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# Isolation of a cellular protein that binds to the human immunodeficiency virus Tat protein and can potentiate transactivation of the viral promoter

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ABSTRACT **The human immunodeficiency virus type 1 Tat protein is a powerful transactivator of the viral long terminal repeat (LTR). We have identified a cellular protein that strongly binds to Tat and can complement Tat transactivation in rodent cells. The cellular protein of about 36 kDa was isolated from extracts of human cells by Tat peptide-affinity chromatography and can form a complex with Tat *in vitro*. Tat transactivation is inefficient in rodent cells microinjected or transfected with the reporter plasmid pHIV-LTRCAT plus the Tat-expressing plasmid pCV-1. Remarkably, coinjection of purified 36-kDa protein with pHIV-LTRCAT plus pCV-1 stimulated Tat transactivation 2.7- to 4.9-fold. Taken together, our findings suggest that the 36-kDa protein may be a transcription factor or modulator that is important for efficient Tat transactivation.**

Human immunodeficiency virus type 1 (HIV-1), the causative agent of AIDS (1–3), is a retrovirus that encodes two classes of genes: structural genes that specify viral capsid and core proteins and regulatory genes that modulate by novel mechanisms the expression and processing of viral genes (for a review, see ref. 4).

The HIV-1 Tat gene encodes an 86-amino acid regulatory protein that is a powerful transactivator of gene expression from the viral long terminal repeat (LTR) and is essential for virus replication (5, 6). The mechanism of action of Tat is complex and poorly understood (for reviews, see refs. 7 and 8). The target sequence for Tat transactivation is a 59-nucleotide RNA stem–loop structure called transactivation response element (TAR) (9) that functions in the form of nascent RNA (10). Recent protein fusion and TAR target swapping experiments have indicated that a major function of TAR is to provide a Tat-binding site (11–13). Consistent with these findings, Tat has been shown to bind TAR RNA *in vitro* (14–17).

Tat comprises several domains; of particular interest is a 9-residue arginine-rich region involved in nuclear localization (18–22). Specific binding to TAR appears to require only the Tat arginine-rich region and eight random amino acids at either end (17). Inasmuch as Tat domains in addition to the arginine-rich region are required for transactivation, it would seem that binding of Tat to TAR is not sufficient for transactivation and that additional steps are involved. It is likely that other Tat domain(s) interact with specific cellular protein(s) that serve important functions in Tat transactivation. Here we describe the identification and purification of a cellular protein that binds to Tat and can complement Tat transactivation in rodent cells. We propose that the 36-kDa cellular protein is a specific cellular factor required for efficient Tat function.

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## MATERIALS AND METHODS

**Preparation of Labeled Cell Extracts.** The human lymphocyte culture Molt3 (from R. Gallo, National Cancer Institute) was grown in suspension in RPMI medium (GIBCO) containing 10% fetal calf serum. Murine cell lines NIH 3T3 (from D. Stacey, Cleveland Clinic Foundation), C127, and Swiss 3T3 (from R. Baserga, Temple University), and Chinese hamster ovary (CHO) cells (from W. Sly, St. Louis University) were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum. Cells were washed and incubated in medium lacking cysteine and methionine for 1 hr at 37°C and labeled with 100 $\mu$Ci of Tran$^{35}$S-label (ICN, 1179 Ci/mmol; 1 Ci = 37 GBq; 70% L-methionine, 15% L-cysteine) at 37°C for 4.5 hr. Cells were washed twice with phosphate-buffered saline (PBS) and lysed at 4°C with RIPA buffer (150 mM NaCl/10 mM Tris, pH 7.4/1 mM EDTA/1% Triton X-100/1% sodium deoxycholate/0.25 mM phenylmethylsulfonyl fluoride/1 trypsin inhibitor unit of aprotinin per ml). Nuclei were ruptured by sonication for 10 sec and the total cell extract was clarified by brief centrifugation.

