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In Vitro and In Vivo Binding of Human Immunodeficiency Virus Type 1 Tat Protein and Sp1 Transcription Factor

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Recent genetic experiments have suggested that *tat* transactivation of the human immunodeficiency virus type 1 (HIV-1) long terminal repeat requires functional upstream enhancer sequences—Sp1 sites, in particular. In these experiments, HeLa cell nuclear extracts were passed over affinity matrices containing chemically synthesized or bacterially expressed HIV-1 Tat. Assay of material that bound to and eluted from the Tat matrices revealed the presence of the Sp1 transcription factor. Other transcription factors (Oct and NF-κB) also bound to Tat matrices but with less efficiency—in parallel with the lower capacities of these binding motifs to confer Tat responsiveness on a basal HIV-1 promoter compared with Sp1 sites. Passage of nuclear extracts over matrices containing other neutral proteins, including bovine serum albumin, ovalbumin, and lysozyme, revealed no or reduced binding. Cross-linking experiments indicated that the purified Sp1 and Tat proteins can form multimeric complexes in the absence of other proteins. The region of Tat responsible for Sp1 binding was localized to a region encompassing residues 30 to 62. Immunoprecipitation experiments with HIV-1-infected T lymphocytes indicated coimmunoprecipitation of Tat and Sp1. These experiments extend previous genetic experiments and suggest a direct interaction between Tat and Sp1 during transactivation.

Human immunodeficiency virus type 1 (HIV-1), the etiological agent of AIDS, encodes a transactivator protein, Tat (36). Tat is expressed early after infection from a doubly spliced mRNA (3, 68), which in turn leads to high-level virus expression. Genetic experiments indicate that Tat is essential for virus infectivity (3, 73), which has made it a target for development of antiviral compounds. Therefore, an understanding of the molecular basis of Tat transactivation is important.

Tat is a small (86-amino-acid [aa]) protein with three domains: proline rich, cysteine rich, and basic (from amino to carboxy terminal). In the HIV-1 genome, Tat-responsive sequences have been mapped to the HIV-1 long terminal repeat (LTR) between positions +1 and +45 (the transactivation-responsive region [TAR]) (55, 58, 62, 63). Tat protein does not bind TAR DNA (24), but it specifically binds to a stem-loop structure in TAR RNA (14, 18, 22, 71, 72), along with one or more cellular proteins (27–29, 53, 67). The consensus is that in binding Tat, TAR RNA tethers it to the transcription complex, where it can interact with other proteins to cause transactivation (5, 8, 65, 69).

Two general mechanisms have been proposed for the action of Tat once it is tethered to the transcription complex. Several experiments have indicated that Tat increases viral expression by increasing the processivity of RNA polymerase (20, 42, 43, 52, 54). Thus, Tat might act as an antiattenuater to prevent premature transcription termination; alternatively, it might mediate formation of a transcription complex that is more processive. Other experiments have indicated that Tat may act by increasing the rate of transcription initiation from the HIV-1 LTR (9, 43, 57, 58, 61). In this case, Tat might interact with cellular transcription initiation factors to increase their efficiency. It should be noted that these two mechanisms are not mutually exclusive, and some studies have supported both mechanisms (43, 51).

Several reports have indicated a role for upstream enhancers in Tat transactivation. The HIV-1 LTR contains an enhancer region consisting of three tandem NF-κB elements followed by two tandem Sp1-binding sites (39, 56). Mutational analyses have indicated that active Sp1-binding sites are important for Tat transactivation, while the NF-κB sites are not (7, 38, 44, 56, 75). Very recently, we and others have shown that a deleted HIV-1 LTR containing a basal HIV-1 promoter and TAR, but lacking NF-κB and Sp1 sites, has significant transcriptional activity (7, 32). However, it was not responsive to Tat (7, 32). Addition of a variety of synthetic enhancer sequences to this basal promoter restored the Tat response (7). Interestingly, different enhancer elements had different efficiencies in conferring Tat responsiveness: Sp1 sites were the most effective, AP1 sites were intermediate, and Oct-1 and NF-κB sites were least effective. These results suggest that Tat may interact with proteins bound at the enhancer sequences during transactivation. Moreover, in the context of the native HIV-1 LTR, interaction with proteins bound at the Sp1 sites (e.g., Sp1) would likely be most important (7, 41, 70).

Sp1 was first identified as a protein factor that bound to the GGGCGG sequence (GC box) in the simian virus 40 early promoter (19, 20). It is a 95- to 105-kDa protein which is ubiquitously expressed in mammalian cells. Sp1-binding motifs are frequently found upstream of cellular and viral promoters, and Sp1 modulates transcription initiation through glutamine-rich domains (15). A functional cDNA clone for Sp1 has been isolated, and Sp1 can be readily assayed in in vitro transcription systems (60). Recently, it has been shown that Sp1 may require physical interaction with other proteins (coactivators) for activity (21, 60). Moreover, other experiments (47) indicate that Sp1 may be a

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direct intermediating protein for a bovine papillomavirus transactivator.

In these experiments, we directly tested the suggestion that Tat may function by interaction with enhancer-binding proteins, in particular, Sp1. Retention of Sp1 protein (and, to a lesser degree, other enhancer-binding proteins) on Tat affinity matrices and coimmunoprecipitation of Tat and Sp1 in infected cells support a direct physical interaction between Sp1 and Tat.

MATERIALS AND METHODS

Plasmid constructions, transfections, and infections. Plasmids were constructed by standard techniques. For in vitro transcription-translation of Sp1 mRNA, a cDNA that encodes Sp1 (40, 64) was placed downstream of a synthetic T7 promoter either in frame (pT7Sp1) or frame shifted by one nucleotide (pT7Sp1fs). In vitro transcription was performed with T7 RNA polymerase (Promega) plus a synthetic cap analog and 1 μg of an appropriately linearized plasmid template; translation of mRNA was performed with rabbit reticulocyte lysate (Promega) along with [^{35}S]methionine and [^{35}S]cysteine. pNF-κBx4 and pSp1x6 were constructed by ligating synthetic NF-κB and Sp1 motifs into the *Xba*I restriction site of p-43CAT (7). pSp1CAT and pmSp1CAT contain three copies of wild-type or mutated Sp1 sites in p-43CAT. Transient transfections were performed as previously described (7). Human T-cell leukemia virus type I (HTLV-I) plasmid pU3RCAT has already been described (10).

Infection of the C81-6645 T4 lymphocyte line with molecularly cloned HIV-1 (pNL4-3) has been described previously (1).

Protein expression, coupling, cross-linking, and chromatography. HIV-1 Tat (86 aa) was produced synthetically (11). Alternatively, a biologically active version (first 72 aa) was expressed in *Escherichia coli* from the thermally inducible coliphage p_L promoter. In the latter, *tat* was cloned into the pL-ner vector (2) in place of *ner*. Tat was purified to homogeneity by urea extraction with successive column chromatography. This *E. coli* Tat was a gift from P. Wingfield and S. Staal (National Institutes of Health).

