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Identification of cellular proteins that bind to the human immunodeficiency virus type 1 trans-activation-responsive *TAR* element RNA

(long terminal repeat/RNA-binding proteins)

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ABSTRACT **Human immunodeficiency virus type 1 (HIV-1) trans-activator protein Tat activates the expression of its viral long terminal repeat (LTR) through a target trans-activation-responsive element termed *TAR*. We have constructed cell lines that constitutively express the HIV-1 Tat protein. Analyses of nuclear proteins from these cells and from matched control cells that do not express Tat have identified three proteins that bind to a radiolabeled HIV-1 *TAR* RNA probe. These polypeptides are 100 kDa, 62 kDa, and 46 kDa in size. Competition experiments using a wild-type *TAR* RNA sequence, a biologically inactive mutant sequence of *TAR*, and an unrelated RNA species demonstrated that these proteins show higher binding affinity to wild-type *TAR* than to the other two non-trans-activatable sequences. We hypothesize that these cellular proteins may mediate a function necessary in Tat-dependent activation of the LTR. The fact that no differences were seen in the binding profiles of nuclear proteins to *TAR* RNA in Tat-producing and Tat-nonproducing cells suggests that Tat does not directly interact with *TAR*.**

Recent molecular studies on the expression of the human immunodeficiency virus type 1 (HIV-1) RNA genome have revealed complex and multifactorial modes of regulation (1, 2). Despite the diverse means for its activation, the HIV-1 long terminal repeat (LTR) can be viewed, in simplified terms, as consisting of a constitutive and a Tat-dependent component for transcription.

Structural dissections of the viral LTR have defined several cis-acting elements important for the constitutive expression of HIV-1. Between nucleotides −340 and −185, relative to the mRNA cap site (nucleotide +1), is a negative regulatory element (3). More proximally, a viral enhancer consisting of a tandemly duplicated GGGACTTTCC sequence is positioned between nucleotides −105 and −80. This sequence is conserved within the immunoglobulin κ light chain (4, 5), the cytomegalovirus (CMV; refs. 6 and 7), and the simian virus 40 (8, 9) enhancers. While it is important for basal genetic activity, the HIV-1 enhancer also responds to mitogenic stimuli such as phytohemagglutinin, phorbol 12-myristate 13-acetate, and calcium ionophores (5, 10–13). A third important group of regulatory sequences, located between the enhancer and the TATA promoter, is the triply reiterated SP1-binding motifs (14, 15). These sequences contribute significantly toward the constitutive expression of the HIV-1 LTR (16).

The cis-acting LTR target required for Tat-activated expression [called the *TAR* (trans-activation-responsive) element; ref. 3] has also been characterized. *TAR* is positioned between nucleotides +1 and +80 (3, 17–19). The combined observations that the *TAR* sequence is present in viral RNA, that *TAR* functions in an orientation-dependent manner (20), and that transcription factors that bind to the DNA *TAR* element play no apparent role in Tat-mediated trans-activation (21, 22) suggest that *TAR* may be functional as a RNA element. In this regard it is noteworthy that the *TAR* RNA sequence exhibits complex intramolecular base pairings and can fold into a hairpin-like secondary structure *in vitro* (17). The integrity of the *TAR* hairpin structure has been shown to be required for *in vivo* activation by Tat (19, 23).

There is, currently, no definitive evidence for a direct interaction between Tat and *TAR*. The lack of sequence-specific nucleic acid-binding properties for Tat (24) suggests that cellular intermediary proteins may be used for its activation of the HIV-1 LTR. Here, we report on the identification of several cellular factors that bind to a radiolabeled *TAR* RNA probe. We find that these factors are present without significant qualitative or quantitative differences in the nuclei of cells that do or do not constitutively express an integrated *tat* gene. We propose that these cellular RNA-binding proteins play a role in mediating Tat-dependent LTR activation.

MATERIALS AND METHODS

Cells and Protein Extracts. Cells expressing HIV-1 Tat were derived by cotransfecting pRSVneo (25) with pSVtat (26). Individual G418-resistant colonies were isolated after 2 weeks of drug selection. HeLaTat, a Tat-producing HeLa cell line, was a gift from Bryan Cullen (Duke University, Durham, NC). Nuclear extracts were made by the procedure of Dignam *et al.* (27).

