs (in kilodaltons) are shown to the left.

Tat32–48 is the core domain of the activation region (Fig. 1A), which is conserved among immunodeficiency viruses and has been shown by mutational analysis to be essential for Tat transactivation and sufficient for activation by the equine infectious anemia virus Tat (1, 6, 12, 20, 23). Because TAP71–279 is the major intracellular form of TAP (26), we analyzed the binding of a TAP71–279 translate to the same Tat ligands. As shown in Fig. 1C, the minimal binding site for TAP71–279 is Tat32–48, the same as for full-length TAP.

The results presented above show that the Tat32–48 sequence is sufficient to bind TAP but do not exclude additional binding sites within the Tat activation region. To determine whether Tat32–48 is the sole contact site for TAP and to confirm further the specificity of TAP interaction with Tat, competition experiments were performed. GST-TAP immobilized on glutathione-agarose was incubated with ^{125}I-labeled Tat in the presence of increasing amounts of Tat32–48 or Tat49–57 peptides. Tat32–48 competes totally with full-length Tat for binding TAP, whereas the basic region does not compete (Fig. 2). We conclude that Tat32–48 includes the single contact site for TAP. The Tat basic region, which might be expected to bind nonspecifically to the highly acidic TAP protein, does not interact under these stringent ionic conditions.

Physical parameters of TAP interaction with Tat in solution. To assess the strength of interaction, the TAP-Tat K_d was determined. First, conditions for solution binding were established. GST-Tat was incubated with a TAP translate in buffer containing increasing amounts of NaCl. The GST-Tat-TAP complex was isolated by addition of glutathione-agarose beads, and bound TAP was quantitated by PhosphorImager analysis. Efficient binding was observed at 500 mM NaCl, and ~50% of maximal binding occurred at 750 mM NaCl (Fig. 3A). Stable binding at high ionic strength is consistent with authentic protein-protein interaction, as opposed to nonspecific ionic interaction. Binding was rapid and reached maximal levels within 30 to 60 min at 4°C (Fig. 3B). To estimate the K_d, the concentration dependence of GST-Tat binding to TAP was determined in buffer containing 500 mM NaCl with an incubation time of 60 min. Significant TAP binding was detected with as little as 5×10^{-8} M GST-Tat (Fig. 3C). Half-maximal binding occurred at (2 to 5) $\times 10^{-7}$ M GST-Tat, which provides a good estimate of the K_d (Fig. 3C).

Single-amino-acid substitutions within the Tat core activation domain markedly reduce TAP-Tat interaction in solution. Tat transactivation in vivo and in vitro is inactivated by substitution within the Tat core domain of Lys-41 with alanine (6, 12, 18, 20, 23). To determine whether this mutation would affect the strength of TAP-Tat interaction, a TAP translate was in-