Coupling of *E. coli* Tat to CNBr-activated Sepharose 4B was performed as recommended by the manufacturer (Pharmacia). A 50-mg portion of CNBr-activated Sepharose 4B was swollen in 1 mM HCl. The swollen beads were equilibrated in 0.1 M $NaHCO_3$–0.5 M NaCl (pH 8.0). After equilibration, the beads were mixed individually with 100 μg of Tat (or Tat peptides), bovine serum albumin (BSA), ovalbumin, or lysozyme (Sigma) for 2 h at room temperature. Subsequently, the beads were washed extensively with 0.1 M sodium acetate–0.5 M NaCl (pH 4.5) and equilibrated in 0.2 M glycine buffer. The beads were then washed with 20 column volumes of 0.1 M $NaHCO_3$–0.5 M NaCl (pH 8.0) and equilibrated with 10 mM *N*-2-hydroxyethylpiperazine-*N*′2-ethanesulfonic acid (HEPES; pH 7.9)–100 mM KCl–0.2 mM EDTA–1 mM dithiothreitol (load buffer). HeLa nuclear lysates, adjusted to load buffer, were mixed with either Tat beads or neutral beads for 1 h at 4°C. The mixture was then washed with 10 column volumes of load buffer; this was followed by elution with 1 column volume of load buffer, 1 column volume of load buffer adjusted to 0.3 M KCl, and 1 column volume of load buffer adjusted to 0.6 M KCl. For synthetic Tat (s-Tat), the affinity column was generated by reacting 1 mg of s-Tat with 1 ml of Reacti-Gel 6x resin (Pierce) in accordance with the manufacturer's specifications.

For the s-Tat affinity column, crude nuclear extracts were prepared from frozen, packed HeLa cells (12 to 18 ml of packed cells) as described by Dignam et al. (17), except that the nuclear extraction buffer was 50 mM HEPES (pH 7.6)–0.42 M KCl–20% glycerol–10% sucrose–5 mM $MgCl_2$–0.1 mM EDTA–1 mM sodium metabisulfite–2 mM dithiothreitol–1 mM phenylmethylsulfonyl fluoride. The nuclear extracts were dialyzed against buffer A (0.25 mM HEPES [pH 7.6], 0.1 M KCl, 12.5 mM $MgCl_2$, 20% glycerol, 10 μM $ZnSO_4$, 1 mM dithiothreitol) and then loaded onto a heparin-agarose column (6 ml; Bio-Rad). The column was washed with 5 to 10 column volumes of buffer A, and bound proteins were eluted with buffer A containing 0.5 M KCl. Column fractions containing protein (measured by A_{280}) were pooled, dialyzed against 50 volumes of buffer A, and loaded onto the s-Tat column in buffer A. Step elutions were done at 0.25 and 0.5 M KCl. Eluates were concentrated and desalted twice into buffer A by centrifugation (5,000 rpm in a Sorvall SS34 rotor at 4°C in Centricon 10 microconcentration tubes [Amicon]). The final volume of the eluted fractions was approximately 200 μl.

For protein-protein cross-linking, Sp1 (25 footprinting units [fpu] as defined by Promega) was incubated with 0.5 μg of purified Tat protein in a 20-μl reaction volume in 0.013% glutaraldehyde (dissolved in 10 mM potassium phosphate [pH 8.0]) for 1 h at room temperature. The cross-linked reaction was electrophoresed in a sodium dodecyl sulfate (SDS)–12% polyacrylamide gel and stained with silver.

UV cross-linking. UV cross-linking was done as described previously (35). Protein fractions were incubated as for gel shift assays with a ^{32}P-end-labeled double-stranded Sp1 oligonucleotide and the nonspecific competitor deoxyinosine-deoxycytidine (dI-dC) in a total volume of 16 μl for 30 min at room temperature in 1.5-ml microcentrifuge tubes. The tubes were then transferred to ice and irradiated with UV light (254 nm) for 5 min in a UVC 1000 Ultraviolet cross-linker (Hoefer). An equal volume of SDS-polyacrylamide gel electrophoresis (PAGE) sample buffer was added, and the samples were heated at 65°C for 10 min and analyzed by SDS-PAGE and autoradiography.

Electrophoretic mobility shift assay. Electrophoretic mobility shifts were performed as previously described (35). The oligonucleotide probes used were as follows: NFκB, 5′TGG GACTTTCC3′ (monomer) or 5′CTAGCAAGGGACTTTC CGCTGGGGACTTTCCA3′ (dimer); Oct, 5′CTAGTCATT TGCATTCATTTGCATA3′ (4); Sp1, 5′GGAGGCGTGGC3′ (monomer) or 5′CTAGGGGAGGCGTGGCCTGGGCGGAC TGGGGAGTGGCG3′ (trimer); Sp1 mutant, 5′CTAGGGGG GGGGGGGCCTGGGGGGACTGGGGGGTGGGG3′ (based upon reference 45). Probes were end labeled with [^{32}P] cATP.

In vitro transcription. In vitro transcription of supercoiled plasmids in *Drosophila* embryo nuclear extract was performed as described by the manufacturer (Promega). Transcripts were assayed by primer extension with a ^{32}P-end-labeled 31-base oligonucleotide primer (5′GCCATTGGGATATATCAACG GTGGTATATCC3′) complementary to *cat* mRNA (6).

RESULTS

Binding of synthetic Tat to nuclear factors. To test the possibility of interactions between Tat and cellular factors that bind to HIV LTR DNA, chromatography on a Tat

affinity column was performed. We previously described the chemical synthesis of biologically active Tat (s-Tat; 13). This approach yielded large quantities, which allowed for ready preparation of an affinity column. A crude HeLa cell nuclear extract (17) was first bound to heparin-agarose to eliminate nucleic acids that might later influence specific or nonspecific binding to s-Tat. The heparin-agarose column was eluted, and the eluate was passed over an s-Tat column. Fractions eluting at 0.25 and 0.5 M KCl were concentrated and tested for binding to HIV-1 LTR DNA fragments in electrophoretic mobility shift assays.

Assays of eluates from the s-Tat affinity column are shown in Fig. 1. The HIV-1 LTR was first divided into fragments containing upstream U3 sequences (*Eco*RV to *Pvu*II [−342 to −21]) and TAR DNA (*Pvu*II to *Hin*dIII [−21 to +77]) (Fig. 1a). As shown in Fig. 1b, the s-Tat eluate contained binding activity for the *Eco*RV-*Pvu*II fragment in the 0.25 M KCl fraction, evident in the presence of 1 μg of poly(dI-dC). The same pattern was also evident in the presence of even higher levels of poly(dI-dC) (5 μg/16-μl reaction), although at reduced intensity. The 0.5 M KCl fraction showed no significant binding to the *Eco*RV-*Pvu*II fragment, even in the presence of low levels of poly(dI-dC). When the *Pvu*II-*Hin*dIII fragment was tested, neither fraction showed efficient binding in the presence of poly(dI-dC) competition (Fig. 1c). These results indicated that s-Tat-binding proteins did not include those with significant affinity for HIV TAR DNA, but they did include those that recognize HIV U3 sequences. Additional mapping with smaller U3 restriction fragments using the 0.25 M KCl fraction (Fig. 1d) demonstrated that the binding activity contained in the s-Tat eluate was directed toward HIV DNA sequences between −141 and −21 (the *Sca*I-*Pvu*II fragment). This region contains the NF-κB- and Sp1-binding sites.

