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CA150, a Nuclear Protein Associated with the RNA Polymerase II Holoenzyme, Is Involved in Tat-Activated Human Immunodeficiency Virus Type 1 Transcription

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Maximal human immunodeficiency virus type 1 (HIV-1) gene expression requires specific cellular factors in addition to the virus-encoded *trans*-activator protein Tat and the RNA element TAR. We developed a functional assay, based on transcriptional activation in vitro, to identify these cellular factors. Here, we describe the purification and molecular cloning of CA150, a nuclear protein that is associated with the human RNA polymerase II holoenzyme and is involved in Tat-dependent HIV-1 transcriptional activation. The sequence of CA150 contains an extensive glutamine- and alanine-rich repeat that is found in transcriptional modulators such as GAL11 and SSN6 in *Saccharomyces cerevisiae* and Zeste in *Drosophila melanogaster*. Immunodepletion of CA150 abolished Tat *trans* activation in vitro. Moreover, overexpression of a mutant CA150 protein specifically and dramatically decreased Tat-mediated activation of the HIV-1 promoter in vivo, strongly suggesting a role for CA150 in HIV-1 gene regulation. Immunoprecipitation experiments demonstrated that both CA150 and Tat associate with the RNA polymerase II holoenzyme. Furthermore, we found that functional Tat associates with the holoenzyme whereas activation-deficient Tat mutants do not. Thus, we propose that Tat action is transduced via an RNA polymerase II holoenzyme that contains CA150.

Human immunodeficiency virus type 1 (HIV-1), the etiologic agent of AIDS, is a complex lentivirus with a highly regulated life cycle. Activation of the HIV-1 promoter absolutely requires the viral RNA element designated TAR (for *trans*-activation response element) and the viral Tat protein (14, 64). The core of the HIV-1 promoter consists of three tandemly repeated binding sites for the cellular transcription factor Sp1, a canonical TATA box, and an initiator element (33). Enhancer sequences upstream of the promoter have been shown to bind many host factors, among them NF-κB (33). In vivo and in vitro studies have shown that NF-κB, Sp1, and TATA-box sequences contribute to basal transcription and Tat-activated transcription (33). In fact, physical interaction between Tat and the TATA-box binding protein (TBP) (34) and between Tat and Sp1 (32) have been reported. Although *trans* activation by Tat requires Sp1 binding sites, they can be functionally replaced by other *cis*-acting elements (2, 3, 10, 47, 67), suggesting that HIV-1 transcriptional activation can be maintained in the absence of specific upstream elements.

The Tat-TAR ribonucleoprotein (RNP) complex is the master switch that controls transcription from the HIV-1 long terminal repeat (LTR). TAR, a highly structured RNA element consisting of the first 60 nucleotides of all viral mRNAs, is absolutely required for Tat-mediated transcriptional activation (14). At present, the mechanism by which Tat activates gene expression is unknown. In vitro and in vivo studies have not definitely distinguished between an effect of Tat on the initiation phase of transcription and an effect on the elongation phase of this process (4, 5, 13, 18, 26, 35, 42, 43, 49, 50, 63, 67, 76).

Extensive studies have suggested the involvement of cellular coactivators in Tat-mediated *trans* activation (6, 8, 29, 46, 66, 69, 76). Recent reports have suggested the occurrence of interactions between Tat and general transcription factors (GTFs) (34, 35), and as well as between Tat and RNA polymerase II (36, 51). Very recently, Zhou and Sharp (77) described the characterization and cloning of Tat-SF1, a human protein that may be involved in Tat *trans* activation. Other cellular factors that interact with Tat have been identified and characterized in some detail (20, 29, 32, 53, 65, 75), but none of these proteins has been conclusively shown to play a role in Tat function.

Given that factors identified solely on the basis of Tat binding may lack biological relevance, as well as the possibility that Tat may function via interactions with macromolecular complexes rather than individual proteins, we set out to identify functionally important Tat coactivators by using a functional assay that measures Tat-mediated *trans* activation in vitro (69, 70). We previously described an activity required for Tat-mediated transcription activation that was depleted by passage through a Tat affinity column (69). As we report here, purification of this activity led to the discovery and isolation of CA150, a nuclear protein that associates with the RNA polymerase II holoenzyme. Here we present results, obtained both in vitro and in vivo, that strongly support a role for CA150 in Tat-mediated transcriptional regulation.

MATERIALS AND METHODS

Identification and purification of CA150. The in vitro transcription system and the generation of active recombinant Tat and glutathione *S*-transferase (GST) fusion proteins have been previously described (69). Specific *trans* activation was calculated as the ratio of the level of the HIV-1 transcript in the presence of Tat to the level of the same transcript in the absence of Tat, with normalization of

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these values to the level of the adenovirus type 2 major late (AdML) transcript in those reactions (49, 69). The specific bands were quantified by using a PhosphorImager (Molecular Dynamics Inc., Sunnyvale, Calif.).

Approximately 3 mg of each GST fusion protein was bound covalently to 1 ml of glutathione-agarose beads with dimethyl pimelimidate (Pierce, Rockford, Ill.) (38). HeLa cell nuclear extract was prepared by the method of Dignam et al. (16) and adjusted to 20 mM HEPES (pH 7.9), 50 mM KCl, 10% (vol/vol) glycerol, 0.2 mM EDTA, 0.5 mM dithiothreitol (DTT), and 50 μg of phenylmethylsulfonyl fluoride (PMSF) per ml (binding buffer). A total of 72 ml of nuclear extract (15 mg of protein/ml) was chromatographed by the following strategy: 9 ml was loaded on the GST-K41A Tat column at a flow rate of 0.05 ml/min, the flowthrough fraction was collected, and the column was washed extensively and eluted with binding buffer containing 2 M KCl. This process was repeated seven times. The flowthrough fractions were pooled, and 18 ml was loaded on the GST-Tat column. Bound proteins were eluted with a 10-ml gradient (from 50 to 500 mM KCl). Fractions (1 ml) were collected, and the column was washed sequentially with 2 M and 50 mM KCl in binding buffer, as described above. This process was repeated three times. Fractions containing Tat *trans*-activation activity (eluting at approximately 150 mM KCl) were pooled and adjusted with binding buffer to 0.5% glycerol. Chromatography on the Q-Sepharose column (0.5 ml) (Sigma, St. Louis, Mo.) was performed as described above except that a 5-ml gradient was used.

Microsequencing of LysC peptides. The CA150 protein peak (eluting at approximately 200 mM KCl) was concentrated with Centriflo (Amicon Inc., Beverly, Mass.) devices. Measurement of the UV absorption at 205 to 210 nm (peptide band) in the final fraction indicated that there was 75 μg of protein. This would indicate a yield of 0.07 μg of CA150 per mg of nuclear extract protein. The total amount of recovered protein was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on a 10% gel and transferred to a polyvinylidene difluoride (PVDF) membrane (0.2-μm pore size; Bio-Rad Laboratories, Hercules, Calif.). The membrane was stained with a 0.1% (wt/vol) solution of Ponceau reagent in 1% acetic acid, and the piece containing the 150-kDa band was excised and destained. Amino acid analysis of approximately 10% of this PVDF piece revealed that there was a minimum of 11 pmol of CA150 present. The remaining 90% of the membrane was submitted to in situ digestion with endopeptidase LysC (Promega Corp., Madison, Wis.) (19). The resulting peptide mixture was separated by narrow-bore high-performance liquid chromatography on a Hewlett-Packard 1090 high-performance liquid chromatograph equipped with a Hewlett-Packard 1040 diode array detector and a 2.1-mm by 150-mm Vydac C_{18} reverse-phase column. The detector outlet was modified with fused-silica capillary tubing to minimize the dead volume and drop size. Optimum fractions from the chromatogram were chosen based on differential UV absorbance at 210 and 292 nm, peak symmetry, and resolution and then submitted to automated Edman degradation on an Applied Biosystems model 477A protein sequencer equipped with a microcartridge. The average initial yield of the four peptides sequenced was 3.58 pmol. Details of the strategies for the selection of peptide fractions and their microsequencing have been previously described (41).