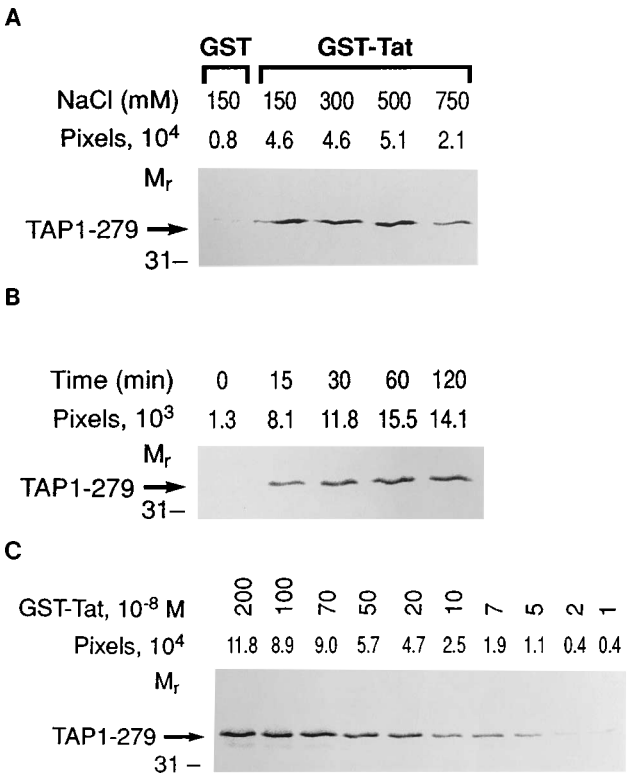

FIG. 3. Parameters of solution binding between Tat and TAP. GST-2TK-Tat or GST-2TK was incubated with a labeled TAP translate in 30 μl of TBST binding buffer containing various amounts of NaCl (A) or for various periods (B). (C) Increasing amounts of GST-Tat were incubated with labeled TAP in 500 mM NaCl for 60 min. GST-Tat-TAP complexes were removed from solution with glutathione-agarose beads and washed extensively, and bound protein was analyzed by SDS-PAGE and quantitated by PhosphorImager analysis. The amount of bound TAP protein is expressed in pixels. M_r is in kilodaltons.

cubated with 1 to 1,000 ng of recombinant GST-Tat1–48 (Fig. 4A, panel A) or GST-Tat1–48, 41Ala (Fig. 4A, panel B). Tat1–48, 41Ala is clearly defective in its efficiency of binding to TAP. To further characterize the Tat41Ala binding defect, competition experiments were performed. GST-Tat was incubated with a TAP translate in solution in the presence of increasing amounts of full-length Tat or Tat41Ala. As shown in Fig. 4B, Tat efficiently competes for binding to TAP whereas Tat41Ala is ineffective. To summarize, the Lys-41 mutation in both the Tat activation region alone (Tat1–48) and in full-length Tat greatly impairs TAP-Tat interaction.

Another amino acid residue within the core domain reported to be essential for Tat transactivation is Phe-38 (20, 23). To establish further the correlation between the Tat activation function and its ability to bind TAP, we tested the ability of recombinant Tat1-48, 38Ala to bind TAP in solution. As shown in Fig. 4C, the Ala-38 mutant is markedly defective in binding TAP. Thus, the in vitro interaction between TAP and two Tat single-amino-acid-substitution mutants in the core domain mirrors the in vivo activity of the Tat mutants and is consistent with the idea that TAP may play a role in Tat transactivation.

The Tat contact site is located within the TAP activation domain. To map TAP sequences required to bind Tat, TAP deletion constructs were cloned into the pGEX-2TK expression vector and purified GST-TAP fusion proteins were ^{32}P labeled with kinase (8). The labeled fusion proteins were incubated under both highly stringent conditions (500 mM NaCl

