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Expression of Human Immunodeficiency Virus Type 1 Tat Proteins in *Escherichia coli* and Application to Study Tat Functions

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The human immunodeficiency virus type 1 (HIV-1), transactivator of transcription (Tat), is one of the viral gene products that is essential for HIV-1 replication. The HIV-1 Tat protein regulates transcription from an HIV-1 long terminal repeat (LTR) and affects the gene expression of cellular proteins during infection. In order to develop an expression system to overexpress and simply purify HIV-1 Tat proteins, the HIV-1 Tat coding sequences that contain one or two exons were amplified using PCR and cloned into a pET vector, which contains a consecutive stretch of six histidine residues at the amino-terminus. The reconstituted vectors were overexpressed in the *E. coli* strain and the soluble recombinant proteins were purified to be homogeneity in a single step by Ni^{+2}-nitrilotriacetic acid Sepharose chromatography under nondenaturing conditions. Recombinant HIV-1 Tat proteins were shown to transactivate the HIV-1 LTR promoter in a dose-dependent manner when introduced into mammalian cells. In addition, treatment of human endothelial cells with purified Tat proteins resulted in a significant increase in the level of vascular cell adhesion molecule-1 (VCAM-1) expression. These results indicate that the recombinant HIV-1 Tat proteins are active in transactivating viral and cellular promoters. The expression and purification system described in this study will facilitate in characterizing the biological functions of the Tat proteins.

Keywords: Expression, HIV-1, Purification, Tat, Transactivation

Introduction

The transactivator of the transcription (Tat) protein of the human immunodeficiency virus type 1 (HIV-1) is one of the viral gene products that are essential for the expression and replication of the viral genome (Arya *et al.*, 1985; Morrow *et al.*, 1994; Sodroski *et al.*, 1986). The 86 amino acids (16 kDa) Tat protein is produced from three doubly or triply spliced mRNAs that are formed by joining one exon preceding the *env* gene with a second exon within *env* (Arya *et al.*, 1985; Sodroski *et al.*, 1985). However, the first coding exon of *tat* is sufficient to direct the synthesis of a fully active, 72 amino acids form (Arya *et al.*, 1985; Muesing *et al.*, 1987).

The mutational analysis of Tat suggests the presence of at least three functional domains: (1) an acidic group of amino acids at the amino terminus, (2) a cysteine-rich cluster consisting of seven cysteine residues, and (3) a positively charged region that is rich in arginine and lysine residues (Rosen, 1992). Mutations in these regions significantly reduce Tat function. The acidic portion of Tat constitutes an activating domain (Ptashne, 1988; Rappaport *et al.*, 1989). The cysteine-rich domain has been shown to function in the formation of a metal-linked Tat dimer (Frankel *et al.*, 1988). The basic domain of Tat is required for both the nuclear and nucleolar localization and for the ability to interact with RNA (Hauber *et al.*, 1989; Ruben *et al.*, 1989).

The target sequence for Tat involves the transactivation response (TAR) element, which is a 59 nucleotide RNA stem-loop and located at the 5' end of all viral mRNAs. Mutational analysis has shown that the TAR function is both position and orientation dependent (Muesing *et al.*, 1987; Rosen *et al.*, 1985). It also requires the presence of an upstream promoter as well as enhancer elements (Cullen *et al.*, 1986).

The HIV-1 Tat protein, which can be secreted from the infected cells, has the ability to enter uninfected cells and exert its activity upon the responsive genes (Ensoli *et al.*, 1993). Previous results indicated that in addition to the HIV-1 promoter, Tat has the capacity to induce transcription of a variety of cellular genes (reviewed by Watson and Edwards,

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1999). Because the Tat protein is readily expressed in insoluble forms in *E. coli.* it has been difficult to obtain sufficient amount of biologically active proteins (Armengaud *et al.*, 1991; Kirsch *et al.*, 1996; Orsini *et al.*, 1996). To establish an expression system to overexpress and simply purify biologically active HIV-1 Tat, expression plasmids containing DNA sequences encoding HIV-1 Tat proteins were constructed, and the recombinant Tat proteins directed with these sequences were efficiently produced in the soluble forms and easily purified from *E. coli* under nondenaturing conditions. These recombinant HIV-1 Tat proteins were shown to be active in the transactivation of the LTR promoter and activation of a cellular gene. That indicates the recombinant HIV-1 Tat proteins are biologically active and useful in the study of HIV-1 Tat function.