To test directly for the presence of NF-κB- or Sp1-like activity in the s-Tat-binding proteins, electrophoretic mobility shift assays were performed with double-stranded oligonucleotides containing a monomeric HIV-1 NF-κB motif or the highest-affinity HIV-1 Sp1 motif. As shown in Fig. 2a, strong binding to the Sp1 oligonucleotide was detected. This binding was competed for by excess unlabeled homologous oligonucleotide. On the other hand, much-reduced binding to the NF-κB probe was seen (see below). These results suggested that Sp1 (more than NF-κB) preferentially bound to the s-Tat affinity column. UV cross-linking with a labeled Sp1 oligonucleotide, followed by SDS-PAGE, was performed on the s-Tat eluate (Fig. 2b). The results confirmed that the binding activity to the Sp1 probe was contained primarily in a protein of ca. 97 kDa (as well as in some lower-molecular-mass species). This molecular mass is consistent with the known molecular size of Sp1 (11). A parallel analysis of a commercial Sp1 preparation revealed a similar protein profile (Fig. 2b). The lower-molecular-mass forms may represent partially degraded forms of Sp1 that still retained specific DNA-binding activity (data not shown).

***E. coli* Tat binds to multiple upstream factors.** We wished to extend the s-Tat results and test their generality. It was possible that the observed Tat-Sp1 interaction might reflect a unique property of chemically synthesized Tat. Also, while elimination of nucleic acids from the nuclear extracts reduced potential artifactual association of DNA-binding proteins bridged by an electrostatic interaction of DNA with the basic domain of Tat, other Tat-binding proteins might have been lost during the heparin-agarose chromatography. Therefore, we repeated the assays with biologically active Tat purified from *E. coli* and a total HeLa nuclear extract.

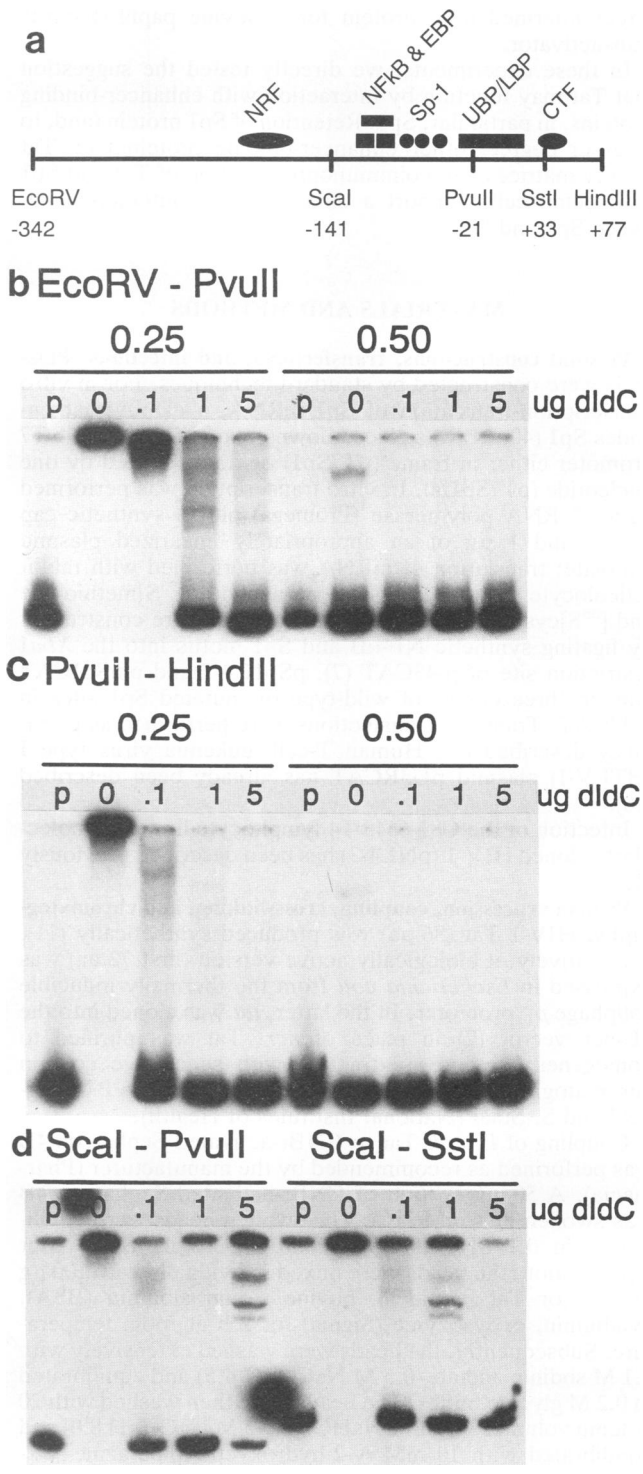

FIG. 1. Binding to HIV U3 DNA by s-Tat-binding proteins. (a) Restriction map of the HIV LTR with representations of binding sites for many host cell nuclear proteins (26, 39, 62). The transcription start is at +1, and the TAR sequence is between +19 and +42. (b) Gel mobility shift assays with a ^{32}P-end-labeled *Eco*RV-*Pvu*II fragment with either the 0.25 or the 0.5 M KCl eluate from the s-Tat affinity column. Different concentrations of nonspecific DNA (dIdC) were added. p, oligonucleotide probe without extract addition. (c) Gel mobility shift assays as in panel b but with the *Pvu*II-*Hin*dIII fragment. (d) Assays with either the labeled *Sca*I-*Pvu*II or the *Sca*I-*Sst*I fragment.