RNA Gel Retardation Assay. Plasmids pGA99, pGA20, and pGA1 (see Fig. 1*A*) were linearized with *Hin*dIII, *Hin*dIII, and *Bam*HI, respectively, and were transcribed *in vitro* by using phage SP6 RNA polymerase without a RNA CAP (m^7GpppG) analog. [^{32}P]UTP-labeled pGA99 RNA (4×10^5 cpm) was mixed with nuclear extracts made from HeLa, HeLaTat, CV12, and CV13 cells. Incubations were conducted in the presence of 20 μg of yeast tRNA in 20 mM Hepes (pH 7.9)/40 mM KCl/1.5 mM $MgCl_2$/1 mM spermidine/0.1% Nonidet P-40 (Sigma) for 10 min at room temperature. The mixtures were then electrophoresed into a 6% polyacrylamide gel in TA buffer (12 mM Tris acetate, pH 7.5/1 mM EDTA/0.1% Nonidet P-40). For competition experiments, unlabeled competitor RNA was added to the extract for 10 min before the addition of radiolabeled probe.

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Abbreviations: HIV-1, human immunodeficiency virus type 1; LTR, long terminal repeat; *TAR* element, trans-activation-responsive element; CMV, cytomegalovirus; Cl, clonal; CAT, chloramphenicol acetyltransferase.

Protein Analysis. RNA, incubated with extract, was irradiated with shortwave UV light for 5 min prior to electrophoresis. Bands from the initial nondenaturing gel were visualized by autoradiography and were excised. The gel slices were first treated with 100 μg of RNase A per ml, then saturated with sodium dodecyl sulfate (SDS) sample buffer, and finally analyzed in a second denaturing polyacrylamide gel containing SDS.

RESULTS

Construction of *TAR* RNA Probes and Tat-Expressing Cell Lines. In a previous study, we demonstrated that Tat, when introduced into mammalian cells, trans-activated the HIV-1 LTR without the need for *de novo* cellular protein synthesis (28). Despite numerous attempts, we have consistently failed to observe specific binding to either the DNA or the RNA form of HIV-1 *TAR* by functional Tat protein (29) synthesized by using a *Baculovirus* vector (our unpublished observations; see Fig. 4*B*). This finding suggests that any direct protein binding to *TAR* is likely to be cellular in origin. Since others have found that the association of cellular proteins with the DNA form of *TAR* did not correlate with Tat-mediated trans-activation (21, 22), we investigated *TAR* RNA for biologically relevant associations with cellular factors.

Our approach used a RNA gel-shift procedure (30, 31) to characterize the interactions between nuclear proteins and a radiolabeled *TAR* RNA. Two sets of reagents were generated to complete these analyses. First, we placed the wild-type HIV-1 *TAR* element (residues +1 to +80) downstream of a procaryotic promoter (pGA99; Fig. 1*A*). This construction permitted the synthesis of a high-specific-activity RNA probe by using SP6 RNA polymerase (34). For controls, similar recombinant plasmids were created to contain either a truncated HIV-1 *TAR* sequence (residues +20 to +80) (pGA20; Fig. 1*A*) or a similarly sized heterologous sequence (pGA1; Fig. 1*A*; ref. 33). In direct trans-activation assays, we verified that the +1 to +80 sequence conferred Tat responsiveness to the expression of a linked chloramphenicol acetyltransferase (CAT) gene that was driven by a CMV promoter (ref. 7; pGA62, Fig. 1*A*), whereas the truncated +20 to +80 TAR sequence (pGA51; Fig. 1*A*) and the *TAR*-unrelated sequence contained in pGA1 (Fig. 1*A*), when similarly tested, were not activated by Tat (results not shown). These *in vivo* findings suggest that the pGA20- and pGA1-generated RNAs do not contain functional *TAR* domains and thus are appropriate *in vitro* negative controls for the pGA99 *TAR* probe.

The three SP6 promoter-containing plasmids shown in Fig. 1 were linearized at appropriate restriction sites and were used to produce a wild-type *TAR* RNA probe and two inactive counterparts. The predicted secondary configurations of these three RNAs in solution based upon free-energy calculations are shown in Fig. 1*B*.