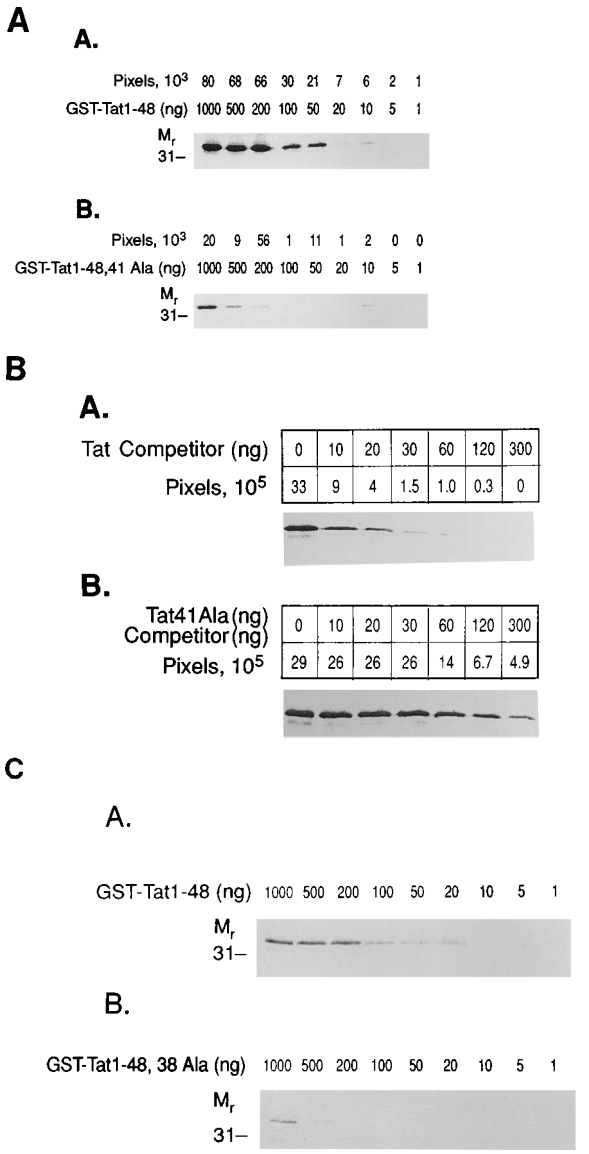

FIG. 4. Single-amino-acid substitutions within the Tat core domain markedly reduce the efficiency of TAP-Tat interaction in solution. (A) From 1 to 1,000 ng of GST-Tat1–48 (panel A) or GST-Tat1–48, 41Ala (panel B) was incubated with ^{32}P-labeled TAP. (B) From 10 to 300 ng of unlabeled recombinant Tat (panel A) or recombinant Tat41Ala (panel B) was mixed with about 1 pmol of GST-2TK-Tat in 30 μl of TBST binding buffer containing 500 mM NaCl and incubated with a labeled TAP translate. (C) GST-Tat1–48 (panel A) and GST-Tat1–48, 38Ala (panel B) were incubated with ^{32}P-labeled TAP. Protein-protein complexes were isolated and bound TAP was quantitated as described in the legend to Fig. 3. M_r is in kilodaltons.

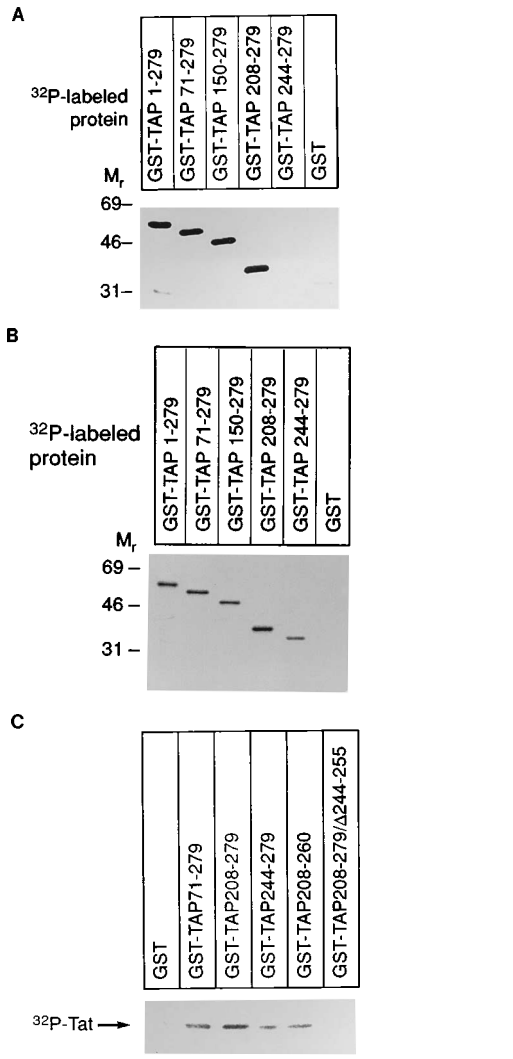

FIG. 5. Mapping of the TAP sequences that bind Tat. (A) TAP208–279 is the minimal binding domain in 500 mM NaCl. ^{32}P-labeled GST-2TK-TAP and GST-2TK-TAP deletion proteins were incubated in TBST binding buffer containing 500 mM NaCl with recombinant Tat (1 μg) immobilized on Affigel 10. After extensive washing, bound ^{32}P-labeled GST-2TK-TAP fusion proteins were eluted and analyzed as described in the legend to Fig. 1. (B) TAP244–279 is the minimal binding domain for Tat in 150 mM NaCl. The experiment described for panel A was performed in TBST binding buffer that contained 150 mM NaCl. (C) The TAP sequence in amino acid residues 244 to 255 is required for binding Tat. ^{32}P-labeled Tat was incubated with the indicated GST-2TK-TAP deletion proteins immobilized on glutathione-agarose in Nonidet P-40 binding buffer containing 300 mM KCl. Agarose beads were washed and bound Tat was measured by PhosphorImager analysis as described in the legend to Fig. 1. M_r is in kilodaltons.