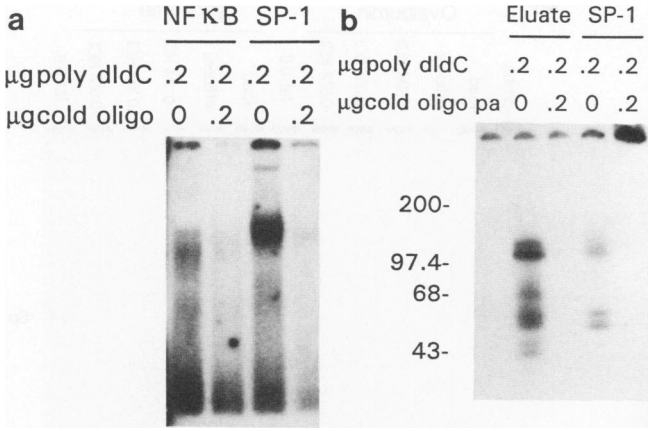

FIG. 2. Binding of Sp1 oligonucleotide to s-Tat-binding proteins. (a) Gel mobility shift assays of the 0.25 M KCl fraction of the s-Tat eluate with end-labeled oligonucleotides specific for the HIV-1 NF-κB (5'TGGGGACTTCC3' and its complement) or the highest-affinity HIV-1 Sp1 (5'GGAGGCGTGGC3' and its complement) site. All reactions contained 0.2 μg of dI-dC. Strong binding to the Sp1 oligonucleotide was observed. There was weak binding to the NF-κB oligonucleotide. Addition of 0.2 μg of a homologous unlabeled oligonucleotide completely eliminated complex formation in both cases. (b) UV cross-linking of the labeled Sp1 oligonucleotide to the 0.25 M KCl s-Tat eluate or to commercially prepared Sp1 protein (Promega). A higher-affinity Sp1 oligonucleotide (5'GATC GGGGCGGGGC3' with complement 5'GATCGCCCCGCCCC3') was used because the binding activity of commercial Sp1 was low. An SDS–7.5% PAGE gel is shown. Addition of an unlabeled homologous oligonucleotide (oligo) effectively competed for formation of the complexes. The numbers to the left are molecular sizes in kilodaltons.

The form of Tat used for these experiments corresponded to the first exon (residues 1 to 72), which has been shown to have full biological activity (23; see below).

We coupled *E. coli* Tat to Sepharose beads (Tat beads). Unlike s-Tat, which was somewhat heterogeneous in SDS-PAGE (11), bacterially produced Tat was homogeneous (Fig. 3A). *E. coli* Tat was also antigenically correct (Fig. 3B) and biologically active when introduced into cells (Fig. 3C). In this series of experiments, we also constructed controls for Tat beads. Neutral proteins (BSA, ovalbumin, and lysozyme) were linked to beads, and these beads were used in parallel to control for nonspecific protein-protein or protein-bead interactions (Fig. 4).

We equilibrated Tat beads or control beads with HeLa nuclear extract. Subsequently, the matrices were exhaustively washed in low-salt buffer (0.1 M KCl); this was followed by successive elutions with low-salt buffer (0.1 M KCl; Fig. 4A, lanes 5 to 12, and 4B, lanes 4 and 10), medium-salt buffer (0.3 M KCl; Fig. 4B, lanes 5 and 11), and high-salt buffer (0.6 M KCl; Fig. 4A, lanes 13 to 20, and 4B, lanes 6 and 13). Samples from each eluate were analyzed for Sp1, NF-κB, and Oct in electrophoretic mobility shift assays by using the corresponding oligonucleotide probes (dimers or trimers of the binding motifs; Fig. 4A and B). All three of the factors tested showed binding to and elution from the Tat beads (Fig. 4A, lanes 18 to 20). By using the BSA matrix to normalize for nonspecific associations (Fig. 4A, lanes 14 to 16), we found that Oct, NF-κB, and Sp1 had specific/nonspecific binding ratios of 5.4, 6.9, and 32, respectively. Moreover, comparison of the amounts of Sp1 versus NF-κB recovered from the Tat beads relative to the amounts in the original sample (Fig. 4A, lanes 1 to 4 [Load]) indicated more efficient binding of Sp1 to Tat. These results paralleled our previous findings that all three factor-binding motifs confer a Tat response on a basal HIV promoter-TAR region, with Sp1 sites being the most efficient (7). As shown in Fig. 4B, the

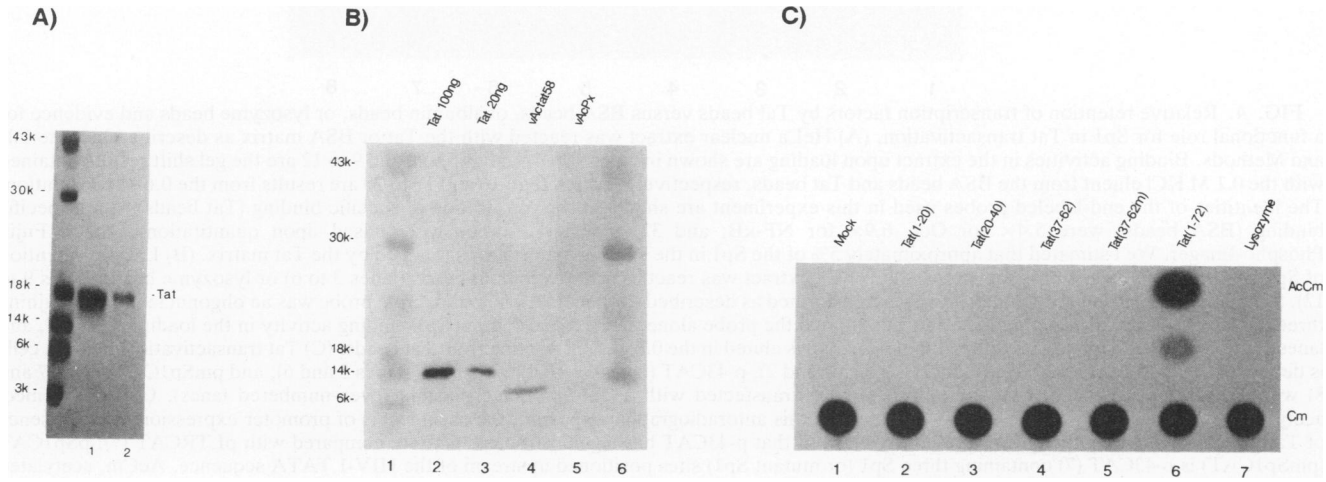

FIG. 3. Purity and biological activity of *E. coli* Tat protein used for coupling to beads. (A) Coomassie blue-stained profiles of Tat after resolution by SDS-PAGE. Lane 1 contained approximately 100 ng of purified *E. coli* Tat protein, and lane 2 contained approximately 20 ng of Tat protein. (B) Reactivity of Tat protein to anti-Tat antibody. Purified *E. coli* Tat protein (lanes 2 and 3) and total cellular proteins (lane 4) from Sf9 cells infected with a baculovirus expressing a 58-aa version of Tat (vActat58) were analyzed by Western blotting with a Tat-specific polyclonal serum. Sf9 cells infected with baculovirus expressing HTLV-I Tax protein (vAcPx, lane 5; 44) were used as a negative control. Prestained molecular size markers were in lanes 1 and 6. (C) *E. coli* Tat is biologically active upon scrape loading (30) into a CV-1 cell line containing an integrated HIV-1 LTR *cat* gene (37). Five micrograms of *E. coli* Tat (Tat 1-72, lane 6) was scrape loaded into 5×10^5 cells, and a chloramphenicol acetyltransferase assay was performed 48 h later. For comparison, different Tat peptides (lane 2 to 5) or a mass-equivalent amount of lysozyme (lane 7) was similarly treated. AcCm, acetylated chloramphenicol; Cm, unacetylated chloramphenicol.