We next constructed cells that constitutively express the Tat protein. Selection of mammalian CV1 cells with G418 after cotransfection of pRSVneo (25) with pSVtat (26) produced several drug-resistant colonies. These were cloned, and individual mass cultures were analyzed for *tat*-specific RNA and protein (Fig. 2). Results from six clonal (Cl) lines revealed four cell lines (Cl 4, Cl 7, Cl 8, and Cl 13; Fig. 2 *Left*) that contained tat mRNA. Three of these four lines (Cl 4, Cl 7, Cl 13; Fig. 2 *Right*) also synthesized Tat protein as verified by a trans-activation assay. Cl 6 and Cl 12 were both negative for Tat RNA and protein (Fig. 2). Cl 8, which showed a modest level of Tat mRNA but did not activate an HIV-1 LTR-CAT gene construct, was not analyzed further. For subsequent experiments, Cl 12 (CV12), Cl 13 (CV13), HeLa, and HeLaTat were used as paired reagents.

Nuclear Proteins in Mammalian Cells Bind to the HIV-1 *TAR* RNA. Nuclear extracts were prepared from HeLa, HeLaTat,

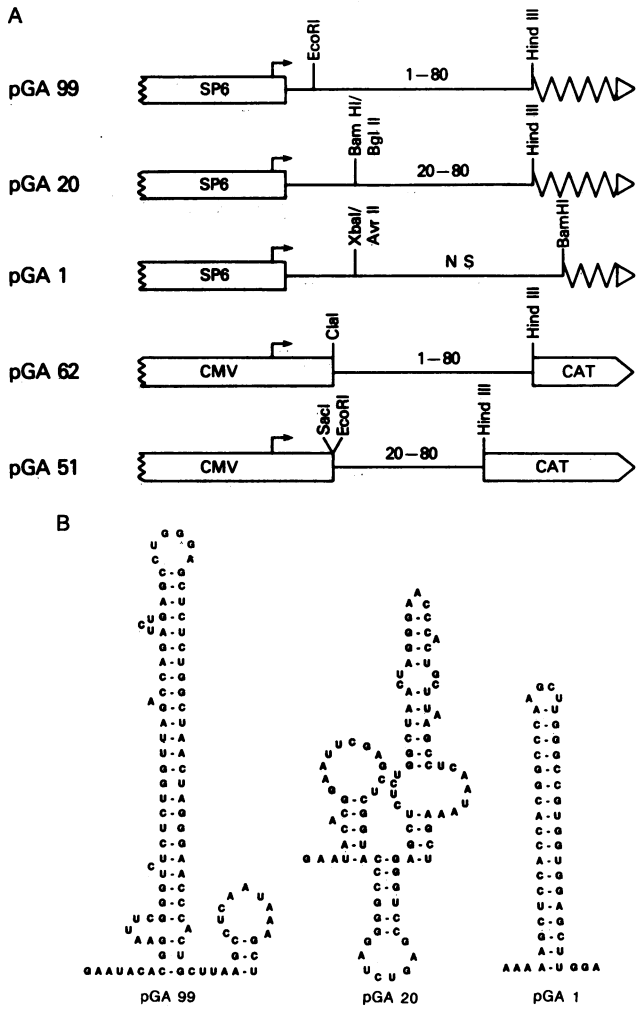

FIG. 1. Molecular cloning of *TAR*-containing plasmids and functional testing for responsiveness to Tat trans-activation. (*A*) Plasmids containing the phage SP6 promoter were made by using the pGEM4 or pGEM3 vectors (Promega). A +1 to +80 or a +20 to +80 HIV-1 LTR-CAT gene sequence (32) was used to create pGA99 and pGA20. A 300-base-pair (bp) *Avr* II fragment from plasmid pB35hp (33) was used to construct pGA1. The CMV promoter is a 700-bp promoter fragment from the *IE94* gene (7). (*B*) Computer-predicted RNA structures of the SP6 transcripts from pGA99, pGA20, and pGA1 after linearization with *Hin*dIII, *Hin*dIII, and *Bam*HI, respectively. NS, nonspecific sequence.