Materials and Methods

Expression vectors The HIV-1 *tat* sequence containing the first exon (72 amino acids), or two exons (86 amino acids), was amplified using *Pfu* DNA polymerase (Clontech) from plasmids coding GST-Tat-1(86R) or GST-Tat-1(72R), respectively (Rhim *et al.*, 1994; Rice and Carlotti, 1990). The sense primer was: 5'-CATATGGAGCCAGTAGATCCTAGACTAGAG-3' and antisense primer (72 amino acids): 5'-GGATCCTTATTGCTTTGATAGAG AAGCTTGATG-3' or antisense primer (86 amino acids): 5'-GGATCCCTATTCCTTCGGGCCTGTCGGGTCCCC-3'. The PCR products containing either Tat86 or Tat72 cDNA fragment were subcloned into the *Nde*I-*Bam*HI site of the 6His fusion vector pET15b (Novagen) in frame. Clones with expected insert were selected using *Nde*I-*Bam*HI restriction analysis and analyzed by sequencing. The resulting vectors were designated pTat86 and pTat72, respectively.

Expression of recombinant Tat proteins and analysis of soluble and insoluble fractions The BL21 *E. coli* (Pharmacia) transformed with pTat were grown overnight at 37°C in LB broth that was supplemented with 100 μg/ml ampicillin. The overnight culture was diluted ten-fold with fresh LB media and grown at 37°C with shaking at 260 rpm until $O.D_{600} = 1.0$. Protein expression was induced by the addition of IPTG to a final concentration of 0.1 mM for 5 hr. The cells were pelleted at 1,500 × g for 30 min and resuspended in a 2 × SDS sample buffer in order to prepare the total cell proteins. Crude soluble and insoluble fractions were prepared by the following procedures. The cell pellet was resuspended in a 1/10 culture volume of 50 mM Tris-HCl pH 8.0, 2 mM EDTA. Lysozyme was added to a concentration of 100 μg/ml and then a 1/10 volume of 1% Triton X-100 was added. The mixture was incubated at 30°C for 15 min and sonicated with a microtip to shear DNA. The mixture was centrifuged at 12,000 × g for 15 min at 4°C. An equal volume of 2 × SDS sample buffer was added to the supernatant, which contained soluble proteins. The insoluble fraction was harvested as a pellet and was resuspended in a 1 × SDS sample buffer. Each fraction, along with the total cell proteins, were analyzed on 15% SDS-polyacrylamide gel followed by Coomassie blue staining.

Purification of the recombinant Tat proteins The recombinant HIV-1 Tat protein, which contained six histidine residues at the N terminus was purified by Ni^{+2} affinity chromatography under native conditions (Kim *et al.*, 1999). The cell pellets, which were resuspended in an ice-cold binding buffer containing 5 mM imidazole, 0.5 mM NaCl and 20 mM Tris-HCl, pH 7.9, were lysed by sonication. After removal of cell debris by centrifugation, the resulting cell extract was then loaded onto the Ni^{+2}-IDA column. The column was washed twice with a wash buffer (80 mM imidazole, 0.5 M NaCl, and 20 mM Tris-HCl [pH 7.9]) followed by elution with an elute buffer containing 1 M imidazole, 0.5 M NaCl and 20 mM Tris-HCl pH 7.9. Fractions were collected. The purified Tat protein that was dissolved in 7 mM DTT and 20% glycerol was then aliquoted and stored at -80°C. The protein concentration were determined with a Bradford protein assay (Biorad) by using BSA as a standard.

Immunoblotting Proteins resolved by SDS-polyacrylamide gel electrophoresis (PAGE) were transferred to a nitrocellulose membrane and analyzed by immunoblotting using sera from AIDS patients (dilution 10,000) and a secondary goat anti-human IgG-horseradish peroxidase (HRP) (dilution 10,000). For the reaction of HRP, the bound antibodies were visualized by enhanced chemiluminescence according to the manufacturer's instruction (ECL; Amersham). When the color reaction had reached the desired intensity, the reaction was stopped and photographed (Choi *et al.*, 1993).