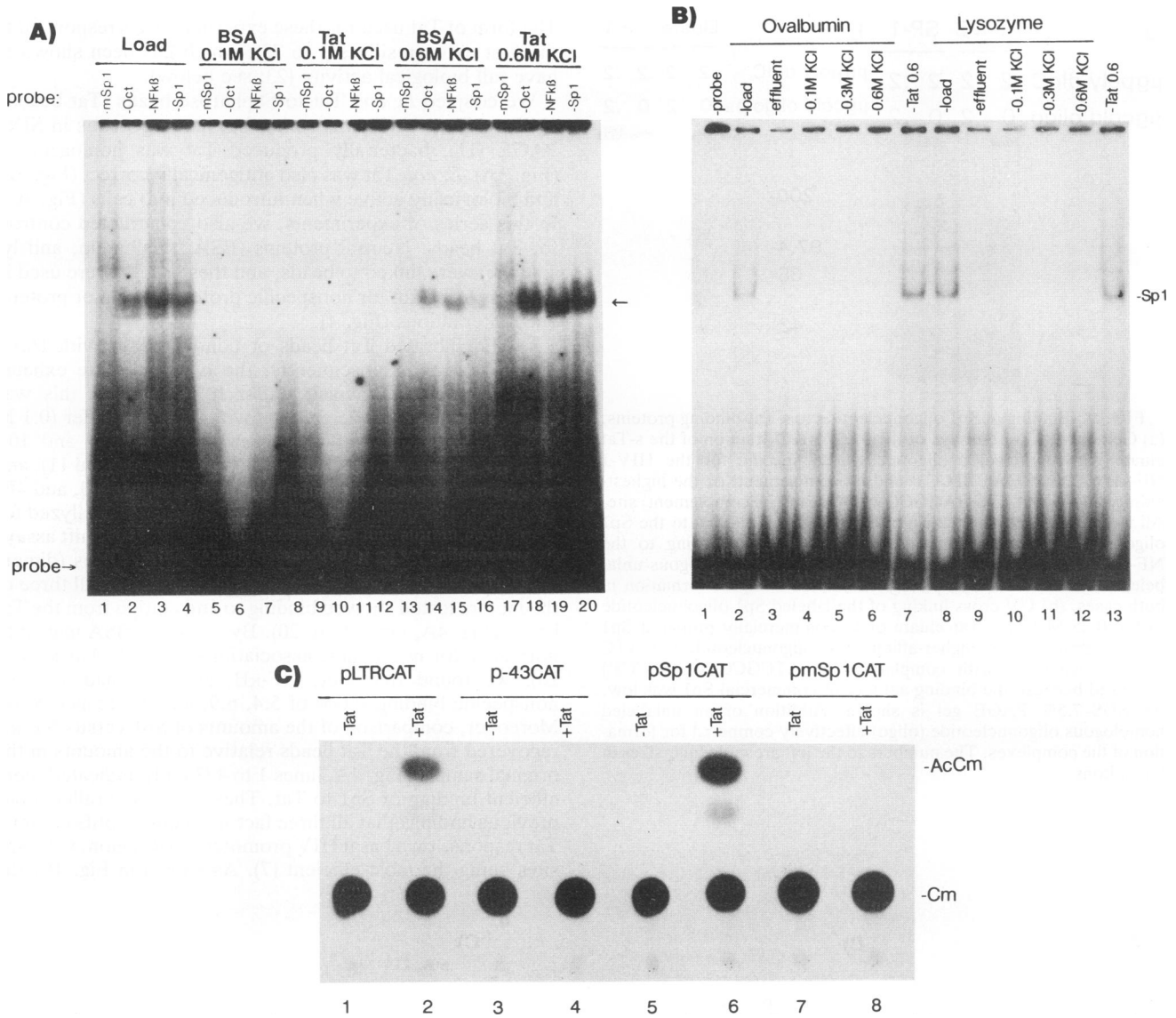

FIG. 4. Relative retention of transcription factors by Tat beads versus BSA beads, ovalbumin beads, or lysozyme beads and evidence for a functional role for Sp1 in Tat transactivation. (A) HeLa nuclear extract was reacted with the Tat or BSA matrix as described in Materials and Methods. Binding activities in the extract upon loading are shown in lanes 1 to 4. Lanes 5 to 8 and 9 to 12 are the gel shift results obtained with the 0.1 M KCl eluent from the BSA beads and Tat beads, respectively. Lanes 13 to 16 and 17 to 20 are results from the 0.6 M KCl elution. The identities of the end-labeled probes used in this experiment are shown at the top. Ratios of specific binding (Tat beads) to nonspecific binding (BSA beads) were 5.4× for Oct, 6.9× for NF-κB, and 32× for Sp1. Ratios were based upon quantitations with a Fujix Phospho-Imager. We estimated that approximately 5% of the Sp1 in the loading material was retained by the Tat matrix. (B) Lack of retention of Sp1 by ovalbumin beads or lysozyme beads. HeLa extract was reacted with ovalbumin beads (lanes 3 to 6) or lysozyme beads (lanes 9 to 12). Stepwise elutions and gel shift analysis were performed as described for panel A. The radioactive probe was an oligonucleotide containing three copies of the Sp1-binding motif. Lane 1 contained the probe alone, lanes 2 and 8 show Sp1-binding activity in the loading material, and lanes 7 and 13 show a comparison of Sp1-binding activities eluted in the 0.6 M KCl fraction from Tat beads. (C) Tat transactivation in HeLa cells is dependent upon functional Sp1. pLTRCAT (lanes 1 and 2), p-43CAT (lanes 3 and 4), pSp1CAT (lanes 5 and 6), and pmSp1CAT (lanes 7 and 8) were transfected alone (odd-numbered lanes) or cotransfected with a Tat-producing plasmid (even-numbered lanes). Chloramphenicol acetyltransferase activities were assayed 48 h later. At this autoradiographic exposure, the basal levels of promoter expression in the absence of Tat were not visible, although we previously showed that p-43CAT has significant basal activity compared with pLTRCAT (7). pSp1CAT (pmSp1CAT) is p-43CAT (7) containing three Sp1 (or mutant Sp1) sites positioned upstream of the HIV-1 TATA sequence. AcCm, acetylated chloramphenicol; Cm, unacetylated chloramphenicol.

ovalbumin and lysozyme matrices confirmed that Sp1 has much higher affinity for Tat than do other, neutral proteins.