Isolation and sequencing of CA150 cDNAs. The sequences of CA150 peptides pk60 and pk74, found in a human EST clone (IMAGE Consortium cDNA clone 38349 5′), were amplified by reverse transcription-PCR of HeLa cell poly(A)$^+$ mRNA. Screening of one oligo(dT)-primed HeLa cell cDNA library and two random-primed T-cell cDNA libraries (Stratagene, La Jolla, Calif.) yielded the full-length CA150 clone. Both strands of the cDNA were sequenced at the University of North Carolina—Chapel Hill Automated Sequencing Facility on a model 373A DNA sequencer (Applied Biosystems, Foster City, Calif.) with the *Taq* DyeDeoxy Terminator Cycle Sequencing kit (Applied Biosystems). The Genetics Computer Group Wisconsin Sequence Analysis Package was used to search the nucleotide and protein databases.

Analysis of CA150 protein expression. To express a truncated CA150 protein in *Escherichia coli*, we amplified a segment of the cDNA, from nucleotides 980 to 3750, using as a template one of the cDNAs obtained in the screening of the libraries. The PCR product was digested, purified, and cloned in frame into the expression vector PGEX2TK (Pharmacia Biotech, Piscataway, N.J.) by using the *Sma*I and *Eco*RI sites of the plasmid. The protein was expressed as a GST fusion under the conditions suggested by the manufacturer. Purified fusion protein was used to generate specific polyclonal antibodies in rabbits (Research Genetics Inc., Huntsville, Ala.). To detect CA150, proteins were separated by SDS-PAGE, transferred to an Immobilon-P (Millipore Corp., Bedford, Mass.) membrane, and then incubated with the specific antiserum (1:2,000 dilution). After being washed, the membrane was incubated with a peroxidase-conjugated secondary antibody (Amersham, Arlington Heights, Ill.), and bound antibodies were detected by enhanced chemiluminiscence (Amersham).

To release proteins from cells, RIPA buffer (150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS, 50 mM Tris [pH 7.5]) was used. Briefly, cells were washed twice with phosphate-buffered saline, and the cell pellets were incubated with cold RIPA buffer containing PMSF at 50 μg/ml for 30 min with gentle hand shaking in a water-ice pocket. After centrifugation at 10,000 × *g* and 4°C for 10 min, the lysate was transferred to a clean tube and used for protein analysis.

In vitro translation of CA150. CA150-encoding DNA sequences were subcloned into pBSK downstream of the T3 RNA polymerase promoter and were expressed by using the coupled transcription-translation rabbit reticulocyte system (Promega) in the presence of [^{35}S]methionine (New England Nuclear, Boston, Mass.) at 30°C for 60 min. Translation products were separated on a 12.5% SDS–polyacrylamide gel and visualized on film after 24 h of exposure.

Indirect immunofluorescence. Immunofluorescence analysis was performed on fixed permeabilized CV-1 cells with a 1:500 dilution of the rabbit CA150-specific serum, 1:500 dilution of the antinucleopore mouse monoclonal antibody 414 (a gift of L. Davis, Brandeis University), and 1:2,000 dilutions of the secondary affinity-purified rhodamine-conjugated goat anti-rabbit and fluorescein-conjugated goat anti-mouse antisera (Jackson Immunoresearch Laboratories, West Grove, Pa.). Antibody incubations were performed at room temperature for 1 h in a dark humidified chamber. Cell nuclei were identified by staining with Hoechst dye. Images were visualized with a Bio-Rad confocal microscope.

Immunoprecipitation with hSRB7 and Tat-specific antibodies. Nuclear extracts were prepared from 10^8 human Molt-4 cells. Cells were harvested, centrifuged for 15 min at 3,000 rpm, washed in 10 ml of ice-cold phosphate-buffered saline, and collected by centrifugation for 15 min at 3,000 rpm. The pelleted cells were resuspended in 4 ml of buffer A (10 mM HEPES [pH 7.8], 10 mM KCl, 2 mM $MgCl_2$, 1 mM DTT, 0.1 mM EDTA, 0.1 mM PMSF) and incubated on ice for 15 min. Then, 250 μl of 10% Nonidet P-40 solution (Sigma) was added, the cells were vigorously mixed, and the suspension was incubated for 30 min at 4°C. The harvested cells were centrifuged for 15 min at 3,000 rpm. Pelleted nuclei were resuspended in 500 μl of buffer C (50 mM HEPES [pH 7.8], 50 mM KCl, 300 mM NaCl, 0.1 mM EDTA, 1 mM DTT, 0.1 mM PMSF, 10% [vol/vol] glycerol), mixed for 30 min, and centrifuged for 15 min at 3,000 rpm and 4°C. The supernatant contained the nuclear proteins (protein concentration, 20 μg/μl).

To carry out the immunoprecipitations, nuclear extract was dialyzed against buffer D 100 (20 mM Tris-HCl [pH 7.9], 10% [vol/vol] glycerol, 100 mM KCl, 1 mM $MgCl_2$, 0.2 mM EDTA, 10 ml $ZnCl_2$, 0.5 mM DTT, 0.5 mM PMSF) plus 1% Triton X-100 (see Fig. 5A) or against the same buffer containing 80 mM KCl (see Fig. 5B). Three-microgram quantities of recombinant GST-Tat and of the GST-Tat mutants K41A and Cys22 were added separately to 100 μl (2 mg) of nuclear extract and rotated for 12 h at 4°C. Then, anti-human SRB7 (anti-hSRB7) polyclonal antibody (11), affinity-purified anti-Tat (Intracel Corp., Cambridge, Mass.), or anti-human transforming growth factor beta (TGF-β) polyclonal antibodies were added, and immunocomplexes were precipitated with protein A-Sepharose. Coimmunoprecipitated proteins were subjected to SDS-PAGE and analyzed by Western blotting with appropriate specific antibodies.

Immunodepletion of CA150. Affinity columns were prepared by binding 1 ml of the preimmune or specific sera to 0.5 ml of protein A beads (Pharmacia Biotech). After extensive washing, immunoglobulins were linked to the matrix with dimethyl pimelimidate as previously described (27). Affinity matrices were built in disposable columns, and uncoupled antibodies were eluted by washing the columns with 0.1 M glycine, pH 3.0. Columns were stored in buffer D (20 mM Hepes [pH 7.9], 20% [vol/vol] glycerol, 0.1 M KCl, 0.2 mM EDTA, and 0.5 mM DTT) containing 0.01% merthiolate. One milliliter of HeLa cell nuclear extract (15 mg of protein/ml) was loaded sequentially on three columns at a flow rate of approximately 0.04 ml/min. The final flowthrough fractions were used in Western blotting and transcription reactions.

Mammalian expression plasmids, cell culture, and transfections. The reporter plasmids pTAR/CAT, containing the HIV-1 promoter, and pcTat, expressing the 86-amino-acid Tat protein under the control of the cytomegalovirus (CMV) promoter, have been previously described (46). pBC12/CMV/IL-2, expressing human interleukin-2 (IL-2) from the CMV promoter, has been previously described (13). pBC12/CMV/ΔCA150 was generated by standard techniques and has a deletion between *Eag*I (+194) and *Pvu*II (+1160) sites of the CA150 sequence.

HeLa cells were grown in Iscove's modified Eagle medium (Gibco/BRL, Grand Island, N.Y.) supplemented with 10% fetal calf serum and 100 μg of penicillin-streptomycin per ml. Transfections were carried out with low-passage-number cells. Cells on 35-mm-diameter plates (~50% confluent) were cotransfected with 1 μg of the reporter plasmid pTAR/CAT and 3 μg (see Fig. 7A and B) or the indicated amounts (see Fig. 7C) of pBC12/CMV/ΔCA150 or pBC12/CMV/IL-2 as a control, in the absence or presence of 3 ng of pcTat, using calcium phosphate. The reporter vector thymidine kinase (TK)-luciferase (0.25 μg) was used as an internal control of transfection. Cells were collected approximately 36 h after transfection. Cell extracts were normalized on the basis of protein concentration as determined by the Bradford method (Bio-Rad Laboratories), and acetylation of chloramphenicol was assayed by the diffusion method of Neumann et al. (54).