[Fig. 5A]) and physiological conditions (150 mM NaCl [Fig. 5B]) with recombinant Tat immobilized on Affigel 10. Strong binding occurred with TAP1–279, TAP71–279, TAP150–279, and TAP208–279 but not with TAP244–279 in 500 mM NaCl (Fig. 5A). Interestingly, TAP244–279 exhibited substantial binding to Tat in 150 mM NaCl (Fig. 5B) and in 300 mM NaCl (see below). We interpret this to mean that TAP244–279 contains the minimal binding domain for Tat and that an upstream TAP sequence can provide additional stability at high ionic strength. Of significance, TAP244–279 contains a strong transcriptional activation element when bound upstream of the core human immunodeficiency virus long terminal repeat promoter in transient expression experiments (26).

To further map the Tat contact site within TAP, GST-TAP constructs lacking amino acid residues 261 to 279 or 244 to 255 were tested for their ability to bind ^{32}P-labeled Tat in buffer containing 300 mM NaCl. As shown in Fig. 5C, GST-TAP208–279, GST-TAP208–260, and GST-TAP244–279 bound Tat, whereas GST-TAP208–279/Δ244–255 was completely defective. Thus, a sequence within TAP residues 244 to 255 is essential for binding Tat.

The general transcription factor TFIIB binds strongly to TAP. What could be the role of TAP in Tat transactivation?

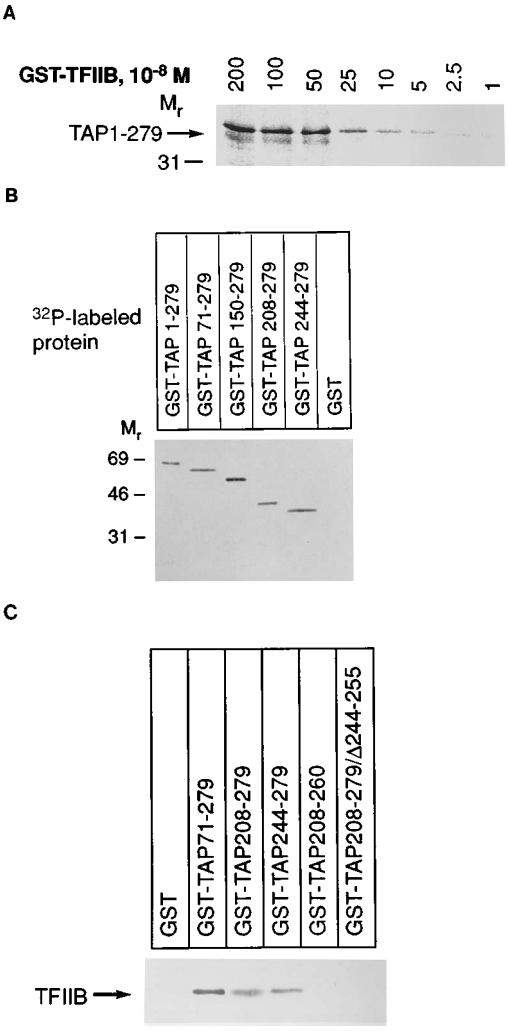

FIG. 6. (A) TFIIB interacts strongly with TAP in solution. From 1×10^{-8} to 2×10^{-6} M GST-TFIIB was incubated with a labeled TAP translate in 30 μl of Nonidet P-40 binding buffer containing 150 mM KCl. GST-TFIIB-TAP complexes were isolated and analyzed as described in the legend to Fig. 3. (B) TFIIB interacts strongly with the TAP activation region. ^{32}P-labeled GST-TAP1–279 and GST-TAP deletion proteins were incubated with 1 μg of TFIIB immobilized on Affigel 10 in 300 μl of Nonidet P-40 binding buffer containing 150 mM KCl. Bound TAP derivatives were isolated and analyzed as described in Materials and Methods. (C) Binding of TFIIB to TAP requires TAP residues within positions 244 to 255 and 260 to 279. T7 epitope-tagged TFIIB (~30 ng) was incubated with agarose-bound GST-TAP deletion mutants (~1 μg) in Nonidet P-40 binding buffer in 300 mM KCl. After extensive washing, bound TFIIB was eluted from beads in SDS-sample buffer and visualized by immunoblot analysis with anti-T7 monoclonal antibody followed by ECL chemiluminescence (Amersham). M_r is in kilodaltons.