These results agreed with the s-Tat findings of Fig. 2. However, the use of multimerized binding motifs as probes conferred greater sensitivity of detection on some proteins (e.g., NF-κB but not Sp1). We also found differences in the optimal salt concentration for binding of heparin-agarose-purified factors to s-Tat versus the binding of factors in crude nuclear extract to *E. coli* Tat. This might reflect different behaviors of proteins after purification on heparin-agarose,

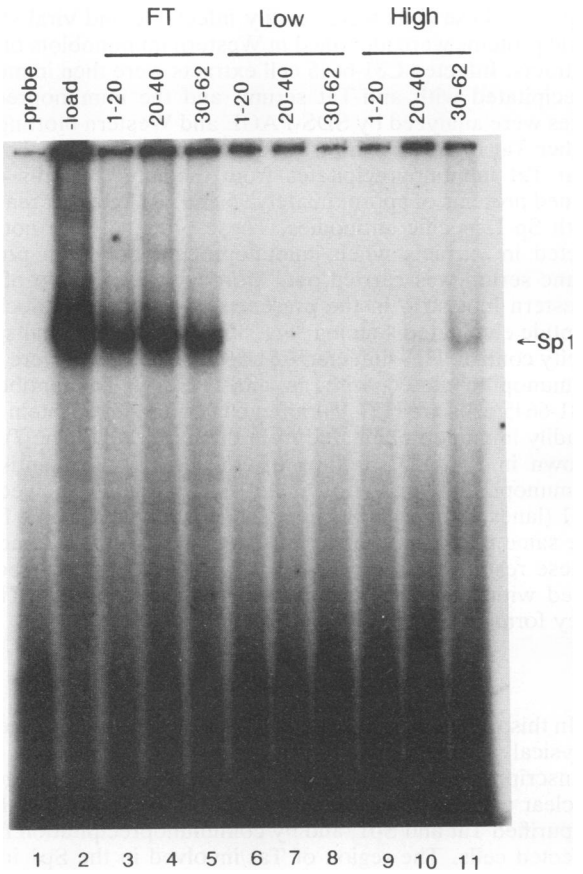

FIG. 5. Assay of Sp1 retention with Tat peptide columns. Tat peptides containing aa 1 to 20 (lanes 3, 6, and 9), 20 to 40 (lanes 4, 7, and 10), or 30 to 62 (lanes 5, 8, and 11) were linked to beads. These beads were equilibrated with HeLa extract and eluted with a low (0.3 M KCl) or high (0.6 M KCl) salt concentration as for Fig. 4. The eluents were reacted with a radiolabeled Sp1 oligonucleotide probe and analyzed by gel mobility shift assay. Lane 1 contained the probe alone, and lane 2 shows Sp1-binding activity in the extract prior to reaction with the respective beads. FT, flowthrough.

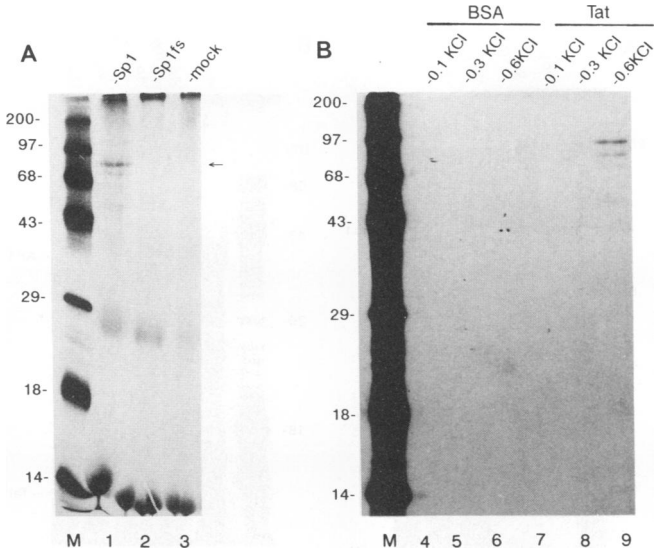

FIG. 6. Retention of Sp1 by Tat beads. (A) In vitro translation of Sp1 mRNA with rabbit reticulocyte lysate. Lanes: 1, translation of T7 RNA polymerase-synthesized Sp1 mRNA; 2, translation of Sp1 mRNA frame shifted by +1 nucleotide immediately following the first AUG; 3, translation of reticulocyte lysate with no added mRNA; M, ^{14}C-labeled molecular size markers. (B) Elution of reticulocyte-synthesized Sp1 from a BSA matrix (lanes 4 to 6) or a Tat matrix (lanes 7 to 9). Whole reticulocyte lysate after translation was divided equally, separately reacted with the two matrices, and eluted stepwise with KCl buffer. Aliquots of each elution were run in an SDS–12% polyacrylamide gel and then autoradiographed. The numbers to the left of each panel are molecular sizes in kilodaltons.

differences between the s-Tat matrix and the Tat beads, or the fact that s-Tat was full length (86 aa versus the 72 aa of *E. coli* Tat).

The ability of Sp1 sites to confer Tat responsiveness on a basal HIV-1 promoter is shown in Fig. 4C. We confirmed that transactivation was indeed dependent upon the addition of functional Sp1-binding sites (pSp1CAT; Fig. 4C, lanes 5 and 6) to a minimal HIV-1 TATA promoter (p-43CAT; Fig. 4C, lanes 3 and 4; reference 7). A control construct containing three mutated Sp1 sites attached to p-43CAT (pmSp1CAT; Fig. 4C, lanes 7 and 8) was not activated by Tat. These intracellular results agree with the in vitro association between Sp1 and Tat (Fig. 4A and B).

We next determined whether there was a definable region within Tat responsible for protein-protein interaction. To do this, peptides encompassing different parts of Tat were fixed to beads (Fig. 5). These beads were tested for the ability to retain Sp1. We detected Sp1-binding activity with a peptide spanning aa 30 to 62 of Tat (Fig. 5, lane 11). Moreover, peptides corresponding to residues 1 to 20 and 20 to 40 had no or very weak activity. Thus, aa 30 to 62 of Tat contain the domain that interacts with Sp1 and, perhaps, other transcription factors. The relative inefficiency of Sp1 binding observed in Fig. 5 compared with Fig. 4 could have been due to the fact that Tat 1-72 (which has the native configuration, allowing biological activity) was used for Fig. 4 while shorter oligopeptides were used for Fig. 5.

Direct association of Sp1 and Tat in vitro. The biochemical experiments of Fig. 2 and 4, as well as genetic experiments (7, 41, 70, 75; Fig. 4C), did not indicate whether Tat and Sp1 interact directly or through a third protein. To address this question, we performed two experiments. First, we prepared Sp1 free of other HeLa nuclear factors by in vitro translation of Sp1 mRNA. Second, we used Sp1 that had been purified to apparent homogeneity. Both protein versions were tested for binding to Tat.

We transcribed Sp1 mRNA from a cDNA clone (40, 66) positioned downstream of a T7 promoter. mRNA synthesized with T7 polymerase and translated in a rabbit reticulocyte lysate yielded a protein doublet of approximately 90 kDa, which is expected for unglycosylated Sp1 (Fig. 6A, lane 1). This radiolabeled protein(s) was mixed with either Tat beads or BSA beads. When both types of beads were separately eluted, there was preferential retention of the Sp1 doublet by the Tat matrix (Fig. 6B, lane 9). Approximately 15 to 20% of the labeled Sp1 bound to and eluted from the Tat beads (data not shown). Given the absence of other nuclear factors, this suggested that translated Sp1 likely bound directly to Tat. However, it was formally possible that a reticulocyte protein(s) bridged the two proteins.