HIV-1 LTR transactivation activity of recombinant HIV-1 Tat proteins The HIV-1 LTR transactivation activity of the recombinant HIV-1 Tat proteins was assessed by using a HeLa cell line containing an integrated HIV-1 LTR with a CAT gene. The HeLa cells were treated with various concentrations of Tat proteins in the presence of 100 μM chloroquine for 12 h in a culture media. Cells were harvested for the preparation of cell extracts in order to perform CAT assays. The cell extracts were assayed for CAT activity by a [^{14}C]chloramphenicol-mediated assay (Sodroski *et al.*, 1986).

Analysis of VCAM-1 expression by an immunocytochemistry assay Human umbilical vein endothelial cells (HUVECs) were prepared using an adaptation of a standard technique (Jaffe *et al.*, 1973) with type II collagenase (Worthington Biochemicals, NJ). The HUVECs were plated in a chamber slide at a density of 1.0×10^4 cells/well. Cells were incubated for 24 hours and washed with a phosphate buffer saline (PBS). Subsequently, cells were incubated for 24 hours as a given dose with either Tat or 10 ng/mL TNF-alpha (Boehringer Mannheim).

The Total cellular expression of VCAM-1 in HUVECs was analyzed by an immunocytochemistry assay as follows. The HUVECs on the chamber slide were fixed with a 10% cold formalin for 10 minutes. The cells were washed with a Tris buffered saline (TBS, pH 7.6) and incubated for 15 minutes in a 10% goat serum in TBS at RT in order to block nonspecific binding. A mouse monoclonal anti-human VCAM-1 (dilution 1 : 75, Novocastra Laboratories, Newcastle upon Tyne, UK) in TBS as a primary antibody was sufficiently added to cells on the

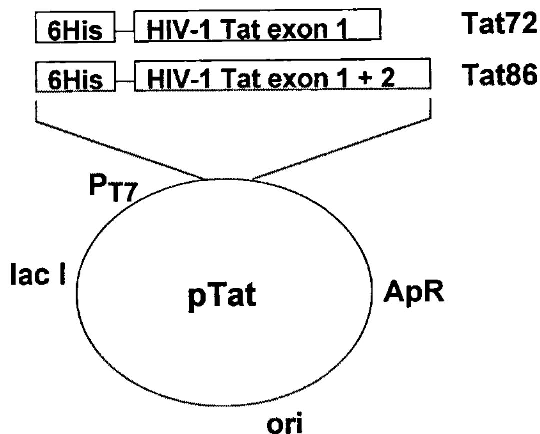

Fig. 1. Schematic diagram of the prokaryotic expression vector pTat containing cDNA fragments for expression of HIV-1 Tat86 or Tat72. The PCR-amplified cDNA fragments containing either Tat86 or Tat72 coding sequences were cloned in frame into pET-15b which contains an N-terminal 6His sequence followed by cloning sites. The resulting vectors were designated pTat86 and pTat72, respectively. The coding frame of the HIV-1 Tat is represented by an open box. ApR, ampicilin resistance gene; ori, plasmid replication origin; lac I, lac repressor expressing gene; PT7, T7 promoter.