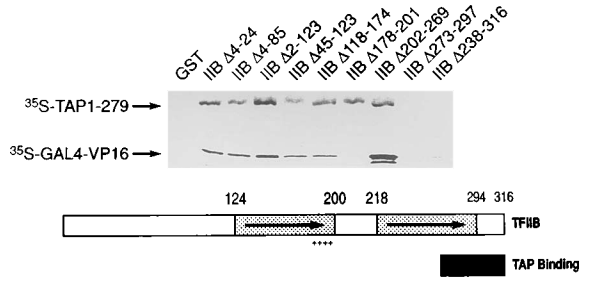

FIG. 7. The C-terminal domain of TFIIB is required for binding TAP. TFIIB deletion mutants expressed as GST fusion proteins and coupled to glutathione-agarose beads were incubated with a TAP translate in Nonidet P-40 binding buffer containing 150 mM KCl. Bound protein was analyzed as described in the legend to Fig. 1. For comparison, a parallel experiment was performed with a translate of GAL4-VP16 as described by Roberts et al. (21). The region of TFIIB required for binding TAP is schematically illustrated (adapted from reference 21).

The TAP C terminus encodes a strong activation domain which binds to the Tat core activation domain. Because of this interaction, we considered the possibility that TAP could function as a coactivator that transmits the Tat activation potential to the general transcription apparatus of the cell. In this regard, several eukaryotic activators have been shown to function, in part, by recruiting TFIIB to the preinitiation transcription complex (2, 14, 15, 21). We therefore asked whether TAP can interact with TFIIB in vitro. GST-TFIIB at concentrations from 1×10^{-8} to 2×10^{-6} M was incubated in solution with a ^{35}S-labeled TAP translate. As shown in Fig. 6A, binding of TAP with TFIIB was detected with as little as 2.5×10^{-8} M TFIIB. Half-maximal binding occurred at (2.5 to 5) $\times 10^{-7}$ M TFIIB, which represents the estimated K_d. We conclude that TAP strongly binds TFIIB.

TFIIB binds to the TAP activation domain. To map the TAP sequences that interact with TFIIB, ^{32}P-labeled GST-TAP deletion proteins were incubated with TFIIB immobilized on Affigel 10. Strong binding to TFIIB occurred with TAP1–279, TAP71–279, TAP150–279, TAP208–279, and TAP244–279 (Fig. 6B); the specificity of these interactions is supported by the lack of binding to GST. Stable interaction between TFIIB and GST-TAP208–279 or GST-TAP244–279 occurred at 500 mM NaCl (data not shown). To further delineate the TAP sequences that interact with TFIIB, additional GST-TAP deletion mutants were incubated with T7 epitope-tagged TFIIB, and bound TFIIB was measured by immunoblot analysis. As shown in Fig. 6C, TAP208–279 and TAP244–279 bound TFIIB, whereas TAP208–279/Δ244–255 and TAP208–260 were defective. Thus, TAP sequences within amino acids 244 to 255 and 260 to 279 are important for binding TFIIB. Significantly, these TAP sequences are also important for the TAP activation function (26).

The carboxy terminus of TFIIB is required for interaction with TAP. A collection of nine GST-TFIIB deletion proteins were incubated with a TAP translate and bound TAP was analyzed by SDS-PAGE. As shown in Fig. 7, the TFIIB C-terminal mutants lacking amino acids 273 to 297 and 238 to 316 were defective in binding TAP. For comparison, the same TFIIB deletion protein constructs were analyzed simultaneously for their ability to bind a GAL4-VP16 translate. Roberts et al. (21) have shown that TFIIB has two sites required for interaction with VP16: one at the C terminus and the second within TFIIB residues 178 to 201. Our results confirm these findings and show, interestingly, that TAP and GAL4-VP16 share a potential contact site at the TFIIB C terminus (Fig. 7).