We next tested whether purified Sp1 could interact with purified Tat. We used a protein cross-linker, glutaraldehyde, to probe formation of Sp1-Tat complexes in solution. On the

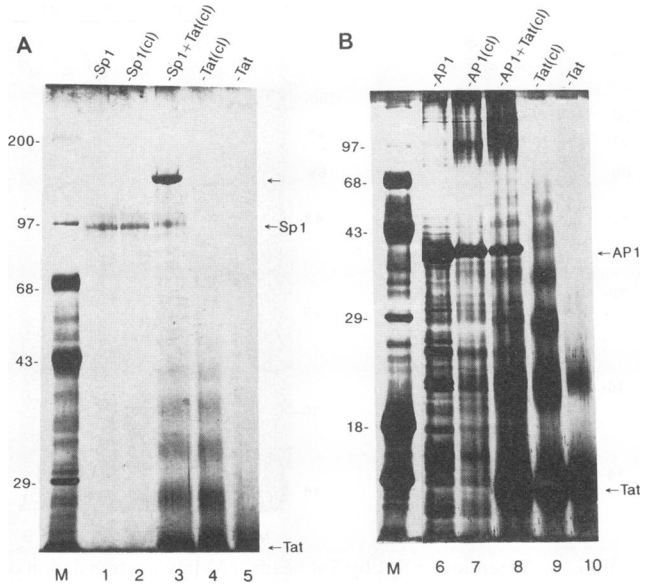

FIG. 7. Cross-linking of Sp1 to Tat. (A) Purified Sp1 and Tat proteins were coupled by using glutaraldehyde. Lanes: 1, Sp1 (25 fpu) alone with no cross-linker added; 2, Sp1 (25 fpu) plus glutaraldehyde in a self-cross-linking reaction; 3, Sp1 (100 fpu) plus Tat (0.5 μg) and glutaraldehyde; 4, Tat (0.5 μg) plus glutaraldehyde; 5, Tat (0.5 μg) alone with no added cross-linker. The samples were resolved in a 12% denaturing gel and then stained first with Coomassie blue and then with silver. The silver-stained profiles are shown. The arrows point to Tat, Sp1, and a novel complex. (B) Cross-linking of AP1 to Tat. Lanes: 6, partially purified AP1 (25 fpu) alone with no cross-linker; 7, AP1 (25 fpu) plus glutaraldehyde; 8, AP1 (100 fpu) plus Tat (0.5 μg) and glutaraldehyde; 9, Tat (0.5 μg) plus glutaraldehyde; 10, Tat (0.5 μg) alone without the cross-linker. (cl) indicates the presence of the cross-linker in the reaction. For some of the cross-linking reactions, more AP1 or Sp1 was necessary to visualize residual unlinked moieties. The numbers to the left of each panel are molecular sizes in kilodaltons.

basis of silver staining, both the Sp1 (Fig. 7A, lane 1) and the Tat (Fig. 7A, lane 5; Fig. 3) preparations were quite homogeneous. Treatment of Sp1 with glutaraldehyde (Fig. 7A, lane 2) produced no homodimers-oligomers. Incubation of Tat alone, however, resulted in homodimers-oligomers, presumably from oxidation of sulfhydryl groups (22; Fig. 7B, lane 10). This effect was accentuated by glutaraldehyde (Fig. 7, lanes 4 and 9). When Sp1 and Tat were incubated together with the cross-linker, we saw a new protein species (Fig. 7A, lane 3, arrow). (Other minor species appeared upon longer development of the gel.) This new polypeptide was compatible in size with a complex containing one Sp1 and two Tat molecules. Parallel experiments were performed with partially purified AP1 (Fig. 7B). Here, we saw some inefficiently cross-linked minor species (for example, Fig. 7B, lane 8, areas between 43 and 68 kDa and above 97 kDa). These findings correlated with the reduced ability of AP1 (compared with Sp1) to support intracellular Tat transactivation (7).

Association of Tat and Sp1 in vivo. As a final test of the biological relevance of the in vitro interactions between Tat and Sp1 described above, we investigated whether Tat-Sp1 complexes could be detected in HIV-1-infected cells. C81-6645 T4 lymphocytes were infected with molecularly cloned HIV-1 (pNL4-3) as described previously (1). As shown in Fig. 8A, these cells were readily infectable and viral structural proteins were identified in Western immunoblots of cell extracts. Infected C81-6645 cell extracts were then immunoprecipitated with anti-Tat serum, and the immunoprecipitates were analyzed by SDS-PAGE and Western blotting for either Tat or Sp1 as shown in Fig. 8B. The results showed that Tat immunoprecipitates from the infected cells contained proteins of approximately 95 and 105 kDa that reacted with Sp-1-specific antibodies. These proteins were not detected in extracts when immunoprecipitation with preimmune serum was carried out. Moreover, incubation of the Western blot strip in the presence of excess Sp1 blocking peptide eliminated staining (Fig. 8C). As an additional specificity control, HIV-infected C81-6645 cell extracts were also immunoprecipitated with an anti-HTLV-I Tax antibody. C81-6645 cells are HTLV-I infected (1), and Tax protein was readily immunoprecipitated from them (Fig. 8D, lane 7). As shown in Fig. 8D, probing of Western blots of anti-Tax immunoprecipitates with the anti-Sp1 antibody showed no Sp1 (lanes 1 and 2) while anti-Tat immunoprecipitates from the same cells showed the presence of Sp1 (lanes 3 and 4). These results indicated that Sp1 protein coimmunoprecipitated with Tat protein in the infected-cell extracts. Thus, they form complexes in infected cells.

DISCUSSION

In this report, we describe in vitro and in vivo evidence for physical interaction between the HIV-1 Tat protein and transcription factor Sp1. This was observed by passage of nuclear extracts over Tat affinity matrices, by cross-linking of purified Tat and Sp1, and by coimmunoprecipitation from infected cells. The region of Tat involved in the Sp1 interaction was in a subregion of the protein including residues 30 to 62. Interactions between Tat and other enhancer-binding proteins were also observed, although in those cases the binding was less efficient.

These results were very consistent with those of genetic experiments described in the introduction and Results, indicating an essential role for enhancer sequences (particularly Sp1) in Tat transactivation. They further suggest that when Tat is tethered to the transcription complex by TAR RNA (or by other artificial means), its ultimate target may be an enhancer-binding protein. In the native HIV-1 LTR, the crucial protein is Sp1, and the experimental results in Fig. 7 suggest that the Tat-Sp1 contact is direct. It should also be noted that since several other enhancer sequences besides Sp1 can confer Tat responsiveness on the basal HIV-1 promoter (7), it is not surprising that Tat would show in vitro binding to these proteins as well.

The finding that the Sp1-binding region of Tat was encompassed in a peptide extending from residues 30 to 62 raised a potential question, because this includes the basic domain and this domain has been shown to be responsible for Tat binding to TAR RNA (14, 71). However, it has been shown that a Tat peptide corresponding to residues 47 to 58 is sufficient for specific TAR RNA binding (12), so other portions of the 30-to-62 region might mediate Sp1 binding.