CV12, and CV13 cells in parallel. These extracts were individually incubated with radiolabeled "+1 to +80 *TAR*" RNA synthesized from pGA99. The resulting reactions were resolved by using a nondenaturing low-ionic-strength polyacrylamide gel. ^{32}P-labeled probe RNA that has bound protein can be seen as bands with retarded migration. We consistently observed electrophoretic profiles consisting of four major groups of slower migrating bands (labeled as 1, 2, 3, and 4; Fig. 3, lane 1). This finding suggests that a minimum of four RNA–protein complexes were detectable in this *in vitro* assay.

The specificity of these RNA–protein complexes was tested in competition experiments with excess amounts of unlabeled homologous or heterologous RNAs. We reasoned that specific protein interactions with the *TAR* RNA should resist competition by heterologous substrates but should be sensitive to homologous competitors. For these experiments, a 20-fold (Fig. 3; lanes 2, 4, and 6) or a 50-fold (Fig. 3; lanes 3, 5, and 7) excess of unlabeled competitor RNA was added first to each incubation reaction. We found that unlabeled

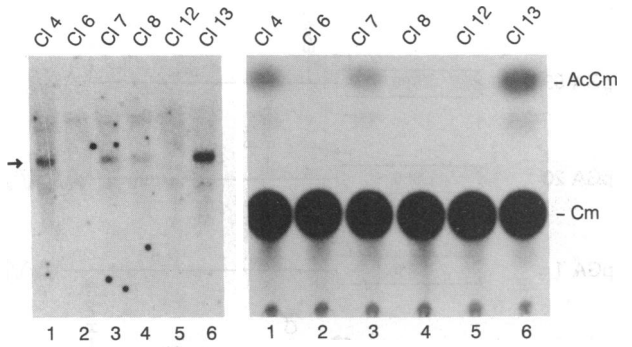

FIG. 2. Cell lines that express *tat* mRNA and protein. (*Left*) Total RNA (10 μg) from six Cl cell lines were resolved in formaldehyde-agarose gel, transferred to nitrocellulose, and probed with a radiolabeled *tat* probe. An arrow points to a *tat*-specific RNA band. (*Right*) CAT assay of the same six cell lines after transfection of each with 1 μg of HIV-1 LTR-CAT gene (pBENNCAT; ref. 32). Cm, chloramphenicol; AcCm, acetylated chloramphenicol.