DISCUSSION

We have performed in vitro protein-protein interaction measurements with the human cellular protein TAP, which was isolated on the basis of its ability to bind to the human immunodeficiency virus type 1 Tat transactivator. Here we show that TAP can bind both to Tat and to TFIIB. Recruitment of TFIIB can be rate limiting for activation by eukaryotic transcription factors (2, 14, 15, 21, 24). Because TAP binds to TFIIB and possesses a strong activation domain, it is possible that TAP may serve as an intermediary in Tat transactivation.

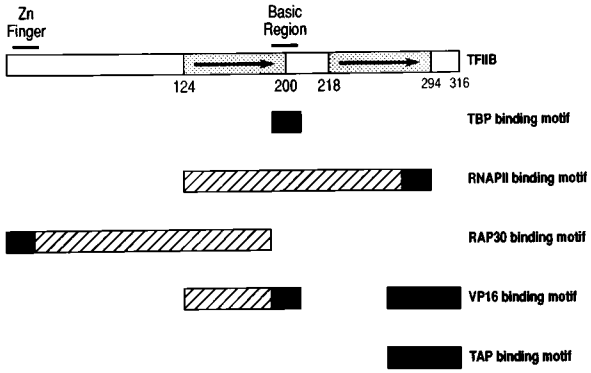

FIG. 8. Summary of binding sites within TFIIB for TAP, VP16 (adapted from reference 21), RNA polymerase II (RNAPII), TBP, and RAP30 (adapted from reference 7).

Thus, it was important to establish the strength and specificity of TAP-Tat and TAP-TFIIB interactions by using purified proteins under stringent binding conditions and to map the protein-protein contact sites.

TAP interaction with Tat in vitro. We show here that TAP binds to a 17-amino-acid sequence, Tat32–48, the core region within the Tat1–48 activation domain conserved among lentivirus Tat proteins. Tat32–48 is the single binding site for TAP, as revealed both by direct binding and by peptide competition. The estimated K_d for TAP-Tat binding in solution is (2 to 5) $\times$ 10^{-7} M, consistent with strong protein-protein interaction. Interaction between TAP and Tat occurred under stringent conditions, and the specificity of this interaction is supported by the stringent binding conditions and by mutational analysis. Single-amino-acid substitutions within the Tat core domain at Lys-41 or Phe-38 markedly reduced binding efficiency in vitro. The same mutations abrogate Tat transactivation in vivo and in vitro (6, 12, 18, 20, 23). The strong correlation between transactivation and in vitro binding suggests that the interaction between TAP and Tat is biologically relevant.

Studies with GST-TAP deletion proteins show that Tat can bind to both TAP208–260 and TAP244–279. The specificity of these interactions is supported by the lack of binding to the internal deletion mutant TAP208–279/Δ244–255. These data suggest that TAP amino acid residues ~244 to 260 are essential for binding to Tat and that residues 208 to 244 and 260 to 279 may lend stability to the TAP-Tat complex.

TAP interaction with TFIIB in vitro. TAP binds strongly to TFIIB, with a K_d of (2.5 to 5) $\times$ 10^{-7} M. The minimal contact sequence for TFIIB is located within the TAP activation region, TAP244–279. Although Tat can bind to TAP208–260 in solution, TFIIB does not bind to this deletion mutant under the same conditions. As shown by deletion analysis, the TAP244–255 sequence is required for binding both Tat and TFIIB. Perhaps TFIIB and Tat bind to overlapping but not identical amino acid sequences. Interestingly, TFIIB covalently linked to Affigel 10 can bind significant amounts of TAP208–260 (data not shown). This suggests the possibility that the TFIIB conformation may play a role in its ability to bind TAP.