Nucleotide sequence accession number. The CA150 cDNA sequence shown in Fig. 2 has been deposited in GenBank and given accession no. AF017789.

RESULTS

Affinity purification of cellular coactivators of HIV-1 Tat. Previously, we and others have shown that novel factors required for *trans* activation by Tat could be identified in vitro in HeLa cell nuclear extracts (69, 70, 76). Nuclear extracts are fully competent to mediate *trans* activation of the HIV-1 promoter in a manner that requires Tat and TAR (26, 35, 49, 69,

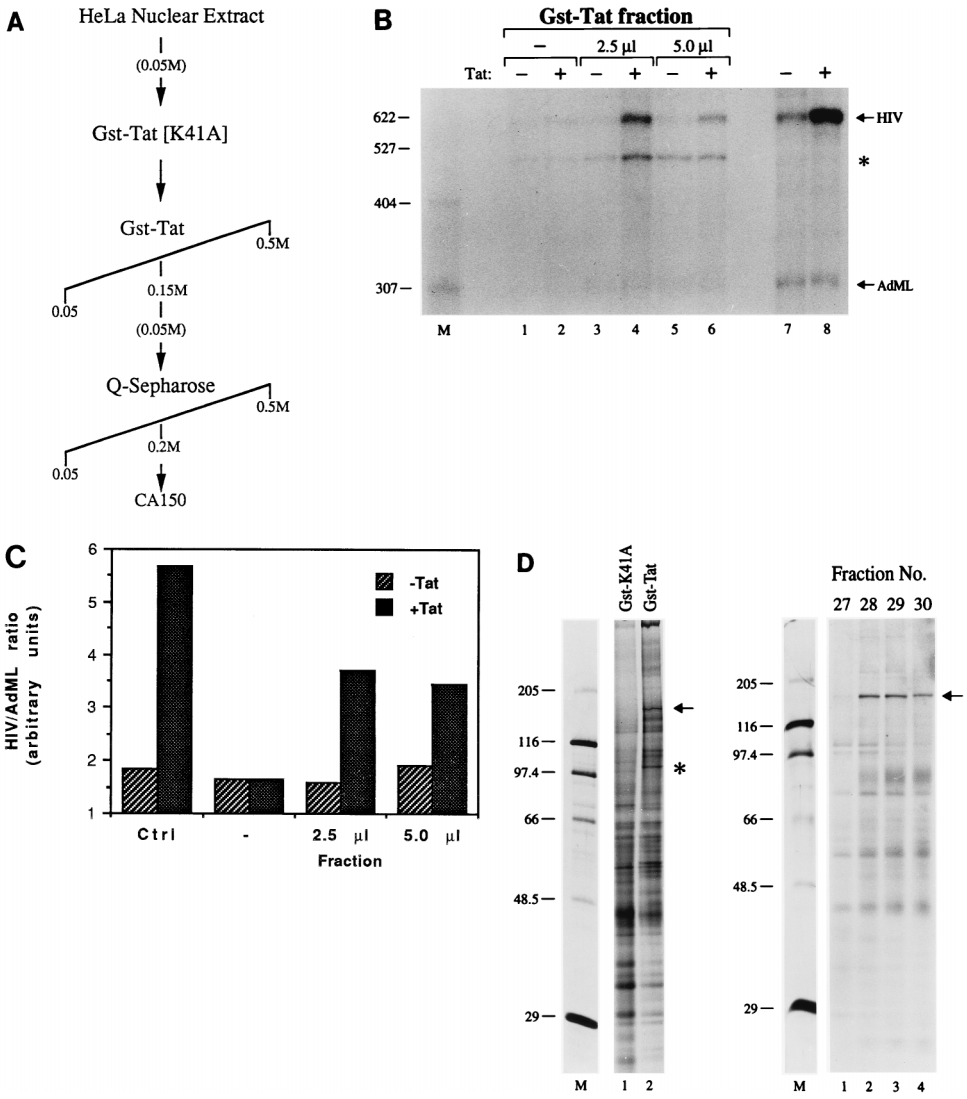

FIG. 1. Purification of CA150. (A) Scheme for the purification of CA150. (B) Reconstitution of Tat *trans* activation with the GST-Tat gradient fraction. Transcription reactions were carried out under experimental conditions with extract containing low-level *trans*-activation activity (50 μg of nuclear extract and 10 ng of TFIIB [69]) (lanes 1 to 6) and control extract (lanes 7 and 8) in the absence (−) or presence (+) of 50 ng of Tat protein or the GST-Tat gradient fraction where indicated. Specific *trans* activation by Tat was calculated as described in Materials and Methods. The origin of the transcript marked with an asterisk is unknown. (C) Experimental data, quantified with the PhosphorImager, of the experiment shown in panel B, shown in graphic form. (D) Silver-stained SDS-polyacrylamide gels of the eluates from the affinity (left) and the Q-Sepharose (right) columns. CA150 and a protein with an M_r of approximately 100 kDa are indicated with an arrow and an asterisk, respectively. Lanes 1 and 2 (left) contained 2 μg of protein from the 2 and 150 mM KCl elution fractions from the GST-K41A Tat and GST-Tat columns, respectively. Lanes 1 to 4 (right) contained 20 μl (approximately 0.5 μg of protein) of fractions 27 to 30 of the Q-Sepharose gradient step. The relative mobility (in kilodaltons) of the molecular mass markers (M) are shown on the left side of the gels in panel D.

70, 76). We have previously identified an activity required for Tat *trans* activation in HeLa cell nuclear extract by using Tat affinity columns (69). In these experiments, the flowthrough fraction from a GST-K41A Tat mutant column supported both basal and Tat-activated transcription, but the flowthrough fraction from a GST-Tat column supported only basal transcription (69). The activity required for Tat *trans* activation could be recovered from the GST-Tat affinity column by elution with 0.5 M KCl (69). Based on these results, we constructed similar Tat affinity columns in which GST fusions were chemically cross-linked to the gel matrix (see Materials and Methods). To purify the functional activity responsible for Tat *trans* activation, HeLa cell nuclear extract was loaded sequentially through GST-K41A Tat mutant and GST-Tat columns as shown in Fig. 1A. Proteins bound to GST-K41A and GST-Tat columns were recovered by elution with 2 M KCl and a 0.05 to 0.5 M KCl gradient, respectively. Chromatographic fractions were tested for their ability to induce Tat-activated HIV-1 transcription in reaction mixtures containing low levels of HeLa cell nuclear extract. Fractions that eluted from the GST-Tat column at approximately 0.15 M KCl displayed *trans*-activation activity (Fig. 1B and C). Elution fractions from the GST-K41A Tat column did not activate HIV-1 transcription (data not shown). The fact that we obtained more activity with 2.5 μl of the GST-Tat gradient fraction may indicate a general inhibition by the larger volumes of this fraction added to the in vitro transcription reaction mixture.

To investigate the protein composition of the active fraction, 2 and 0.15 M KCl fractions from mutant and wild-type Tat columns were subjected to SDS-PAGE and silver staining.