**Peptide-Affinity Chromatography.** Affi-Gel 10 was prepared as recommended (Bio-Rad) and 0.5-ml amounts were coupled for 2 hr at room temperature with 0.5 ml of PBS containing 0.5 mg of the chemically synthesized Tat86 peptide (19), bovine serum albumin (BSA), or the chemically synthesized human papillomavirus type 16 (HPV16) E7 98-amino acid polypeptide (23). After treatment with 0.1 M ethanolamine to block residual active ester groups, gels were packed by gravity into 0.8 cm$^2$ × 5 cm columns, washed sequentially with column buffer (20 mM Tris·HCl, pH 8.1/5 mM $MgCl_2$/1 mM EDTA/1 mM 2-mercaptoethanol/10% glycerol) containing 2 M NaCl and 0.05 M NaCl, and stored at 4°C in column buffer containing 0.05 M NaCl and 0.2% sodium azide.

For affinity chromatography, 1 ml of $^{35}$S-labeled whole cell extract was circulated through the column at 4°C for 3 hr or overnight at a flow rate of 1 ml/hr. Columns were washed sequentially with 6–20 column volumes of column buffer containing 0.05 M, 0.1 M, 0.2 M, 0.4 M, 0.6 M, and 2 M NaCl at a flow rate of 3 ml/hr and 1-ml fractions were collected. Appropriate fractions were precipitated with trichloroacetic acid/deoxycholate, heated in SDS/PAGE sample buffer, and electrophoresed on a discontinuous 15% SDS/polyacrylamide gel (24). Fluorograms were prepared by incubating the gel in 1 M sodium salicylate for 1 hr, drying, and exposing the gel to Kodak X-Omat film at −70°C with x-ray intensifying screens.

**Preparative Purification of 36-kDa Protein.** Extracts of unlabeled Molt3 cells (5 × 10$^7$ cells) were prepared and the

Abbreviations: HIV-1, human immunodeficiency virus type 1; LTR, long terminal repeat; TAR, transactivation response element; BSA, bovine serum albumin; CAT, chloramphenicol acetyltransferase; HPV16, human papillomavirus type 16; CHO, Chinese hamster ovary.

36-kDa protein was purified by Tat peptide-affinity chromatography, as described above, followed by SDS/PAGE using multiple gel lanes. Gels were reversibly stained with $CuCl_2$ (25), eluted (26), and renatured (27).

**Plasmids.** pCD12 (pHIV-LTRCAT), which contains the HIV-1 promoter fused upstream of the chloramphenicol acetyltransferase (CAT) gene (28), and pCV-1, which expresses HIV-1 Tat constitutively (29), were provided by S. Josephs (National Cancer Institute).

**Coimmunoprecipitation of a Tat86/36-kDa Complex.** $^{35}S$-labeled 36-kDa protein was purified by elution from a Tat peptide-affinity column with 0.6 M and 2.0 M NaCl, concentrated by ultrafiltration with a Centricon-10 tube (Amicon), and resuspended in PBS. SDS/PAGE showed that the 36-kDa protein was the predominant radioactive species in the preparation (see Fig. 2, lane 1). Recombinant Tat 86 (100 or 500 ng) was incubated with 4000 cpm (10 ng, roughly estimated) of the purified $^{35}S$-labeled 36-kDa protein in 50 $\mu$l of PBS for 3 hr at 37°C. Immunoprecipitation buffer [50 mM Tris·HCl, pH 7.4/150 mM NaCl/5 mM EDTA/0.05% Nonidet P-40 (NP-40)/0.1% BSA] was added to a final volume of 1.5 ml followed by addition of 10 $\mu$l of Tat86 antibody (prepared in rabbits and purified by Tat peptide-affinity chromatography). After incubation overnight at 4°C, 20 $\mu$l of protein A-Sepharose (5 mg) in NET (50 mM Tris·HCl, pH 7.4/150 mM NaCl/5 mM EDTA) was added and the mixture was incubated at 4°C for 1 hr. Beads were washed five times in NET containing 0.5% NP-40, boiled for 5 min in sample buffer (24), and analyzed by SDS/PAGE and autoradiography.

**Mammalian Cell Microinjection and *in Situ* Hybridization Assay for Tat Transactivation.** Cells were grown on coverslips and nuclei were microinjected as described (19, 23, 30). Plasmids pCD12 and pCV-1 were dissolved in 30 mM Tris·HCl (pH 8.1; 100 $\mu$g/ml) and coinjected in the absence or presence of purified 36-kDa protein. At 5–8 hr after injection, cells were fixed in paraformaldehyde and CAT RNA was measured by *in situ* hybridization using as probe CAT DNA labeled with dCTP[$^{35}S$] (19). After posthybridization washes, cells on coverslips were dehydrated in ethanol, dried, mounted on microscope slides, dipped in Kodak NTB-2 emulsion, and developed after 2–3 days of exposure. The percentage of microinjected cells that hybridized with the CAT DNA probe was scored by microscopic examination; positive cells were classified as giving low- or high-density response as described (19).