Four lines of evidence support biological relevance for the observed Tat-Sp1 interaction. First, when nuclear extracts were passed over affinity matrices containing other neutral proteins (BSA, lysozyme, and ovalbumin), reduced or undetectable binding of the transcription factors was observed (Fig. 4). Second, other nuclear DNA-binding proteins that bind to other regions of the HIV-1 LTR (e.g., UBP/LBP and CTF; references 25, 39, and 74) were not retained on the

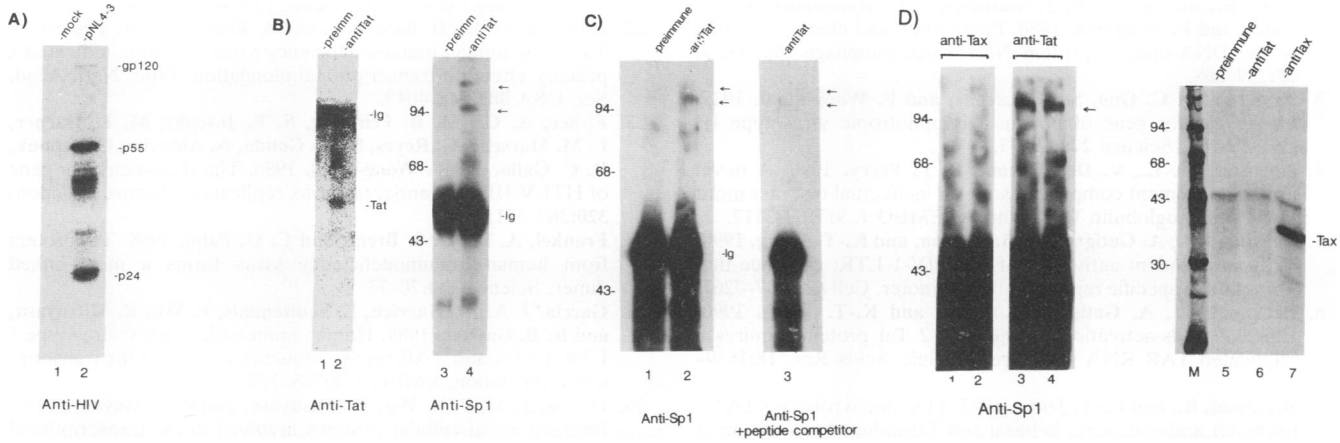

FIG. 8. Coimmunoprecipitation of Tat and Sp1 from infected cells. (A) C81-6645 cells were infected with 5×10^6 reverse transcriptase units of molecularly cloned HIV-1 (pNL4-3). After 4 days, cell extracts were prepared from mock-infected or infected cultures and analyzed by SDS-PAGE and Western blotting with serum from an HIV-1-infected patient. (B) An infected-cell extract was immunoprecipitated with anti-Tat sera (a mixture of rabbit anti-Tat and rat anti-Tat sera) (lanes 2 and 4) or preimmune sera (lanes 1 and 3). The immunoprecipitates were divided in two, resolved in an SDS–16% polyacrylamide (lanes 1 and 2) or an SDS–7% polyacrylamide (lanes 3 and 4) gel, and transferred to polyvinylidene difluoride membranes. The membranes were then incubated with anti-Tat (lanes 1 and 2) or anti-Sp1 (Santa Cruz Biotechnology) antibodies. Antibody binding was visualized by [^{125}I]protein A for anti-Tat antibodies and by chemiluminescence (TROPIX, Bedford, Mass.) for anti-Sp1 antibodies. The 17-kDa Tat-specific band in the immunoprecipitate is indicated in lane 2. Two Sp1-specific bands of approximately 95 and 105 kDa were evident in the Tat immunoprecipitates as indicated in lane 4. (C) Immunoprecipitation of an infected-cell extract with anti-Tat or preimmune sera was done as described for panel B, except that the anti-Tat immunoprecipitate was divided and loaded into two adjacent lanes of an SDS-polyacrylamide gel (lane 2 and 3). After electrophoresis and transfer to polyvinylidene difluoride, the two lanes were cut apart. One was reacted with anti-Sp1 antibody (lanes 1 and 2), and the other was reacted with anti-Sp1 antibody preincubated with 20 μg of blocking Sp1 peptide (lane 3). All strips were visualized by chemiluminescence. (D) Immunoprecipitation of an infected-cell extract with anti-HTLV-I Tax serum (lanes 1 and 2) or anti-Tat serum (lanes 3 and 4) was carried out, and Sp1 was detected by Western blot analysis as for panel B. Duplicate immunoprecipitations with each antiserum are shown (lanes 1 and 2 and 3 and 4, respectively). The Sp1 apparent in the anti-Tat immunoprecipitates is indicated by the arrow. Immunoprecipitation of [^{35}S]methionine-labeled uninfected C81-6645 cells, followed by SDS-PAGE and autoradiography, is shown in lanes 5 to 7. HTLV-I Tax protein was readily evident when the labeled extracts were immunoprecipitated with anti-Tax serum (lane 7). No HIV proteins would be expected in the uninfected C81-6645 cells (lane 6). Lane M contained radioactive molecular weight markers. The numbers to the left of some of the panels are molecular sizes in kilodaltons.

s-Tat affinity column, while Sp1 (and, to a lesser degree, NF-κB) was (Fig. 1). Third, our previous genetic experiments indicated that a variety of enhancer motifs can confer Tat response on a basal HIV-1 promoter but with differing efficiencies (7). Sp1 motifs were the most effective, while AP1, Oct, and NF-κB sites, in descending order, were less effective. As shown in Fig. 4 and 7, the relative efficiencies of Tat binding to the different enhancer-binding proteins mirrored this order, with the Tat-Sp1 interaction being the strongest. Fourth, the coimmunoprecipitation experiment of Fig. 8 demonstrated that Tat-Sp1 complexes can be detected in HIV-1-infected cells. Combined, these results provide persuasive support for the biological relevance of Tat-Sp1 binding.

Protein-protein interactions in transcriptional regulation are well known (for reviews, see references 31, 46, 48, and 59). For example, acidic transactivators such as Gal4 or VP-16 can directly contact TFIID (34) or TFIIB (49, 50; reviewed in reference 66). The TATA-binding protein component of TFIID can also bind at least six separate protein coactivators (21; reviewed in reference 48). Some of these coactivators are necessary to mediate transcriptional effects of factors such as Sp1 (60). Other activators, such as adenovirus E1A, possibly contact the TATA-binding protein (31, 33) directly, and others, such as bovine papillomavirus E2 (47), indirectly affect TATA through Sp1. Thus, there are precedents for Tat functioning by contact with Sp1 or other enhancer-binding proteins. These results do not exclude the possibility that Tat may also contact other cellular proteins during transactivation, and other Tat-binding proteins have also been described (16). The initial tethering of Tat to the transcription complex is through binding to the stem-loop structure of TAR RNA. Highly structured RNA leaders are also common in cellular genes, which raises the possibility that regulation at the promoter by proteins that initially bind to 5′ leader RNAs may occur for cellular genes as well.

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