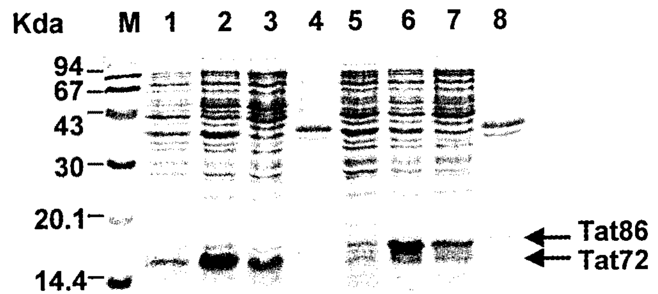

Fig. 2. Determination of solubility of recombinant HIV-1 Tat proteins. *E. coli* BL21 transformed with pTat86 or pTat72 were induced with 0.1 mM IPTG for 5 h. Cell pellets from induced bacterial culture were lysed directly in 50 mM Tris-HCl, pH 8.0 containing lysozyme (100 μg/ml) and fractionated into soluble and insoluble fractions. Bacterial proteins in each fractions were analyzed by 15% polyacrylamide-SDS gel electrophoresis and detected by Coomassie blue staining. Lane M, Molecular weight markers, lane 1 and 5, pre-induction culture of *E. coli* BL21 transformed with pTat86 or pTat72, respectively; lane 2 and 6, expression culture *E. coli* BL21 transformed with pTat86 or pTat72, respectively 5 h after induction with IPTG; lane 3 and 7, supernatant; lane 4 and 8, pellet.

chamber slide and incubated for 1 hour at RT. Alkaline phosphatase conjugate-goat anti-mouse IgG (dilution 1 : 64, Sigma Co.) in TBS was used as a secondary antibody and applied for 1 hour at RT for VCAM-1 staining. Each step was followed by a thorough wash in TBS for 10 minutes. Colorization was performed for 10 minutes with a chromogenic substrate of nitro blue tetrazolium (Sigma Co.) and 5-bromo-4-chloro-3-indoyl phosphate p-toluidine (Sigma Co.) in an alkaline phosphatate buffer (100 mM Tris-HCl, 5 mM $MgCl_2$, pH 9.5). After counterstaining with accustain eosin Y, stained cells were sealed with a coverslip in a mount solution (Biomeda Corp., CA) for light microscopy. Photomicroscopy was performed with an SC35 camera-attached Olympus CH2.

Results and Discussion

Construction and expression of recombinant HIV-1 Tat proteins The HIV-1 Tat gene is separated into two coding exons. The HIV-1 Tat protein composed of 86 amino acids is produced from mRNAs containing two coding exons. The Tat protein composed of 72 amino acids and produced from mRNAs containing the first coding exon. Both forms of Tat have been shown to be capable of directing the transactivation of an HIV-1 LTR promoter (Arya *et al.*, 1985; Muesing *et al.*, 1987). To facilitate the purification, both HIV-1 Tat72 and Tat86 were designed to be expressed as polyhistidine-tagged fusion proteins. To construct expression vectors in ordee to produce both forms of HIV-1 Tat in *E. coli*, cDNAs encoding either one exon or two exons of HIV-1 Tat were amplified and subcloned into pET15b, thereby generating pTat72 and pTat86, respectively (Fig. 1). The N-terminally tagged Tat fusion protein that was encoded from one exon was then designated Tat72. The N-terminally tagged Tat fusion protein that was encoded from two exons was then designated Tat86.

The HIV-1 Tat protein is easily aggregated upon overexpression and purification. This is probably due to the property of oligomerization. Thus, we examined whether or not the recombinant Tat fusion proteins that are expressed by pTat vectors in *E. coli* were synthesized as soluble or insoluble proteins. The *E. coli* that was transformed with pTat86 or pTat72 was induced with IPTG and lysed by sonication. The soluble and insoluble cell fractions from cell lysates were prepared and analyzed by a gel electrophoresis with Coomassie blue staining (Fig. 2). Both forms of the recombinant HIV-1 Tat proteins, Tat72 and Tat86, were found to be present mainly in a soluble fraction following lysis of bacterial cells. The presence of expressed HIV-1 Tat proteins in a soluble fraction will be helpful in purifying and concentrating these proteins under native conditions without denaturants or reducing agents.

The HIV-1 Tat has been functionally divided into three major domains. The first contains the acidic region at the amino terminus, which plays a role in transcriptional activation. The second is the cysteine-rich region, which includes a conserved core domain and functions in the formation of the Tat dimer. The third is the basic region, which is required for both nuclear and nucleolar localization and for binding of Tat to TAR. Both Tat86 and Tat72, which are expressed in this experiment, have these three functional domains.