## RESULTS

**Identification of a Cellular Protein That Strongly Binds to Tat Peptide-Affinity Columns.** To identify cellular proteins that could form a complex with Tat and thus might function in Tat-mediated transactivation, we determined whether specific cellular proteins would bind to chemically synthesized full-length Tat (Tat86). Extracts of Molt3 cells, metabolically labeled with [$^{35}S$]methionine/[$^{35}S$]cysteine were circulated through a Tat/Affi-Gel 10 column in buffer containing 0.05 M NaCl. Bound proteins were eluted with column buffers containing increasing NaCl concentrations and identified by SDS/PAGE and autoradiography. As controls, affinity columns were used that contained immobilized BSA or the 98-amino acid HPV16 E7 protein [which can transactivate the adenovirus E2 promoter (23) but not the HIV-1 promoter (data not shown)]. As shown in Fig. 1, most cellular proteins did not bind to the Tat or BSA column and were found in the flow-through and 0.05 M NaCl fractions. Low levels of several labeled proteins were eluted with 0.1 M, 0.2 M, and 0.4 M NaCl. A prominent protein of 36 kDa (±2 kDa) was eluted in the 2.0 M NaCl fraction (and variable amounts were eluted in the 0.6 M fraction) from the Tat column but not from

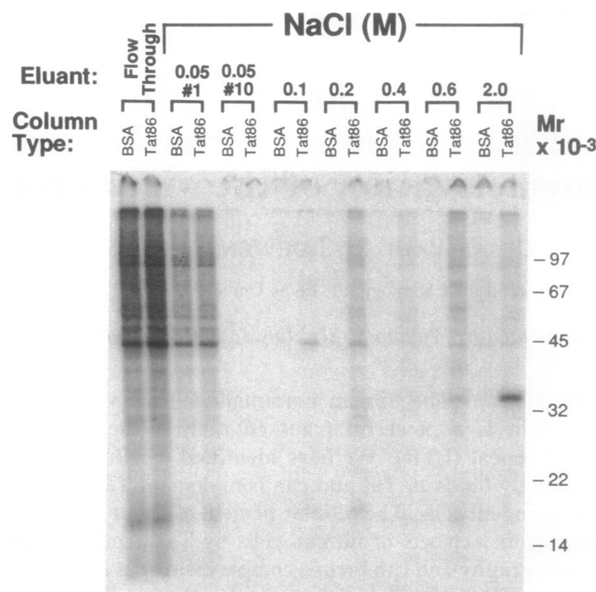


FIG. 1. Identification of a human cellular 36-kDa protein that binds to a Tat peptide-affinity column. An extract of $1.5 \times 10^7$ Molt3 cells, labeled with [$^{35}S$]methionine/[$^{35}S$]cysteine ($4.4 \times 10^8$ cpm), was split into two portions, one-half circulated for 12 hr through a 0.5-ml Affi-Gel 10 column containing 500 $\mu$g of immobilized Tat86 peptide and the second portion circulated through a similar BSA column. The columns were eluted stepwise with ten 1.0-ml amounts of column buffer containing 0.05 M NaCl and 3-ml amounts of buffer containing 0.1, 0.2, 0.4, 0.6, and 2.0 M NaCl. The first and tenth 1.0-ml fractions of the 0.05 M eluate, 1 ml each of the 0.1, 0.2, 0.4, 0.6, and 2.0 M eluates, and 50 $\mu$l of the flow-through were precipitated with trichloroacetic acid/deoxycholate and analyzed by SDS/PAGE and autoradiography. Molecular mass protein markers (shown in kDa), as in Fig. 2, were run in a parallel lane.

the BSA column (Fig. 1) or the HPV16 E7 column (data not shown). The fact that the 36-kDa protein elutes only at high NaCl concentrations from the Tat peptide-affinity column indicates that it binds Tat with high affinity. This is consistent with the possibility that the 36-kDa protein might play a role in Tat transactivation. The 36-kDa protein was detected readily by peptide-affinity chromatography in three additional human T-lymphocyte cell lines and at lower levels in human HeLa and A-549 cells (data not shown).