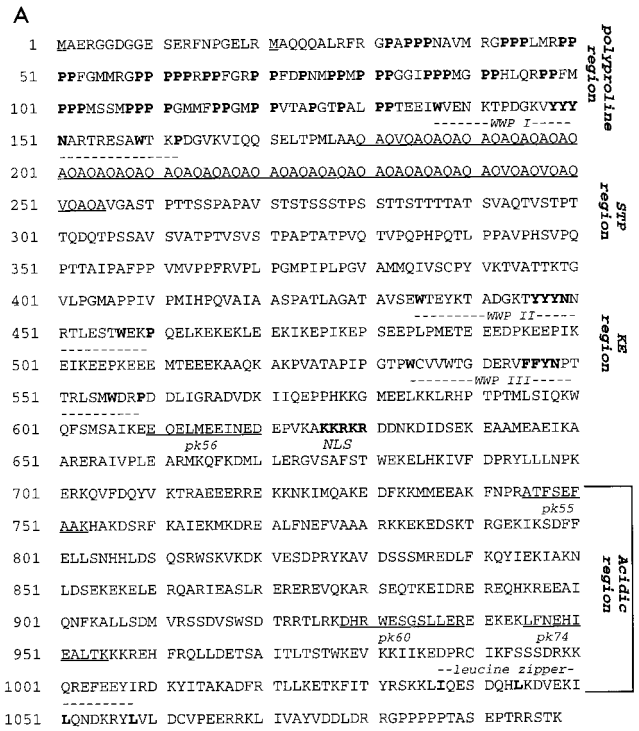

FIG. 2. Sequence analysis of CA150. (A) The predicted amino acid sequence of human CA150. The underlined methionines at the beginning of the sequence represent two putative translation initiation sites. The interesting domains and motifs are highlighted and include (i) a proline-rich region in the amino-terminal portion of the protein (many of these prolines are found in runs of three or more); (ii) three WWP domains; (iii) a glutamine-alanine (QA) repeat, which spans 76 amino acids; (iv) a region rich in serine, threonine, and proline (STP); (v) a region rich in lysine and glutamic acid (KE) spanning 60 residues; (vi) a putative leucine zipper in the carboxy-terminal sequence; and (vii) a putative nuclear localization signal (KKRKR). The peptide sequences obtained by microsequence analysis (pk) are also indicated. The amino acid sequences 299 to 326 and 379 to 399 are not present in all of the cDNA clones analyzed and possibly represent alternatively utilized exons. (B) The domains and motifs in CA150. *CA150-I* cDNA of 4.3 kb as well as the protein codified by its open reading frame are shown in a schematic form. aI and aII represent possible alternatively utilized exons. The domains and motifs in the protein are as for panel A. NLS, nuclear localization signal. (C) The WWP domains in CA150. Shown here are the three WWP domains of CA150 and the consensus derived from these and other proteins with homologous domains (9). Conserved residues are underlined. aa, amino acids.

Analysis of the salt fractions from the GST-Tat affinity columns revealed an unexpectedly complex pattern, probably due to the sticky nature of Tat. Specific binding of several proteins could be seen over the nonspecific binding. Two of these proteins, with apparent molecular masses (M_r) of 150 and 100 kDa (Fig. 1D, left panel), reproducibly bound to the GST-Tat matrix in many chromatographic experiments. Thus, we focused our efforts on following these proteins through further chromatographic purification steps. We named these potential coactivator proteins CA150 and CA100 because of their apparent M_rs of 150 and 100 kDa, respectively. The finding that several proteins were present in the active CA150 fraction raised the possibility that the activity could represent a multicomponent complex. Further separation of these proteins by several approaches yielded inactive fractions (data not shown). One interpretation of this finding is that a complex consisting of multiple proteins was responsible for the Tat *trans*-activation activity and that this complex was disrupted during the final chromatographic step.

In order to identify and characterize specific proteins that bound to the GST-Tat column, CA150 was purified to near homogeneity on Q-Sepharose. The CA150 peak fractions were pooled (Fig. 1D, right panel), subjected to SDS-PAGE, and transferred to a PVDF membrane. A single band detected by staining with Ponceau reagent was excised and subjected to in situ LysC digestion. The amino-terminal sequences of four LysC peptides purified by high-performance liquid chromatography were obtained: pk55 (ATFSEFAAK), pk56 (ExELmEeiNEd), pk60 (DHRxESGSLLEr), and pk74 (LFNEHIEA LTK); the lowercase letters indicate low-confidence results, and x's stand for unidentifiable residues. The peptide sequence data suggested that CA150 was a novel gene product and provided the information needed to isolate cDNA clones encoding it.

Sequence of CA150 cDNA. Fortuitously, the LysC peptides pk60 and pk74 were found to be adjacent in an open reading frame predicted by the sequence of a cDNA clone among the library of expressed sequence tags (EST) (Washington University and Merck EST project). The EST cDNA was used to probe an oligo(dT)-primed HeLa cell cDNA library and two random-primed human T-cell cDNA libraries. Several related clones were isolated, and cDNA *CA150-I*, consisting of ~4.3 kb, was chosen for further study. Northern blot analysis revealed a single major transcript of approximately ~4.5 kb present in all human tissues examined (data not shown). Assuming the presence of a poly(A) tail of 100 to 200 nucleotides, the size of *CA150-I* (4.3 kb) is consistent with it being a full-length cDNA. The presence of a stop codon 5′ of the putative translation start site in *CA150-I* confirmed that we had most likely obtained the complete coding region.

CA150-I encoded a predicted 1,097-amino-acid protein (Fig. 2A). A search of the nucleic acid and protein databases, using both the BLAST and FASTA algorithms (58, 59), revealed CA150 to be unique and novel. The predicted sequence contained motifs and domains found in other proteins, many of them transcription factors (Fig. 2A; a schematic representation is shown in Fig. 2B). CA150 begins and ends with regions that contain runs of prolines; the region encompassing amino acids 32 to 132 is rich in proline residues (48%), and many of these prolines are found in runs of three or more. This polyproline region is similar to sequences found in formins, nuclear phosphoproteins involved in the development of the kidneys and

limbs in mice (31, 72). These domains in formins have been shown to interact with SH3 and WWP domains (9). Interestingly, CA150 has three WWP domains (68) (Fig. 2A); the two N-terminal WWP domains (I and II) are very similar to each other, whereas the carboxy-terminal domain (III) is more divergent (Fig. 2C). These recently recognized domains are involved in protein-protein interactions (7, 68) and have been identified in proteins involved in a wide variety of cellular functions (7). Thus, it is possible that CA150 dimerizes via WWP and polyproline domain interactions or interacts with other proteins through these domains.

Possibly the most salient feature of CA150 is a 76-amino-acid-long glutamine-alanine (QA) repeat found between amino acids 180 and 255 (Fig. 2A and B). This type of QA repeat has not been found among mammalian proteins, although runs of glutamines are observed in some transcription factors, such as TBP (30), and runs of glutamines have been shown to act as activation domains (23). QA repeats have been found in other transcription factors from *Saccharomyces cerevisiae* (GAL11 and SSN6) (62, 71) and *Drosophila melanogaster* (Zeste) (61). SSN6 and GAL11 show low levels of homology to CA150 in regions other than the QA repeats, and both scored among the best five when the nonredundant sequence databases were searched by using the BLAST algorithm (58, 59) (data not shown). Interestingly, a human TBP-associated repressor (Dr1) contains a glutamine- and alanine-rich motif (distinct from the QA repeat found in CA150) which has been shown to be important for its repressor function (74).

The CA150 gene product contains a region rich in serine, threonine, and proline (STP) that immediately follows the QA repeat (Fig. 2A). An STP motif is associated with a Q run in TBP (30), raising the possibility that this association has functional significance. Other interesting features of CA150 are a region of highly charged amino acids spanning 60 residues (KE region [Fig. 2A]), which is remarkable for containing 24 glutamic acid, 1 aspartic acid, and 11 lysine residues; a putative nuclear localization signal; and a putative leucine zipper (40) in the C-terminal part of the sequence (Fig. 2B).