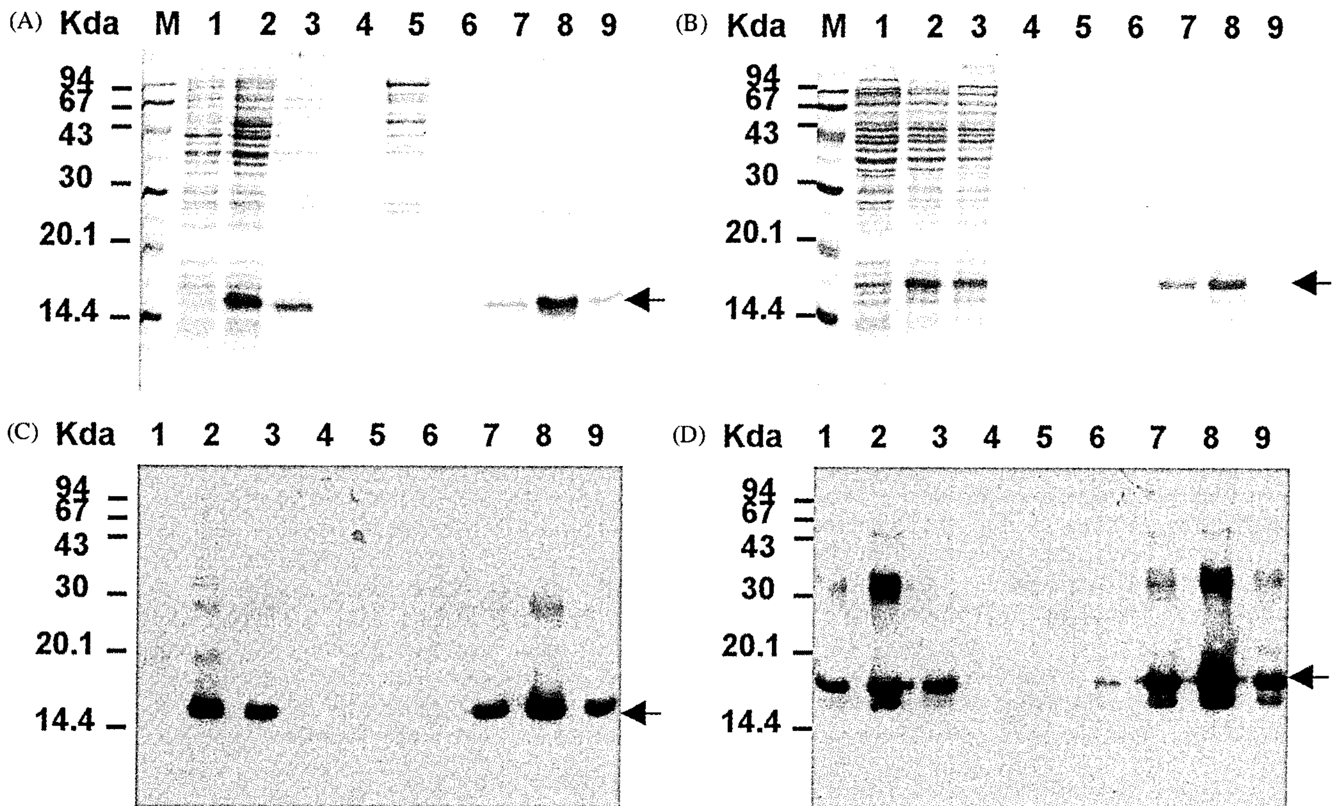

Fig. 3. Analysis of recombinant HIV-1 proteins during the purification steps. *E. coli* BL21 transformed with pTat72(A) or pTat86(B) were induced with 0.1 mM IPTG for 5 h and fractionated to purify the recombinant fusion protein by affinity chromatography on a nickel sepharose column. Aliquots of each fractions were removed from several steps of the purification and analyzed by 15% SDS-polyacrylamide gel electrophoresis and stained with Coomassie blue. In panel A and B, lane 1, expression culture after induction; lane 2, supernatant; lane 3, pellet; lane 4, flow-through fraction from NTA column; lane 5, fraction eluted with binding buffer; lane 6, fraction eluted with washing buffer; lane 7-9, proteins eluted with elution buffer containing 400 mM imidazole. The fractions from each steps of the purification procedure were tested for the presence of HIV-1 specific proteins. Proteins were separated as in Fig. 3A(C) or 3B(D) and then transferred to nitrocellulose membrane. Membranes were incubated with AIDS patient serum as the first antibody and then peroxidase conjugated goat-anti human IgG as the secondary antibody. The blot was developed by ECL method. In panel C and D, lane 1, expression culture after induction; lane 2, supernatant; lane 3, pellet; lane 4, flow-through fraction from NTA column; lane 5, fraction eluted with binding buffer; lane 6, fraction eluted with washing buffer; lane 7-9, proteins eluted with elution buffer containing 400 mM imidazole.