**The 36-kDa Protein Forms a Complex *in Vitro* with Tat86.** Since the 36-kDa protein bound strongly to a Tat peptide-affinity matrix, we next asked whether purified the 36-kDa protein could form a complex with Tat in solution. Purified $^{35}S$-labeled 36-kDa protein was incubated with approximately a 10- and 50-fold molar excess of recombinant Tat86 followed by immunoprecipitation with anti-Tat antibody. Most of the 36-kDa protein (60–80%) was coimmunoprecipitated with Tat (Fig. 2, lanes 2 and 3, respectively; lane 1 shows the 36-kDa protein prior to immunoprecipitation). No 36-kDa protein was immunoprecipitated by anti-Tat antibody in the absence of Tat (data not shown). Additionally, no 36-kDa protein was coimmunoprecipitated when incubated with the 98-amino acid HPV16 E7 polypeptide followed by immunoprecipitation with antibody to the E7 protein (lanes 4 and 5). These data demonstrate that purified 36-kDa protein can form a strong complex with Tat *in vitro*.

**Purification of the Cellular 36-kDa Tat-Binding Protein.** To purify quantities of 36-kDa protein sufficient for functional studies, we exploited the ability of the cellular 36-kDa protein to form a tight complex with Tat86. Fig. 3 shows a Coomassie blue-stained gel of the combined 0.6 M/2.0 M NaCl eluate after preparative Tat peptide-affinity chromatography of a Molt3 cell extract. A major 36-kDa protein band was visu-

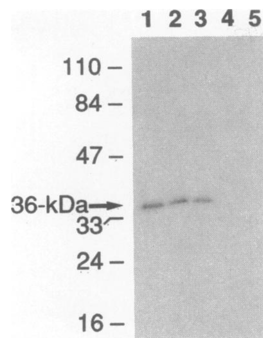


FIG. 2. Formation of a complex between the 36-kDa protein and Tat86 *in vitro*. The $^{35}S$-labeled 36-kDa protein was incubated with an ≈10-fold (lanes 2 and 4) or ≈50-fold (lanes 3 and 5) molar excess of recombinant Tat86 (lanes 2 and 3) or HPV16 E7 polypeptide (lanes 4 and 5) and immunoprecipitated with antibody to Tat86 (lanes 2 and 3) or HPV16 E7 polypeptide (lanes 4 and 5), followed by SDS/PAGE and autoradiography. Lane 1, the 36-kDa protein prior to immunoprecipitation. Molecular masses are shown in kDa.

alized under reducing and nonreducing conditions. The 36-kDa protein after SDS/PAGE was reversibly stained with $CuCl_2$, eluted, renatured, and used for the cell microinjection studies described below.

**The Purified 36-kDa Protein Complements Tat Transactivation of HIV-LTRCAT in Rodent Cells.** Fractionation studies with Molt3 cells showed that the 36-kDa protein was present mainly in the cell nucleus (data not shown). Nuclear localization is consistent with a role for the 36-kDa protein as a transcription factor involved in Tat transactivation. Of interest in this regard, CHO cells and murine NIH 3T3 cells were reported to support poorly Tat transactivation, whereas rodent–human cell hybrid clones containing human chromosome 12 or 6 were reported to exhibit increased levels of transactivation (31, 32). These findings suggest that chromosome 12 or 6 might encode a factor required for efficient Tat transactivation. We therefore determined whether the 36-kDa protein would stimulate Tat transactivation in murine and CHO cell lines.

To measure the ability of the 36-kDa protein to complement Tat transactivation in rodent cells, we used a sensitive