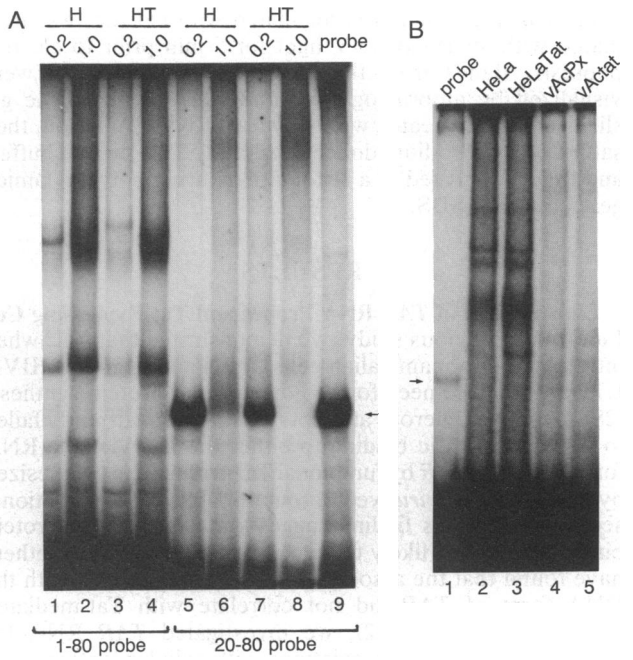

FIG. 4. RNA gel retardation assay using a nonfunctional *TAR* RNA probe and using a Tat-containing SF9 cell extract. (*A*) A +1 to +80 *TAR* (lanes 1–4) or a +20 to +80 *TAR* (lanes 5–9) ^{32}P-labeled RNA probe was incubated with 0.2 μg (lanes 0.2) or 1 μg (lanes 1.0) of HeLa (lanes H) or HeLaTat (lanes HT) nuclear extract and was resolved in a nondenaturing gel. Lane 9 contains the +20 to +80 *TAR* probe alone with no added extract. (*B*) Incubations of HeLa extract (lane 2), HeLaTat extract (lane 3), Tax-containing SF9 extract (ref. 28; vAcPx, lane 4), or Tat-containing SF9 extract (vActat, lane 5) with the +1 to +80 *TAR* probe were respectively resolved in a nondenaturing gel. Lane 1 contains the +1 to +80 *TAR* probe alone. Arrows point to bands formed because of the secondary structures of the RNAs.

homologous RNA cleanly competed with band 4, which was not affected by heterologous RNAs (Fig. 3). In contrast, the intensity of band 3 was virtually unchanged with the addition of excess unlabeled wild-type +1 to +80 *TAR* RNA (Fig. 3, lanes 2 and 3), suggesting that this protein–RNA interaction is likely to be less specific. Bands 1 and 2 showed variably reduced intensities with heterologous competitors (Fig. 3). Because these bands were more sharply reduced in intensity in the presence of pGA99 RNA as compared with that synthesized from pGA20 or pGA1, we inferred that these proteins show some degree of increased binding affinity for the +1 to +80 *TAR* RNA over their affinity for the truncated or the unrelated RNA species.

A potentially provocative observation from this experiment is the virtual identity in electrophoretic profiles of *TAR* RNA-binding proteins in Tat-expressing and Tat-nonexpressing cells (compare HeLa to HeLaTat and CV12 to CV13; Fig. 3). Since these paired cell lines are clearly different in Tat function (Fig. 2), the lack of detectable quantitative and qualitative differences between the extracts in *TAR* RNA-binding proteins is consistent with Tat acting indirectly through preexisting cellular factors (28).