Protein expression analysis of CA150. To further characterize CA150, we produced a GST fusion with the C-terminal 772 amino acids of CA150 and generated specific rabbit antisera against it. Western blot analysis with two independent anti-CA150 sera revealed the presence of a 150-kDa protein in HeLa cell nuclear extract and in the fractions from the GST-Tat and Q-Sepharose columns used in the purification (Fig. 3A). Protein expression analysis revealed the presence of CA150 in all human cell lines tested (Fig. 3B). This ubiquitous pattern of distribution was in agreement with the results of Northern blot analysis (data not shown). In vitro transcription and translation of the *CA150-I* cDNA yields a protein with an apparent M_r of 150 kDa (Fig. 3C) which comigrates with CA150. Although the expected M_r of the encoded protein is approximately 120 kDa, recombinant CA150 migrated in SDS-polyacrylamide gels with an apparent M_r of 150 kDa. The aberrant mobility observed on gels is probably due to sequences near the N terminus, between amino acids 194 to 1160, which include the QA repeat. Transient in vivo overexpression in HeLa cells of a truncated form of CA150 that does not include this region migrated with the expected mobility (data not shown).

The approach employed in the purification of relevant Tat cofactors did not require direct binding of Tat to these factors. We sought, however, to test the possibility that a direct interaction between CA150 and Tat occurs. For these experiments, we used in vitro-translated [^{35}S]CA150 and GST-Tat to carry out pull-down experiments in which the pellets were analyzed

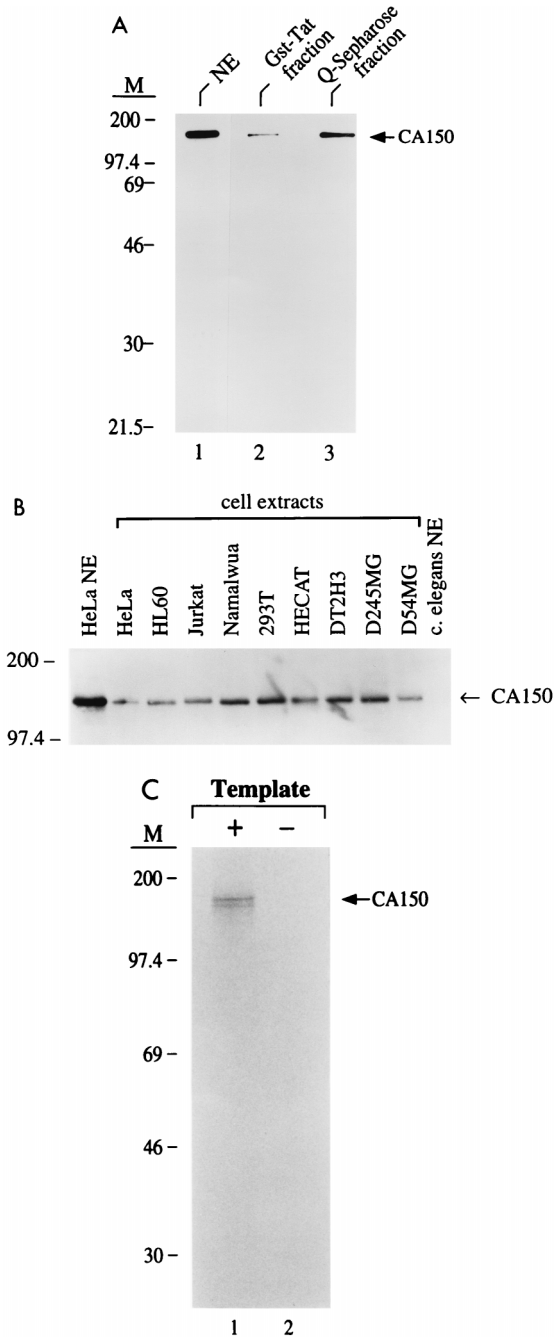

FIG. 3. Analysis of CA150 protein expression. (A) Antibodies generated against a truncated recombinant CA150 protein recognize a 150-kDa protein in HeLa cell nuclear extract (NE) as well as in the GST-Tat and Q-Sepharose purification step fractions. The positions of the CA150 protein (indicated by an arrow) and the relative mobility (in kilodaltons) of the molecular mass markers (M) are shown on the right and left sides of the figure, respectively. (B) CA150 protein expression in different cell lines. Forty micrograms of HeLa cell (lane 1) and *Caenorhabditis elegans* (lane 11) nuclear extracts and the same amount of the epithelial HeLa cell (lane 2), macrophage HL60 (lane 3), T-cell Jurkat (lane 4), B-cell Namalwua (lane 5), embryonic kidney cell 293T (lane 6), keratinocyte HECAT (lane 7), blastoma DT2H3 (lane 8), and glioblastoma D245MG (lane 9), and D54MG (lane 10) extracts were subjected to SDS-PAGE and Western blot analysis. Antiserum against CA150 was used to localize the protein (indicated by an arrow). (C) The product of in vitro transcription and translation of CA150 is a protein that migrates with a mobility corresponding to that of a protein of 150 kDa. Reactions were carried out in the presence (+) or absence (−) of DNA template. Translation products were separated on a 12.5% SDS–polyacrylamide gel and visualized after exposure for 24 h. Symbols are as for panel A.

by SDS-PAGE and fluorography. Under these conditions, only a very weak interaction between these proteins was observed (data not shown). In addition, we failed to detect any interaction when using the yeast two-hybrid system (data not shown). Taken together, these data do not support a direct interaction between CA150 and Tat.

CA150 is a nuclear protein that associates with RNA polymerase II holoenzyme. Although not an absolute requirement, it was expected that a protein involved in Tat *trans* activation would be nuclear. In order to localize endogenous CA150, we performed indirect immunofluorescence with anti-CA150 antibodies on CV-1 cells. CA150 was localized in the nucleoplasm of these cells and was excluded from the nucleoli (Fig. 4).

The low-level but intriguing homology with GAL11, a component of the RNA polymerase II holoenzyme (herein termed holoenzyme) in the yeast *S. cerevisiae*, and the finding that holoenzyme has been shown to respond to activators led us to investigate whether CA150 might be associated with the human holoenzyme, which has been recently characterized (11, 48, 56). Molt-4 nuclear extracts were supplemented with GST-Tat, GST-K41A Tat, or GST-Cys22 Tat (a second inactive Tat mutant) and were subjected to immunoprecipitation with antibodies against hSRB7, a component of the human holoenzyme (11). CA150 was efficiently immunoprecipitated by anti-hSRB7 antibodies (Fig. 5A). The pellet fractions also contained proteins known to associate with holoenzyme, such as RPB1, TFIIF, and MO15/Cdk7 (Fig. 5A), and to have activity in transcription assays as described by Chao et al. (11) (data not shown). As expected, however, anti-hSRB7 antibodies did not precipitate TFIIB, TBP, or NFκB(p65) (Fig. 5A). CA150, RPB1, TFIIF, and MO15/Cdk7 were pelleted by anti-hSRB7 antibodies in all extracts regardless of whether a Tat protein was present (Fig. 5A and data not shown). We conclude from these data that a fraction of CA150 in nuclear extracts was associated with holoenzyme and that this association was Tat independent. CA150 copurified with RNA polymerase II in a complex with a size similar to that of holoenzyme in gel filtration chromatography, further supporting this conclusion (25).

We further explored the association of Tat with holoenzyme by immunoprecipitating the extracts with an anti-Tat serum which could bring down all three GST-Tat fusion proteins. Holoenzyme components, such as hSRB7, MO15/Cdk7, and TFIIF, as well as CA150 were precipitated by anti-Tat antibodies if the extract was supplemented with GST-Tat (Fig. 5B), but not if it was supplemented with GST-K41A Tat or GST-Cys22 Tat (Fig. 5B) or if no Tat was added (data not shown). Taken together, these experiments strongly suggest that Tat can associate with holoenzyme in a way that depends on the critical Lys-41 and Cys-22 residues within the activation domain of Tat. It is also intriguing to note that Tat, but not the Tat mutants, associated with TBP and TFIIB (Fig. 5B). It is unlikely that these two proteins are holoenzyme components given their absence from the pellets of the anti-hSRB7 immunoprecipitates (Fig. 5A).