TFIIB is pivotal in the formation of a transcription initiation complex (for a review, see reference 27). Figure 8 illustrates the binding sites within TFIIB for TAP, VP16, RNA polymerase II, TBP, and RAP30 (the small subunit of TFIIF) (7, 21). Analysis of TFIIB deletion mutants shows that sequences near the C terminus of TFIIB are required for binding TAP. It may be significant that the TFIIB C terminus is also required for binding the acidic transcription activator VP16, as reported by Roberts et al. (21) and confirmed here. The binding site for RNA polymerase II also maps to the TFIIB C terminus, whereas TBP binds to the basic region and RAP30 binds to the N-terminal region (7) (Fig. 8).

What could be the role of TAP in Tat transactivation? We speculate that the normal function of TAP may be as a coactivator for unidentified cellular activators (for reviews, see references 13 and 19). Several lines of evidence are consistent with the possibility that TAP may serve as a coactivator for Tat transactivation. (i) TAP binds strongly and specifically to the Tat core activation element. (ii) TAP binds strongly to TFIIB. (iii) TAP possesses a strong transcriptional activation domain (26). (iv) The TAP activation domain is required for binding Tat and TFIIB in solution. (v) Cotransfection experiments show that Tat can recruit TAP to the promoter (26). (vi) Coimmunoprecipitation analysis shows that Tat can associate with endogenous TAP in vivo (26). The simplest model is that a species of TAP serves as an coactivator which interacts with TAR-bound Tat and results in the efficient recruitment of TFIIB to the transcription initiation complex through its interaction with TAP. Tat has been recently reported to also bind TBP (10), a second component of the general transcription machinery. Perhaps the role of Tat as a potent transcriptional activator is reflected by its ability to interact both with TFIIB via TAP and with TBP. This possibility is in keeping with the idea that multiple contacts between transcriptional activators and the general transcription machinery facilitate preinitiation complex formation and efficient transcriptional activation (2).

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REFERENCES

1. **Carroll, R., L. Martarano, and D. Derse.** 1991. Identification of lentivirus Tat functional domains through generation of equine infectious anemia virus/human immunodeficiency virus type 1 *tat* gene chimeras. J. Virol. **65:**3460–3467.
2. **Choy, B., and M. R. Green.** 1993. Eukaryotic activators function during multiple steps of preinitiation complex assembly. Nature (London) **366:**531–536.
3. **Cullen, B. R.** 1993. Does HIV-1 Tat induce a change in viral initiation rights? Cell **73:**417–420.
4. **Desai, K., P. M. Loewenstein, and M. Green.** 1991. Isolation of a cellular protein that binds to the human immunodeficiency virus Tat protein and can potentiate transactivation of the viral promoter. Proc. Natl. Acad. Sci. USA **88:**8875–8879.
5. **Formosa, T., J. Barry, B. M. Alberts, and J. Greenblatt.** 1991. Using protein affinity chromatography to probe structure of protein machines. Methods Enzymol. **208:**24–45.
6. **Green, M., M. Ishino, and P. M. Loewenstein.** 1989. Mutational analysis of HIV-1 Tat minimal domain peptides: identification of trans-dominant negative mutants that suppress HIV-LTR-driven gene expression. Cell **58:**215–223.

6a. **Guan, K., and J. E. Dixon.** 1991. Eukaryotic proteins expressed in Escherichia coli: an improved thrombin cleavage and purification procedure of fusion proteins with glutathione S-transferase. Anal. Biochem. **192:**262–267.

7. **Ha, I., S. Roberts, E. Maldonado, Z. Sun, L.-U. Kim, M. R. Green, and D. Reinberg.** 1993. Multiple functional domains of human transcription factor IIB. Genes Dev. **7:**1021–1032.
8. **Kaelin, W. G., Jr., W. Krek, W. R. Sellers, J. A. DeCaprio, F. Ajchenbaum, C. S. Fuchs, T. Chittenden, Y. Li, P. J. Farnham, M. A. Blanar, D. M. Livingston, and E. K. Flemington.** 1992. Expression cloning of a cDNA encoding a retinoblastoma-binding protein with E2F-like properties. Cell **70:**351–364.
9. **Kamine, J., T. Subramanian, and G. Chinnadurai.** 1991. Sp1-dependent