Purification of the recombinant Tat fusion proteins by affinity chromatography To obtain purified recombinant Tat proteins, an 86 residue Tat protein and a 72 residue Tat protein were overexpressed in *E. coli*. Since the recombinant Tat protein was designed to contain six histidines at the amino-terminus, it would be easy to purify recombinant proteins in the soluble fraction of bacterial lysates by metal-chelate affinity chromatography. The induction of the culture was scaled up in volume and the cell lysate was prepared to purify and concentrate the recombinant Tat protein in the soluble fraction. In a single step, recombinant Tat proteins were purified from cell lysates under nondenaturing conditions by Ni^{++} affinity chromatography. Proteins in the fractions from each step of the purification procedure were analyzed by SDS-polyacrylamide gel electophoresis with Coomassie blue staining (Fig. 3A and 3B). The recombinant HIV-1 Tat72 and Tat86 proteins have a calculated molecular mass of 9,784 and 11,000 Da, respectively. However, it was observed that the recombinant Tat proteins migrated to the bands with a higher molecular weight rather than to those of the expected sizes in SDS-PAGE, which is consistent with the previous results (Fenrick *et al.*, 1989; Rice and Carlotti, 1990). The yields of Tat proteins in our experimental conditions were approximately 1 mg per liter culture. The preparation of the Tat proteins was used for further biological experiments.

To evaluate whether or not the HIV-1 Tat proteins expressed in *E. coli* retain immunoreactivity against AIDS patient serum, proteins in the fractions from each step of the purification procedure were separated on SDS-PAGE, transferred to a nitrocellulose membrane, and probed with the AIDS patient serum. Fig. 3C and 3D present the results of a typical immunoblot analyses. The HIV-1 specific proteins were detected on the bands with the expected molecular weight of HIV-1 Tat72 and Tat86. Recombinant Tat that were

proteins evaluated by Coomassie blue staining and immunoblot analysis appeared to be present largely in a monomeric form on denaturing gels, but a fraction of proteins were also detected in oligomeric forms consisting mainly of dimer. This result indicates that the purified recombinant Tat proteins are capable of self-association into the homodimer. Previous studies using HIV-1 Tat protein expressed in *E. coli* demonstrated that the HIV-1 Tat protein binds two metal ions per monomer and that it forms a metal-linked dimer in vitro (Frankel and Pabo, 1988). The cysteine-rich region of the Tat protein was shown to serve as metal ligands, causing Tat to form metal linked dimers. Whether or not the dimer formation plays a role in the function of Tat in infected cells is still unanswered. However, recent work with synthetic HIV-1 Tat protein, which can form multimers including dimers and trimers, suggests that only the monomeric form of exogenous Tat is the relevant functional form acting in cells (Tosi *et al.*, 2000).

Transactivation activity of purified Tat proteins on HIV-1 LTR promoter Previous studies have demonstrated that extracellular HIV-1 Tat protein can be secreted from the infected cells and has the ability to enter uninfected cells freely (Frankel and Fabo, 1988; Ensoli *et al.*, 1993; Mann and Frankel, 1991). A basic domain of Tat protein rich in arginine and lysine residues has been identified to be responsible for this property (Vives *et al.*, 1997). Due to this property, extracellular HIV-1 Tat protein is able to regulate the expression of a variety of cellular genes during infection as well as the HIV-1 promoter. To evaluate whether or not the recombinant Tat fusion proteins that are expressed in this study retain the biological activity, the transactivation activity of Tat proteins on the HIV-1 LTR promoter and the expression of cellular protein, VCAM-1 was examined.