Nuclear extracts depleted of CA150 are incapable of Tat-mediated *trans* activation. The results of the purification approach used support a critical role for CA150 or CA150-associated factors in *trans* activation by Tat. The most important prediction that could be made was that depletion of CA150 should lead to an abrogation of Tat *trans* activation. In order to examine the role of CA150, we subjected HeLa cell nuclear extract to immunoaffinity chromatography with anti-CA150 or preimmune antibodies. The flowthrough fraction from the anti-CA150 column showed a 90% depletion of CA150 (Fig. 6A and data not shown) but no effect on the levels of polypyrimidine tract binding protein, a protein involved in pre-mRNA splicing (21). The flowthrough fraction from the column containing the preimmune antibodies had levels of CA150 similar to those in the original load (Fig. 6A). In vitro transcription reactions were carried out with the flowthrough fractions from the immunoaffinity columns in order to evaluate the effect of the depletion on basal and Tat-activated transcription. Immunodepletion of CA150 from the extract affected Tat *trans* activation of the HIV-1 LTR, whereas it had relatively little effect on basal transcription of the HIV-1 or the AdML promoter (Fig. 6B). Quantification of this (Fig. 6C) and similar experiments showed that *trans* activation by Tat was diminished by 45 to 87%. The flowthrough fraction from the preimmune column behaved like the load and supported basal and Tat-activated transcription (Fig. 6B). The CA150-immunodepleted extract showed a residual *trans*-activation activity. Recent data indicate that CA150 is present in HeLa cell nuclear extracts in complexes of different molecular sizes. A CA150-containing complex of the same size described for and with components of the human holoenzyme was present in low abundance (25). The fact that we could not completely eliminate CA150 from the extract (Fig. 6A), together with the data mentioned above, may explain the residual *trans*-activation activity observed in the in vitro-immunodepleted extracts. To date, we have not been able to express a full-length recombinant CA150 protein for testing in reconstitution experiments (data not shown). Nevertheless, the immunodepletion results suggest a role for CA150 and/or associated proteins in Tat-mediated transcriptional activation.

Overexpression of a truncated CA150 protein affected Tat-dependent transcription from the HIV-1 promoter in HeLa cells. The results cited above suggested a role for CA150 in Tat-mediated transcriptional activation. To confirm those data, we tested the ability of CA150 to regulate HIV-1 promoter activity in regular transfection experiments. We were unable to express full-length CA150 via transient transfections (data not shown). We reasoned that if CA150 was playing a critical role in Tat-mediated *trans* activation, it might be possible to interfere with Tat activation by overexpressing truncated forms of CA150 in HeLa cells. To assess this possibility, a truncated CA150 protein named ΔCA150, which lacks part of the polyproline-rich region, the first N-terminal WWP domain, and the QA repeat, was constructed (Fig. 2B). Transient transfection of ΔCA150 DNA resulted in the overexpression of a protein of the expected electrophoretic mobility as assessed by Western blotting (data not shown). In vivo overexpression of ΔCA150 by transient transfection resulted in a decrease in Tat-mediated transcriptional activation of the HIV-1 promoter but had little effect on basal HIV-1 transcription or transcription from the TK promoter in a dose-dependent manner (Fig. 7 and Table 1). The effect of ΔCA150 overexpression on transcriptional activation by the acidic activation domain of VP16 and the proline-rich activation domain of CTF in the GAL4-VP16 and GAL4-CTF fusion proteins was assayed. No decrease in the transcriptional activity of the reporter gene was observed upon cotransfection of ΔCA150 (data not shown). Thus, these results suggest that CA150 specifically regulates the activation of HIV-1 gene expression by Tat.

DISCUSSION

However critical the role of upstream activators in modulating transcription from the HIV-1 LTR, the master regulator of activation is the Tat-TAR RNP. Thus, we have focused our studies on the structure and function of this RNP. Whereas the viral components of this RNP, Tat and TAR, have been well

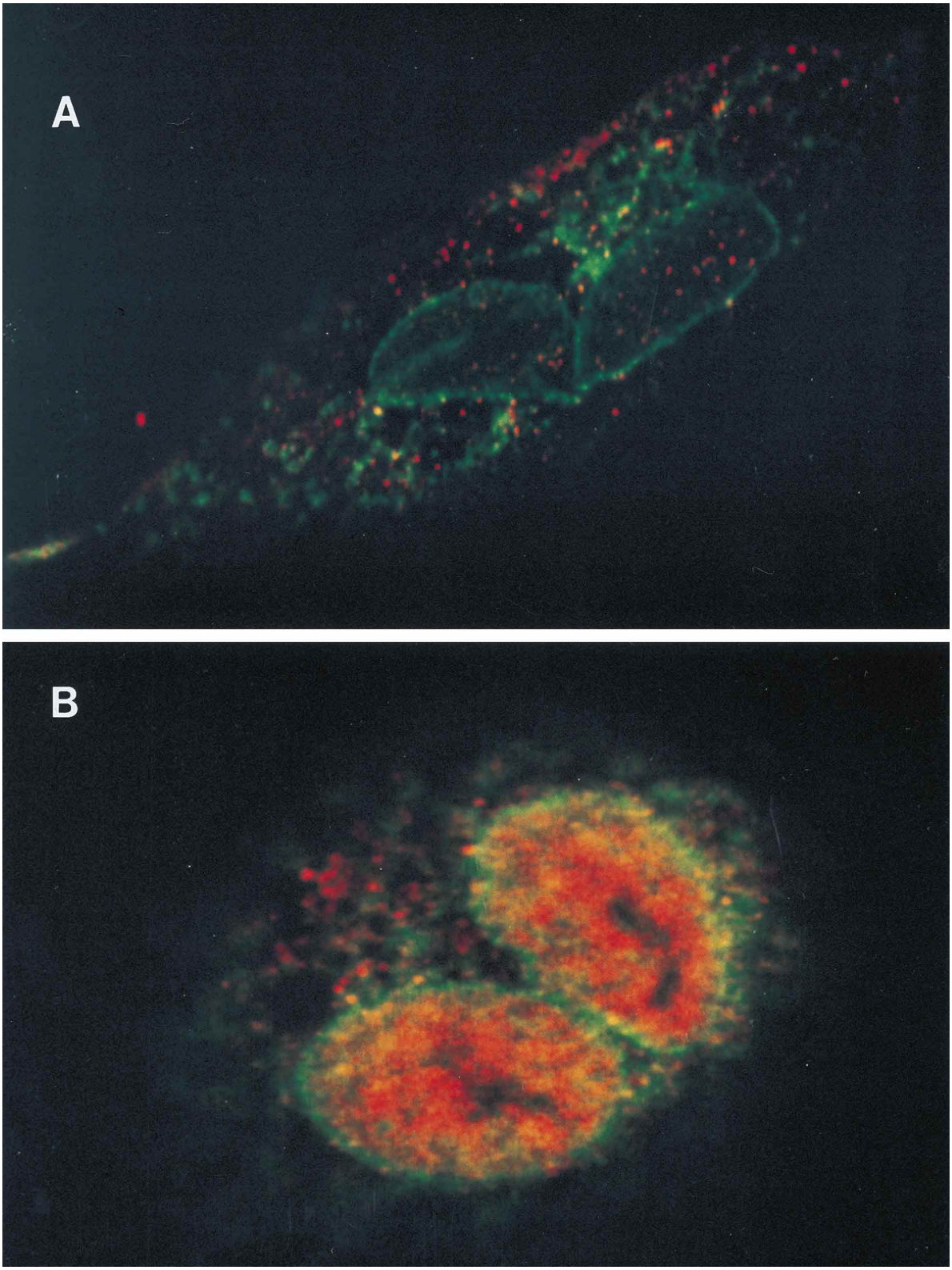

FIG. 4. CA150 is present in the nucleoplasm of CV-1 cells. Cells were prepared as described in Materials and Methods and stained with preimmune (A) or anti-CA150 (B) sera as well a secondary anti-rabbit immunoglobulin coupled to rhodamine (red). The same cells were stained with anti-nucleopore-specific antibody and a secondary antimouse serum coupled to fluorescein (green).