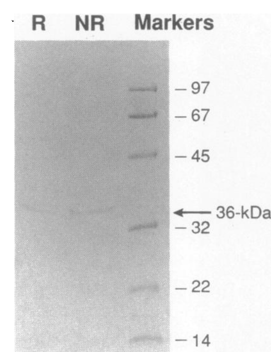


FIG. 3. Preparative purification of the Tat-binding cellular 36-kDa protein. An extract of $5 \times 10^7$ Molt3 cells was circulated for 3 hr on a Tat peptide-affinity column and eluted with column buffers as described in the legend to Fig. 1. The 0.6 M and 2.0 M NaCl eluates were pooled and concentrated by ultrafiltration with a Centricon-10 tube (Amicon), split into two portions, one treated with SDS sample buffer containing 2-mercaptoethanol (reducing conditions, R) and the second treated with SDS sample buffer lacking 2-mercaptoethanol (nonreducing, NR). Samples were subjected to SDS/PAGE and stained with Coomassie blue. The marker lane contained 2 μg each of phosphorylase B (97 kDa), BSA (67 kDa), ovalbumin (45 kDa), carbonic anhydrase (32 kDa), trypsin inhibitor (22 kDa), and lysozyme (14 kDa).

cell microinjection assay (19). The reporter plasmid pHIV-LTRCAT and the Tat-expressing plasmid pCV-1 were coinjected into cell nuclei, and the formation of HIV-LTR-directed CAT RNA was measured by *in situ* hybridization with a CAT $^{35}S$-labeled DNA probe. Positive cells were scored microscopically as "low-density" cells, which contain 50–200 silver grains per cell, or "high-density" cells, which contain >200 grains (19) (negative cells generally contain <5 grains). By this assay, >70% of microinjected human HeLa cells were positive for Tat transactivation and about 90% of these were high density (Table 1) (19). Coinjection of the 36-kDa protein did not alter significantly the response of HeLa cells (Table 1).

In contrast to the strong response of human cells, only 10–15% of murine C127, murine Swiss 3T3, and CHO cells were positive for Tat transactivation when the cells were coinjected with pHIV-LTRCAT and pCV-1; further, only about one-third of positive cells were high density (Table 1). The percentage of total positive cells was increased 2.7- to 4.9-fold by comicroinjection of the purified 36-kDa protein at 500 ng/ml (see Table 1 and Fig. 4). A dose–response was observed; coinjection of 50 ng of 36-kDa protein per ml induced a 1.6- to 3.2-fold increase in Tat transactivation, whereas 5 ng/ml did not provide significant stimulation (Table 1). Similar results were obtained with murine NIH 3T3 and BALB/c 3T3 cell lines (data not shown). Further, the 36-kDa protein did not stimulate HIV-LTRCAT expression when injected in the absence of the Tat-expressing plasmid pCV-1 (Table 1). In addition, the 36-kDa protein did not affect the expression of coinjected pSV2CAT (data not shown). We

Table 1. Potentiation of Tat transactivation by coinjection of purified 36-kDa protein with plasmids pHIV-LTRCAT and pCV-1

| Microinjected material | Conc., ng/ml | Transactivated cells, % Total | High/low* | Fold increase† |
|---|---|---|---|---|
| Human HeLa cells | | | | |
| pHIV-LTRCAT | | 2 | 0/2 | — |
| + pCV-1 | | 73 | 66/7 | 1.0 |
| + 36-kDa protein | 500 | 79 | 57/22 | 1.1 |
| Murine C127 cells | | | | |
| pHIV-LTRCAT + pCV-1 | | 15 | 4/11 | 1.0 |
| + 36-kDa protein | 500 | 40 | 22/18 | 2.7 |
| + 36-kDa protein | 50 | 24 | 12/12 | 1.6 |
| + 36-kDa protein | 10 | 20 | 9/11 | 1.3 |
| pHIV-LTRCAT | | 0 | 0/0 | — |
| + 36-kDa protein | 500 | 0 | 0/0 | — |
| Murine Swiss 3T3 cells | | | | |
| pHIV-LTRCAT + pCV-1 | | 10 | 3/7 | 1.0 |
| + 36-kDa protein | 500 | 49 | 28/21 | 4.9 |
| + 36-kDa protein | 50 | 32 | 17/15 | 3.2 |
| + 36-kDa protein | 10 | 10 | 2/8 | 1.0 |
| pHIV-LTRCAT | | 0 | 0/0 | — |
| + 36-kDa protein | 500 | 0 | 0/0 | — |
| CHO cells | | | | |
| pHIV-LTRCAT + pCV-1 | | 14 | 4/10 | 1.0 |
| + 36-kDa protein | 500 | 58 | 10/48 | 4.1 |
| + 36-kDa protein | 50 | 46 | 10/36 | 3.2 |
| + 36-kDa protein | 10 | 14 | 2/12 | 1.0 |
| pHIV-LTRCAT | | 0 | 0/0 | — |
| + 36-kDa protein | 500 | 2 | 0/2 | — |

Cell nuclei were injected with 100 μg of pHIV-LTRCAT per ml with or without 100 μg of pCV-1 per ml and the indicated concentration of 36-kDa protein. Cells were fixed 5–8 hr later and analyzed for HIV-LTR-directed expression by *in situ* hybridization with $^{35}S$-labeled CAT DNA. Positive cells were scored as described in ref. 19.