activation of a synthetic promoter by human immunodeficiency virus type 1 Tat protein. Proc. Natl. Acad. Sci. USA **88:**8510–8514.
10. **Kashanski, F., G. Piras, M. F. Radonovich, J. F. Duvall, A. Fattaey, C.-M. Chiang, R. G. Roeder, and J. N. Brady.** 1994. Direct interaction of human TFIID with the HIV-1 transactivator Tat. Nature (London) **367:**295–299.
11. **Korman, A. J., J. D. Frantz, J. L. Strominger, and R. C. Mulligan.** 1987. Expression of human class II major histocompatibility complex antigens using retrovirus vectors. Proc. Natl. Acad. Sci. USA **84:**2150–2154.
12. **Kuppuswamy, M., T. Subramanian, A. Srinivasan, and G. Chinnadurai.** 1989. Multiple functional domains of Tat, the trans-activator of HIV-1, defined by mutational analysis. Nucleic Acids Res. **17:**3551–3561.
13. **Lewin, B.** 1990. Commitment and activation at Pol II promoters: a tail of protein-protein interactions. Cell **61:**1161–1164.
14. **Lin, Y.-S., and M. R. Green.** 1991. Mechanism of action of an acidic transcriptional activator. Cell **64:**971–981.
15. **Lin, Y.-S., I. Ha, E. Maldonado, D. Reinberg, and M. R. Green.** 1991. Binding of general transcription factor TFIIB to an acidic activating region. Nature (London) **353:**569–571.
16. **Loewenstein, P. M., M. Ishino, N. Abdullah, and M. Green.** 1991. Inhibition of HIV-1 replication by mutant Tat peptides, p. 151–172. *In* A. Kumar (ed.), Advances in molecular biology and targeted treatment for AIDS. Plenum Press, New York.
17. **Malim, M. H., J. Hauber, R. Fenrick, and B. R. Cullen.** 1988. Immunodeficiency virus rev trans-activator modulates the expression of the viral regulatory genes. Nature (London) **335:**181–183.
18. **Marciniak, R. A., B. J. Calnan, A. D. Frankel, and P. A. Sharp.** 1990. HIV-1 Tat protein *trans*-activates transcription *in vitro*. Cell **63:**791–802.
19. **Ptashne, M., and A. A. F. Gann.** 1990. Activators and targets. Nature (London) **346:**329–331.
20. **Rice, A. P., and F. Carlotti.** 1990. Mutational analysis of the conserved cysteine-rich region of the human immunodeficiency virus type 1 Tat protein. J. Virol. **64:**1864–1868.
21. **Roberts, S. G. E., I. Ha, E. Maldonado, D. Reinberg, and M. R. Green.** 1993. Interaction between an acidic activator and transcription factor TFIIB is required for transcriptional activation. Nature (London) **363:**741–744.
22. **Song, C.-Z., P. M. Loewenstein, and M. Green.** 1994. Transcriptional activation *in vitro* by the HIV-1 Tat protein—evidence for specific interaction with a coactivator(s). Proc. Natl. Acad. Sci. USA **91:**9357–9361.
23. **Southgate, C. D., and M. R. Green.** 1991. The HIV-1 Tat protein activates transcription from an upstream DNA-binding site: implications for Tat function. Genes Dev. **5:**2496–2507.
24. **Sundseth, R., and U. Hansen.** 1992. Activation of RNA polymerase II transcription by the specific DNA-binding protein LSF. J. Biol. Chem. **267:**7845–7855.
25. **Vaishnav, Y. N., and F. Wong-Staal.** 1991. The biochemistry of AIDS. Annu. Rev. Biochem. **60:**577–630.
26. **Yu, L., Z. Zhang, P. M. Loewenstein, K. Desai, Q. Tang, D. Mao, J. S. Symington, and M. Green.** 1995. Molecular cloning and characterization of a cellular protein that interacts with the human immunodeficiency virus type 1 Tat transactivator and encodes a strong transcriptional activation domain. J. Virol. **69:**3007–3016.
27. **Zawel, L., and D. Reinberg.** 1993. Initiation of transcription by RNA polymerase II: a multistep process. Prog. Nucleic Acid Res. Mol. Biol. **44:**67–108.