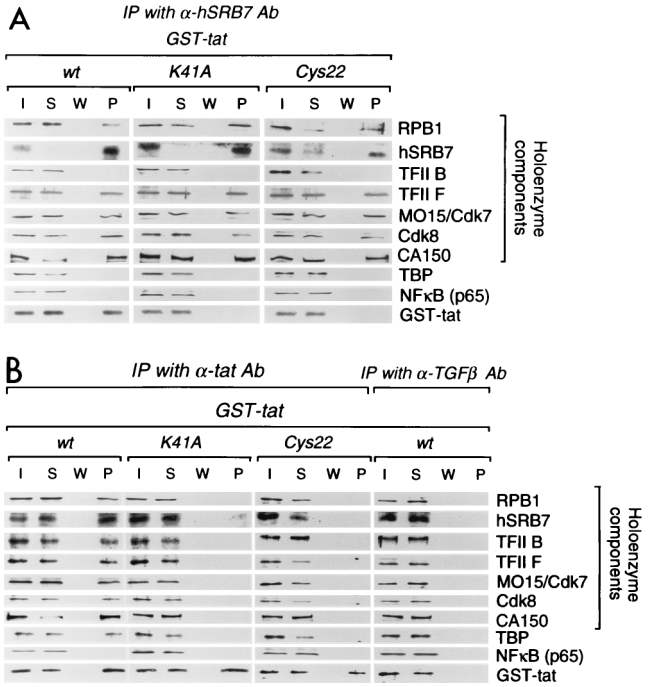

FIG. 5. Association between CA150, Tat, and holoenzyme. (A) Coimmunoprecipitation of human RNA polymerase II holoenzyme components with Tat transcription activator, using hSRB7-specific polyclonal antibodies (Ab). Nuclear extract prepared from Molt-4 cells was incubated with the indicated GST-Tat fusion proteins, and immunoprecipitations (IP) were carried out with anti-hSRB7 (α-hSRB7) polyclonal antibodies. One-fortieth of the input (I) and supernatant (S) fractions and 1/40 of the last wash (W) and pellet (P) fractions were subjected to SDS-PAGE and analyzed by Western blotting with appropriate specific antibodies. (B) Coimmunoprecipitation of Tat transcription activator with human RNA polymerase II holoenzyme, using Tat-specific polyclonal antibodies. A nuclear extract prepared from Molt-4 cells was incubated with the indicated GST-Tat fusion proteins, and immunoprecipitations were carried out with anti-Tat (α-tat) polyclonal antibodies or a control antiserum (anti-human transforming growth factor beta [TGF-β]). One-fiftieth of the input (I) and supernatant (S) fractions and 1/50 of the last wash (W) and pellet (P) fractions were subjected to SDS-PAGE and analyzed by Western blotting with appropriate specific antibodies.

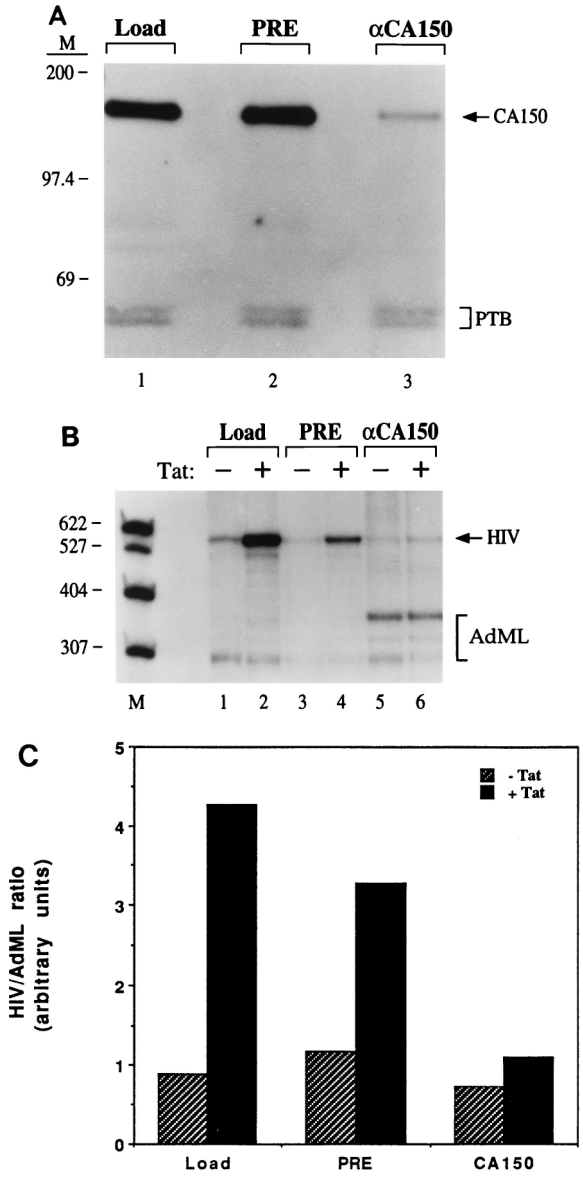

FIG. 6. Effect of the immunodepletion of CA150 from nuclear extract in Tat *trans* activation. (A) Immunodepletion of CA150 from HeLa cell nuclear extract. Shown are the results of Western blot analysis of the load (lane 1) and the flowthrough fractions from the preimmune (PRE) (lane 2) and immune (anti-CA150 [αCA150]) (lane 3) antibody affinity columns. Specific antibodies against CA150 and polypyrimidine tract binding protein (PTB) (24) were used to localize the proteins. The positions of the proteins and the relative mobilities (in kilodaltons) of the molecular mass markers (M) are shown on the right and left sides of the panel, respectively. (B) Analysis of basal and Tat-activated transcription in the immunodepleted extracts. Transcription reactions were performed with the indicated fractions in the absence (−) or presence (+) of 50 ng of Tat. Specific transcripts are indicated by arrows. Molecular size markers (M; in base pairs) are also indicated. Note that lanes 3 and 4 were underloaded. (C) Experimental data, quantified with the PhosphorImager, from the experiment shown in panel B are provided in graphic form.

characterized, cellular factors that associate with Tat and TAR to form the complete and functional RNP have not been identified. Previously, we showed that at least some of these cellular coactivators are not GTFs such as TFIIB and TBP but rather are factors not normally required for basal transcription (69). Consequently, we set out to identify factors required for Tat-dependent activation of HIV-1 transcription. The methodology employed did not require the direct binding of Tat by these factors or that these factors act as single components. In this paper, we have described the purification and initial characterization of CA150, which is associated with the RNA polymerase II holoenzyme, and we posit that it is a coactivator of Tat action based on in vitro and in vivo data.

Glutamine runs, coactivators, and multiprotein complexes. The predicted sequence of CA150 revealed notable motifs and domains, many of which are hallmarks of transcription factors. Perhaps the most salient of these is the QA repeat. To our knowledge, this is the first report of a mammalian protein containing this distinctive repeat of glutamines and alanines, which has been previously found in the *S. cerevisiae* transcriptional factors GAL11 and SSN6 and in the *D. melanogaster* protein Zeste (61, 62, 71). These factors are not GTFs required for all RNA polymerase II transcription but rather are needed for maximal or regulated expression of many genes. As such, they have been dubbed coactivators or transcriptional couplers. In some cases, these factors can also act as repressors of transcription, such as SSN6 (37). The presence of the QA motif in CA150, as well as the functional data implicating CA150 in the process of *trans* activation by Tat, suggests a role for CA150 as a transcriptional coactivator.