*High- and low-grain density cells (see text).

†Effect of coinjected 36-kDa protein.

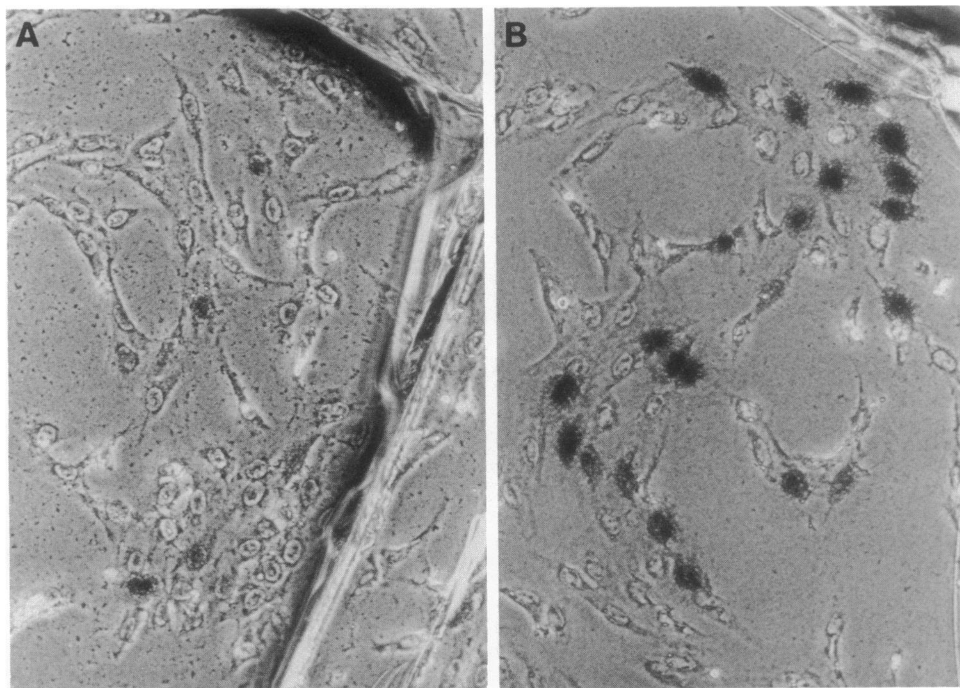


FIG. 4. Effect of the cellular 36-kDa protein on Tat transactivation of pHIV-LTRCAT in murine cells. Swiss 3T3 cells on a coverslip were microinjected with pHIV-LTRCAT plus pCV-1 in the absence (*A*) or presence (*B*) of the 36-kDa protein (500 ng/ml). At 6 hr after microinjection, cells were fixed and analyzed by *in situ* hybridization using $^{35}$S-labeled CAT DNA as probe. (×400.)

conclude that the 36-kDa protein does not by itself activate the HIV-LTR and that it most likely acts together with Tat to potentiate transactivation specifically of the HIV-LTR promoter.

These findings suggest that the rodent analogue of the human 36-kDa protein is either absent from rodent cells or present at levels insufficient for efficient Tat transactivation. To examine these possibilities, extracts of $^{35}$S-labeled mouse NIH 3T3 cells were analyzed by Tat peptide-affinity chromatography. The 36-kDa protein was not detected in NIH 3T3 cell extracts under conditions that readily identified the 36-kDa protein in the 2.0 M NaCl fraction from human Molt3 cell extracts (Fig. 5). Similar results were obtained in four additional experiments with different cell extracts. These results suggest that NIH 3T3 cells do not synthesize appreciable levels of the Tat-binding 36-kDa protein. Analysis of other murine and rodent cell lines is necessary.