Two additional control experiments were performed. In

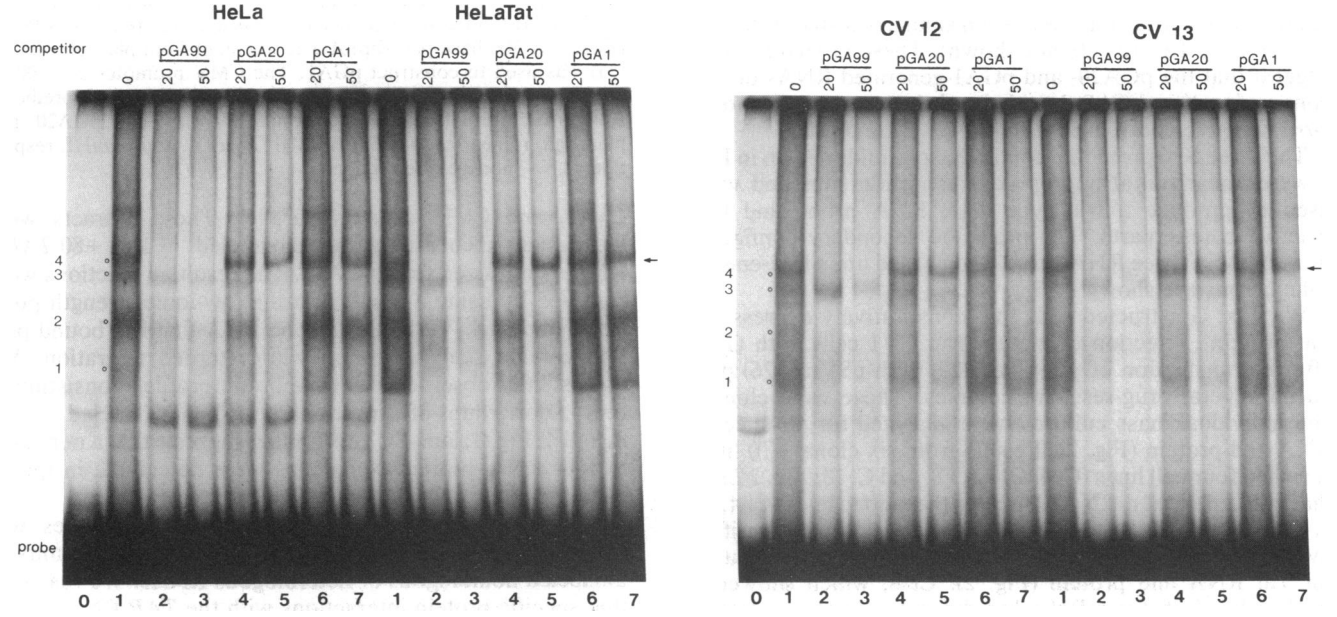

FIG. 3. RNA gel retardation assay of pGA99 *TAR* transcript. Nuclear extracts were from HeLa, HeLaTat, CV12, and CV13 cells. Lanes: 0, probe alone; 1–7, probe incubated with nuclear extract from the cell lines as indicated; 2–7, addition of unlabeled competitor RNAs (lanes 2 and 3, pGA99; lanes 4 and 5, pGA20; lanes 6 and 7, pGA1) to the incubations prior to the addition of labeled pGA99 probe at either a 20-fold (lanes 2, 4, and 6) or a 50-fold (lanes 3, 5, and 7) excess. ○, Four major migration-retarded bands labeled 1, 2, 3, and 4. The arrow on the right points to a single band (band 4) that is most clearly erased by competition with homologous but not with heterologous RNA.

the first we synthesized from plasmid pGA20 (Fig. 1*A*) a ^{32}P-labeled nonfunctional "+20 to +80 *TAR*" RNA. In incubations using HeLa or HeLaTat nuclear extracts, cellular proteins that bound to the functional +1 to +80 *TAR* RNA (Fig. 4*A*, lanes 1–4) showed no avidity for the biologically inert +20 to +80 *TAR* probe (Fig. 4*A*, lanes 5–8). This finding agrees with the specificity of recognition by cellular factors for a functional *TAR* RNA target. To test that Tat protein does not itself specifically bind to the +1 to +80 *TAR* RNA, we made a Tat-containing extract (vActat; Fig. 4*B*, lane 5) using an overexpressing SF9 cell system (29). Compared to a control extract prepared from SF9 cells that overexpressed the human T-cell leukemia virus type I Tax protein (vAcPx; Fig. 4*B*, lane 4), no Tat-specific RNA–protein interaction was detected in the vActat (Fig. 4*B*, lane 5) extract. The absence of the +1 to +80 *TAR*-binding activities found in the mammalian cell (HeLa and HeLaTat; Fig. 4*B*, lanes 2 and 3) extracts from the SF9 cell extracts (Fig. 4*B*, lanes 4 and 5) is consistent with the inability of the latter cell to support Tat activation of the HIV-1 LTR (28, 29).

Molecular Sizes of the *TAR* RNA-Binding Proteins. We and others have previously used nondenaturing gels to separate covalently linked DNA–protein complexes that were generated as a result of UV-irradiation (35, 36). These complexes are excisable from the nondenaturing gel, and after trimming with nuclease, can be reelectrophoresed into a second gel containing SDS to size the protein component of each complex. We applied this approach to the four RNA–protein complexes seen in Fig. 3. A typical electrophoretic profile of the four described bands in nondenaturing gel after UV-irradiation is seen in Fig. 5*A Left*. This profile resembles that seen in experiments conducted without UV-irradiation (Fig. 3). We individually excised bands 1 and 2. Bands 3 and 4 migrated in close proximity to each other and were analyzed together in a single gel slice. After limited treatment with RNase A to remove excess tagged RNA, the bands were reelectrophoresed into an SDS/polyacrylamide gel. An autoradiographic exposure of the SDS gel revealed proteins that became radiolabeled by virtue of residually linked RNA probe molecules (Fig. 5*A Right*).