The role of the QA repeat in the yeast coactivators has not

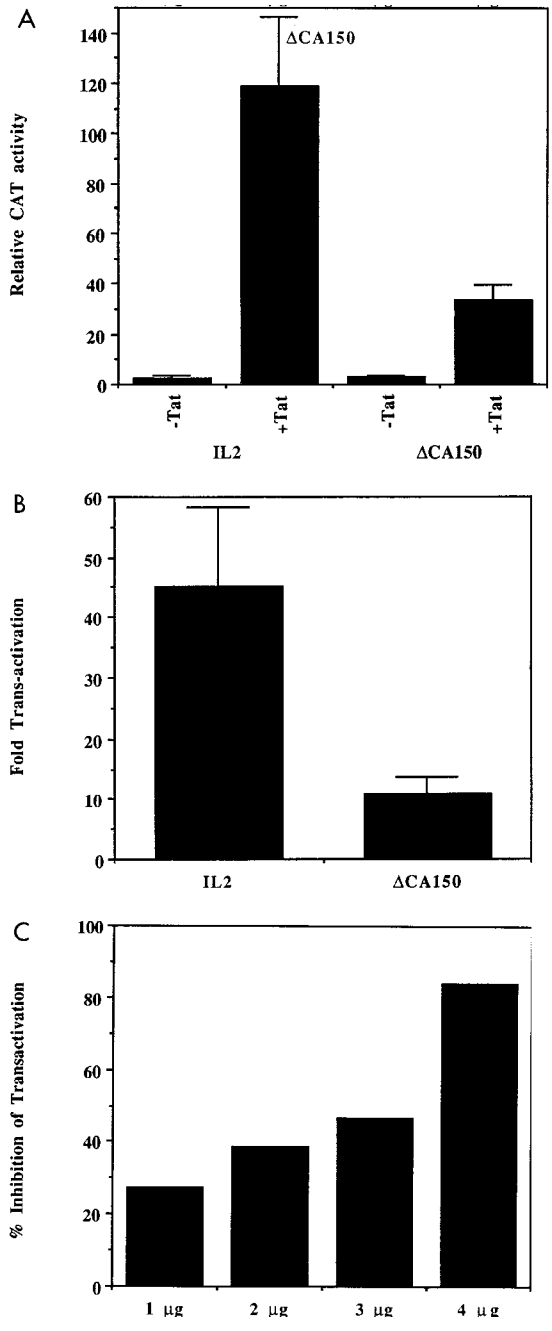

FIG. 7. Effect of ΔCA150 on basal and Tat-activated transcription from the HIV-1 promoter. (A) The level of Tat activation in a chloramphenicol acetyltransferase (CAT) reporter assay induced by overexpression of ΔCA150 is compared with that induced by a control vector (IL-2) in the absence (−) and presence (+) of Tat. (B) *trans* activation was calculated as the ratio of the CAT activity (slopes) in the presence and in the absence of Tat. Data represent the average of four experiments performed in duplicate. Where shown, error bars represent the standard deviation from the mean. (C) Effect of different amounts of ΔCA150 on *trans* activation by Tat. Values are from one experiment after normalization for the internal control luciferase activity of the TK-luciferase reporter vector (TK-luc). Similar results were obtained in two independent transfection experiments.

been completely elucidated. It is not absolutely required for the repressive functions of SSN6; however, to our knowledge, mutants lacking the QA repeat have not been tested for complementation in activation-dependent assays. The GAL11 homolog in *Kluveromyces lactis* (Kl-GAL11) can partially complement *S. cerevisiae* lacking GAL11. Kl-GAL11 does not have a QA repeat; nevertheless, it has a similar sequence in which the alanines are replaced by prolines and other residues (52). It is intriguing to note that both GAL11 and SSN6 have been shown to be part of higher-order macromolecular complexes (1, 73). Extensive glutamine runs are found in TBP (30), the core of the multicomponent TFIID, and in SRB8, a member of the mediator complex (28), suggesting an association between extensive runs of glutamines and membership in large transcriptional complexes.

CA150, RNA polymerase II holoenzyme, and Tat *trans* activation. Several lines of evidence suggest that CA150 plays a role in Tat *trans* activation. First, CA150 was purified by monitoring an activity in a functional assay (Fig. 1). Second, immunodepletion of CA150 from nuclear extracts affected Tat *trans* activation of the HIV-1 promoter while having little effect on basal transcription of the HIV-1 and the AdML promoters (Fig. 6B). Third, in vivo overexpression of a truncated CA150 protein resulted in a decrease in Tat activation of the HIV-1 promoter but had little effect on basal HIV-1 transcription or transcription from the TK promoter (Fig. 7 and Table 1). Previously, no single factor had been implicated both in vivo and in vitro in Tat-activated transcription. The most compelling candidate to date had been Tat-SF, a protein which is required for Tat-mediated *trans* activation in vitro and leads to decreased basal HIV-1 transcription upon overexpression in vivo (77). Although no relationship of Tat-SF1 with holoenzyme has been reported, it will be of interest to determine if this protein and CA150 are components of the same complex (see below).

The in vitro experiments described in this paper indicate that a CA150-associated activity is required for Tat-activated HIV-1 transcription. The facts that we lost activity in the last step of the purification and that we could only recover very low levels of Tat-mediated *trans* activation in CA150-immunodepleted extracts by addition of the most purified fractions of CA150 (data not shown) suggest that CA150 may be a component of a complex functional activity. The presence of multiple proteins that copurified with the functional activity (Fig. 1C) supports this, and we therefore hypothesized an association of CA150 with a higher-order complex, which may serve as a bridge between Tat and the general transcriptional machinery. The finding that CA150 coimmunoprecipitated with the holoenzyme argues that this complex could be holoenzyme itself or a complex associated with holoenzyme, which supports the recent report by Cujec et al. (12).

The implications of the association of Tat with holoenzyme are several. Basal transcription by the HIV-1 promoter may be carried out by a core enzyme assembled with GTFs but without all holoenzyme components. This polymerase may initiate in a TATA-box-dependent (60) or -independent (3, 45, 55) fashion and synthesize short transcripts. Thus, on the one hand, in the absence of the Tat-TAR RNP, this promoter seems incapable of efficiently recruiting an elongation-competent polymerase. On the other hand, Tat may *trans* activate the HIV-1 promoter by recruiting an elongation-competent RNA polymerase II holoenzyme via direct interactions with the Tat-TAR RNP. Transcription by this elongation-competent holoenzyme results in high levels of full-length transcripts. The finding that holoenzyme has been shown to respond to activators supports this model (1, 17, 39). The Tat effect could be also mediated by

TABLE 1. Effect of overexpression of ΔCA150 on basal and Tat-activated transcription

Reporter gene	Expt	Transcriptional activity after transfection with[a]: Vector (IL-2) − Tat	Vector (IL-2) + Tat	Fold activation with vector	ΔCA150 − Tat	ΔCA150 + Tat	Fold activation with ΔCA150
CAT	1	2.1	119.6	56.9	2.4	33.1	13.7
	2	4.0	149.8	37.4	3.5	27.4	7.8
	3	2.7	83.5	30.9	3.2	41.6	13
	4	3.1	122.7	39.5	3.4	32.4	9.5
LUC	1	459,738	398,447	0.8	465,921	439,295	0.9
	2	149,516	138,143	0.9	102,064	72,146	0.7
	3	167,411	165,705	0.9	114,421	206,946	1.8
	4	299,891	246,115	0.8	322,798	246,593	0.7

[a] Activity from the pTAR-CAT (CAT) and TK-LUC (LUC) reporter genes is shown for four independent experiments. All results shown represent the averages of data from duplicate specimens. CAT activity was the value of the slope of the linear function obtained by plotting cpm of acetylated chloramphenicol versus time. Relative luciferase (LUC) activity was obtained by measuring light emission in the same samples with a luminometer.

modification of holoenzyme factors, such as Tat-mediated phosphorylation of the C-terminal domain of the largest subunit of RNA polymerase II (57). This scenario is in agreement with the proposed model in which Tat enhances dramatically the efficiency of elongation of HIV-1 transcripts by recruiting more elongation-competent transcription complexes (15). Nevertheless, Tat-mediated *trans* activation depends on a functional TAR element. TAR RNA might modify the Tat complex to activate transcription. The interaction between Tat and holoenzyme (this paper and reference 12) and the finding that Tat must be present during preinitiation complex formation in order to *trans* activate the HIV-1 promoter (22, 44) are consistent with this model.

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