## DISCUSSION

We have identified and purified a 36-kDa protein from human cells that strongly binds to the HIV-1 Tat protein. The tight binding and elution only at high NaCl concentrations facilitated purification of the 36-kDa protein. The 36-kDa protein described here is distinctly different from a 46-kDa protein (TBP-1) encoded by a cDNA clone that was isolated from a GT11 fusion protein library on the basis of its ability to bind biotinylated Tat (33). Not only is there a clear difference in molecular masses but there also is no amino acid sequence homology between TBP-1 (33) and two recently sequenced 36-kDa tryptic peptides of 28 and 19 amino acids (unpublished data). Furthermore, a computer search with the sequences of the 36-kDa tryptic peptides reveals that the 36-kDa protein is not encoded by a cellular gene contained in current DNA and protein data bases.

We describe here findings that suggest a role for the 36-kDa protein in Tat transactivation. Rodent cells are deficient in Tat transactivation (31, 32). By a cell microinjection complementation assay we show that the 36-kDa protein increases the efficiency of Tat transactivation in murine and CHO cells by 2.7- to 4.9-fold. Of interest, the presence of human chromosome 12 in human–rodent hybrid cells has been reported to increase Tat-mediated transactivation to about the same extent, 2.3- to 7-fold (31, 32). Experiments to determine whether chromosome 12 encodes the 36-kDa protein are necessary. Although we did not observe a protein of the same size as the 36-kDa protein in murine NIH 3T3 cells

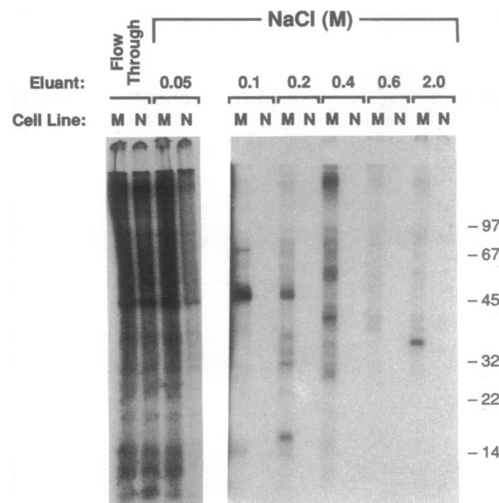


FIG. 5. Comparison of 36-kDa protein levels in human Molt3 (M) and murine NIH 3T3 (N) cells. Extracts of [$^{35}$S]methionine/[$^{35}$S]cysteine-labeled Molt3 cells ($1.4 \times 10^8$ cpm) and NIH 3T3 cells ($1.8 \times 10^8$ cpm) were chromatographed on the same Tat peptide-affinity column, essentially as described in the legend to Fig. 1. All of the eluate from the 0.1–2.0 M fractions, 50 μl of the flow-through, and 250 μl of the 0.05 M fraction were precipitated with trichloroacetic acid/deoxycholate and analyzed by SDS/PAGE on the same gel. The first four lanes were exposed for 8 hr and the remaining lanes were exposed for about 48 hr. Molecular mass protein markers (shown in kDa), as described in the legend to Fig. 2, were run in a parallel lane.

by Tat peptide-affinity chromatography, it will be important to analyze its expression in other murine and rodent cell lines to determine the generality of this observation.

One can only speculate on the function of the 36-kDa protein in Tat transactivation. Diverse reports implicate effects of Tat on transcription initiation, transcription elongation, and translation (for reviews, see refs. 7 and 8). The first step in Tat transactivation probably involves binding Tat through its basic domain to TAR RNA, perhaps in cooperation with the 68-kDa TAR-binding cellular protein (34). It is possible that the 36-kDa protein binds to a Tat domain not involved in TAR binding. Our preliminary findings indicate that the Tat cysteine-rich region and basic region are not essential for binding the 36-kDa protein (data not shown). The formation of a ternary complex could facilitate formation of a transcription complex between TAR-bound Tat and the transcription machinery of the cell. This might facilitate transactivation during second and subsequent rounds of initiation of transcription (7). Alternatively, the complex might facilitate, in some manner, transcription elongation (35). Our preliminary data suggest that the 36-kDa protein does not by itself bind to TAR RNA. Possibly the 36-kDa protein is a general transcription factor or binds to a specific transcription factor involved in HIV-LTR transactivation.

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