In this fashion, we characterized the molecular sizes of the *TAR*-binding proteins. When band 1 was analyzed by SDS/PAGE, no discretely labeled protein was seen (Fig. 5*A*, band 1). The reason for this is unclear. However, it is possible that the polypeptide associated with this complex is refractory to UV-induced covalent bonding. Alternatively, the protein may bind in such a way to the RNA site that RNase treatment cleaves away all labeled nucleotides. Band 2 contained a 62-kDa polypeptide (Fig. 5*A*, band 2). Bands 3 and 4 together comprised three proteins of 100, 62, and 46 kDa (Fig. 5*A*, bands 3 and 4). In other experiments, we separated the 62-kDa and 46-kDa proteins (Fig. 5*B*, lane 1) from the 100-kDa protein (Fig. 5*B*, lane 2), using heparin-agarose column chromatography. We found that the 0.4 M KCl column fraction gave rise to RNA–protein complexes that comigrated with bands 2 and 3 and that the 0.5 M KCl fraction produced a complex that comigrated with gel-shift band 4 (results not shown).

DISCUSSION

A major focus of molecular studies on Tat-mediated activation of the HIV-1 LTR is an understanding of the mechanistic contributions of the host cell. Although a posttranscriptional function has been attributed to Tat (37–40), it is currently understood that the major effect of this viral trans-activator is to increase the rate of transcription from the HIV-1 LTR (19, 26, 28, 41). Extrapolating from the examples of other