First, in assessing the biological function of purified Tat72 and Tat86 on the HIV-1 LTR promoter, the transcriptional activation of HIV-1 LTR by Tat proteins was evaluated using a HeLa cell line containing the HIV-1 LTR promoter that is linked to a chloramphenicol acetyltransferase (CAT) gene. Varying concentrations of Tat proteins were added into the target cells and incubated for 4 hr in order to introduce the protein into cell monolayers. A monolayer culture of the cells was then removed from the surface of the culture dish with a rubber policeman. Cells were centrifuged, and CAT assays were performed by using [^{14}C]chloramphenicol. The basal level of CAT expression from the LTRCAT in HeLa cells was detected to be very low, while the addition of either recombinant Tat86 or Tat72 proteins into cells increased CAT expression in a dose-dependent manner (Fig. 4A). As graphically demonstrated, the CAT gene expression was markedly increased in cells that treated with Tat86 or Tat72 with similar efficiency (Fig. 4B). These results indicate that both Tat86 and Tat72 proteins can efficiently transactivate HIV-1 LTR promoter and are consistent with the previous reports that the second exon of HIV-1 Tat protein is not essential for transactivation of LTR promoter (Arya *et al.*, 1985; Muesing *et al.*, 1987).

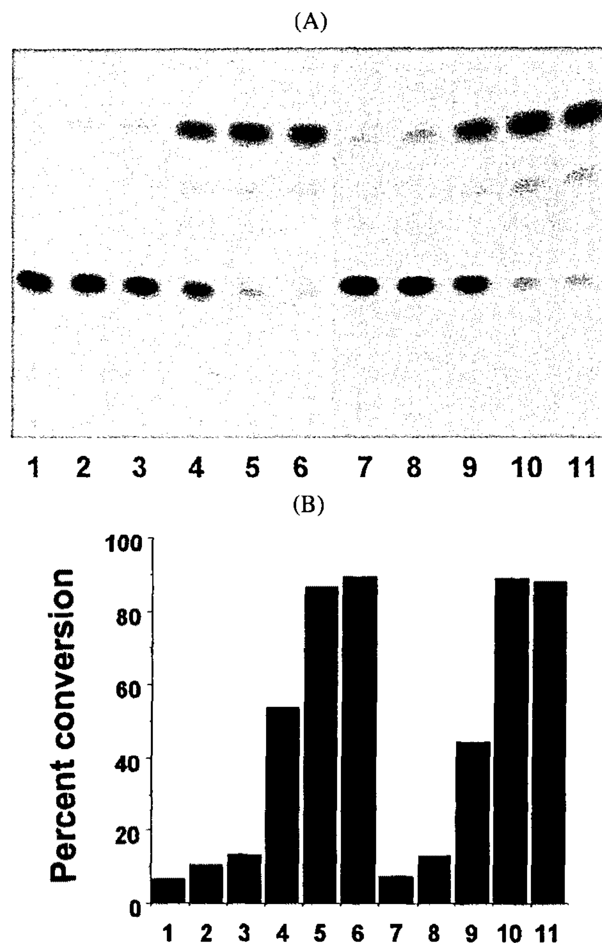

Fig. 4. Transactivation activity of purified Tat protein on HIV-1 LTR transcription. (A) The biological activity of purified Tat protein was evaluated by treatment of Tat protein into the monolayer of a HeLa cell line that contained integrated HIV-1 LTRCAT gene in the presence of 100 μM chloroquine. CAT assays were performed 12 h after addition of protein. (B) Graphical representation of CAT activity activated by Tat relative to basal CAT activity. The percent conversions of [^{14}C]chloramphenicol from results obtained in panel A were determined by Phosphorimager quantitation. Lane 1, no Tat; lanes 2-6, Tat72; lanes 7-11, Tat86; lane 2 and 7, 0.1 μg of Tat/ml; lane 3 and 8, 0.5 μg of Tat/ml; lane 4 and 9, 1 μg of Tat/ml; lane 5 and 10, 10 μg of Tat/ml; lane 6 and 11, 50 μg of Tat/ml. These results are representative of three independent experiments.