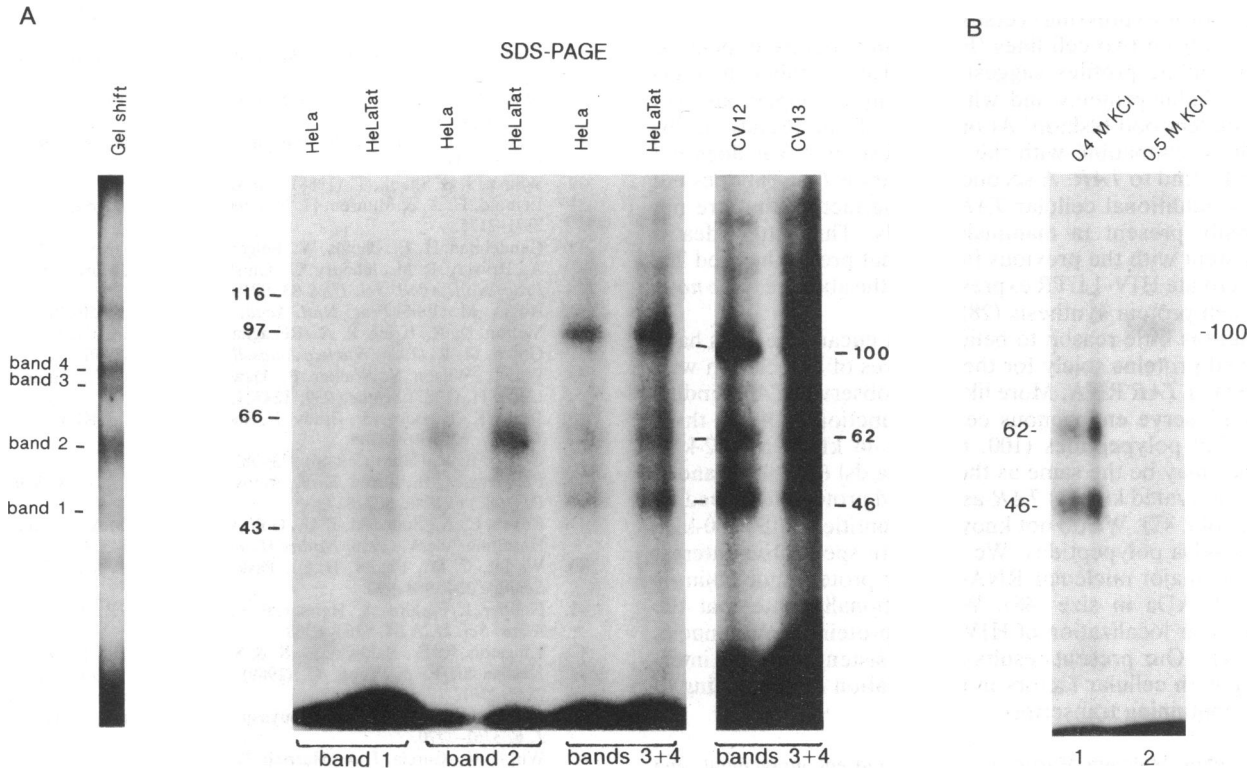

FIG. 5. SDS/PAGE analysis of the RNA–protein complexes after covalent coupling induced with UV. (*A Left*) Typical profile of the RNA–protein complexes in a gel-shift assay after UV-irradiation. Bands 1 to 4 were excised. (*A Right*) SDS/PAGE of bands 1, 2, and 3 plus 4. Each band was removed from the nondenaturing gel and was resolved in a second SDS/polyacrylamide gel. We analyzed the RNA–protein complexes formed using HeLa, HeLaTat, CV12, and CV13 extracts. Three proteins of 100, 62, and 46 kDa were seen for bands 3 and 4. A 62-kDa polypeptide was seen for band 2. No discretely labeled protein was seen in the analysis of band 1. (*B*) Separation of the 46-kDa and 62-kDa proteins from the 100-kDa protein. HeLa nuclear extract was chromatographed over a heparin-agarose column. *TAR*-binding proteins in the 0.4 M KCl (lane 1) and 0.5 M KCl (lane 2) elution fractions were visualized by UV-crosslinking to a radiolabeled +1 to +80 *TAR* probe.

viral trans-activators (see review in ref. 42), Tat can either interact directly with its responsive target sequence (*TAR*) or exert its effect through the mediation of cellular proteins.

The aim of our study was to identify candidate cellular polypeptides that may functionally cooperate with Tat. We investigated the interactions between host cell nuclear factors and the *TAR* RNA molecule for two reasons. First, we considered it unlikely that Tat binds directly to *TAR* in a biologically relevant manner. It has been reported that Tat has no sequence-specific binding affinities for either DNA or RNA (24). We, despite the availability of overexpressed functional protein (29), have never observed a reproducible specific binding of Tat to either the DNA or RNA forms of *TAR* (our unpublished observations; Fig. 4*B*). In addition, the simultaneous presence of Tat and *TAR* within a procaryotic cell does not provide for functionally significant trans-activation (43), suggesting a requirement for eucaryotic host cell proteins. Second, the previously described binding of cellular factors to *TAR* DNA (21, 44, 45) is apparently irrelevant for Tat-mediated trans-activation. Two studies have verified that mutations in *TAR* DNA that abolished Tat-responsiveness did not affect binding by cellular proteins and that mutations that affected protein binding to *TAR* DNA did not affect trans-activation (21, 22). In view of recent genetic evidence that the nascent *TAR* RNA is the functional target for Tat (B. Berkhout and K.-T.J., unpublished data), we considered it important to characterize cellular proteins that recognize the *TAR* RNA element.

We have identified four RNA–protein complexes, using nuclear extracts from cells that do or do not synthesize Tat. It is perhaps noteworthy, given the nuclear localization of Tat (41), that no *TAR*-binding protein was detected in the corresponding cytoplasmic extracts (data not shown). The fact that paired (Tat-expressing versus Tat-nonexpressing) nuclear extracts from two cell lines (HeLa and CV1) gave identical RNA-binding profiles suggests that Tat probably interacts with cellular proteins and with *TAR* in a complex and not well-understood fashion. At one level of interpretation, this result is compatible with the hypothesis that Tat does not directly bind to *TAR*. A second corollary is that Tat does not induce additional cellular *TAR*-binding factors that are not normally present in mammalian cells. This latter idea is consistent with the previous finding that presynthesized Tat can activate HIV-1 LTR expression in the absence of *de novo* host cell protein synthesis (28).

There is little reason to believe that eucaryotic cells have evolved proteins solely for the purposes of association with the HIV-1 *TAR* RNA. More likely the observed *TAR*-binding proteins serve endogenous cellular functions. Of the three identified polypeptides (100, 62, and 46 kDa), the 62-kDa species may be the same as the 66-kDa dsl (double stranded RNA-activated kinase) *TAR*-associated protein described by others (46, 47). We do not know the identities of the 100-kDa and 46-kDa polypeptides. We note with speculative interest that the major nucleolar RNA-binding protein, nucleolin, is also 100 kDa in size (48). We additionally note that the subnuclear localization of HIV-1 Tat protein is in the nucleolus (41). Our present results are consistent with Tat interacting with cellular factors in the initiation or processing of *TAR*-containing transcripts.

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