Also, among the cellular genes that are known to be affected by the HIV-1 Tat proteins, vascular cell adhesion molecule-1 (VCAM-1) was chosen to assess whether or not the purified HIV-1 Tat proteins are capable of activating expression of this gene. Previous studies have shown that HIV-1 Tat exerts its activity on the expression of various adhesion molecules including the vascular cell adhesion molecule-1 (VCAM-1), endothelial leukocyte adhesion

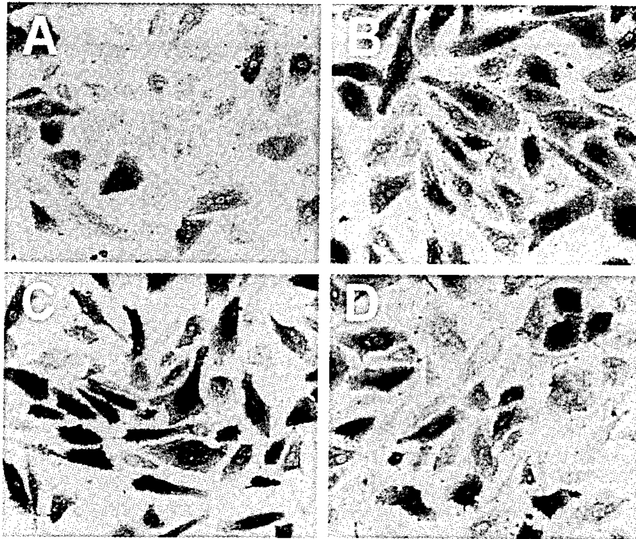

Fig. 5. Effect of recombinant HIV-1 Tat protein on VCAM-1 expression of HUVECs. Confluent HUVECs were incubated in a endothelial cell growth medium and treated with Tat proteins (1 and 5 μg/ml) or 10 ng/mL TNF-α for 24 hr. Expression of VCAM-1 in HUVECs was analyzed by an immunocytochemistry assay using mouse monoclonal anti-human VCAM-1. Magnification × 150. (A) control; (B) 10 ng/mL TNF-α ; (C) 5 μg/ml Tat86; (D) 1 μg/ml Tat86.

molecule-1 (ELAM-1) and the intercellular adhesion molecule-1 (ICAM-1) in human endothelial cells (Dhawan *et al.*, 1997). The expression of VCAM-1 by purified HIV-1 Tat86 and cytokine TNF-α was examined on the cell surface of HUVECs. Treatment of HUVEC with purified HIV-1 Tat86 induced the VCAM-1 expression in a dose-dependent manner (Fig. 5). The expression levels of VCAM-1 in cells treated with purified HIV-1 Tat86 at 5 μg/ml were comparable with that of cells treated with 10 ng/ml TNF-α which is known to induce the expression of VCAM-1. This result shows that the HIV-1 Tat proteins are capable of affecting the expression pattern of cell surface proteins. Induction of cell surface molecules, including VCAM-1 by HIV-1 Tat protein, may play a role in HIV-induced pathogenesis such as the extravasation of HIV-infected cells.

In summary, biologically active recombinant Tat proteins were produced by overexpression in *E. coli* and purified by a single-step Ni^{+2}-nitrilotriacetic acid Sepharose chromatography from the soluble fraction of bacterial extracts under nondenaturing conditions. Purified recombinant HIV-1 Tat proteins were shown to transactivate the HIV-1 promoter and cellular promoter when introduced into mammalian cells.

The HIV-1 Tat proteins were recently shown to serve as a carrier to direct the uptake of heterologous proteins including ovalbumin, beta-galactosidase, horseradish peroxidase into cells (Fawell *et al.*, 1994; Watson and Edwards, 1999). A simple purification system that was developed in our study will facilitate the study of the structural and functional characterization of the HIV-1 Tat proteins. In addition, the recombinant HIV-1 Tat proteins that were obtained in our study can be used as a delivery vehicle of biologically active molecules that are non-permeable into cells.

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