Gene, 150 (1994) 307–311

GENE 08339

Biological activity and intracellular location of the Tat protein of equine infectious anemia virus

(Recombinant protein; EIAV; lentiviruses; *trans*-activation; His tag; pET prokaryotic expression vector)

Rina Rosin-Arbesfeld[a], Pnina Mashiah[a], Dieter Willbold[b], Paul Rosch[b], Steven R. Tronick[c], Abraham Yaniv[a] and Arnona Gazit[a]

[a]*Department of Human Microbiology, Sackler School of Medicine, Tel Aviv University, Tel Aviv 69978, Israel;* [b]*Lehrstuhl fur Struktur und Chemie der Biopolymere, Universität Bayreuth, D-95440 Bayreuth, Germany. Tel. (49-921) 55-3540; and* [c]*Laboratory of Cellular and Molecular Biology, National Cancer Institute, Bethesda, MD, 20892, USA. Tel. (1-301) 496-9683*

Received by A. Kohn: 8 April 1994; Revised/Accepted: 9 May/15 May 1994; Received at publishers: 11 August 1994

SUMMARY

The Tat protein of equine infectious anemia virus (EIAV) was synthesized in *Escherichia coli* using the inducible expression plasmid, pET16b, which contains a His.Tag leader, thus allowing for rapid and efficient enrichment of the histidine-tagged protein by metal affinity chromatography. Yields of up to 20 mg of Tat were obtained from 10^{11} bacterial cells. The recombinant Tat protein was shown to potently *trans*-activate the EIAV long terminal repeat (LTR) following its introduction into canine cells by 'scrape loading'. The EIAV Tat protein was found to localize predominantly within the cytoplasm, in contrast to HIV-1 Tat. The availability of large amounts of purified functional EIAV Tat protein should greatly facilitate detailed structure-function analyses.

INTRODUCTION

Equine infectious anemia virus (EIAV), a lentivirus, is the etiologic agent of a chronic relapsing infectious disease in horses, characterized by symptoms of fever, anemia, glomerulonephritis and uremia (Montelaro et al., 1992). The EIAV genome contains three major ORFs, *gag*, *pol* and *env*, which encode the major viral structural proteins, as well as three additional short ORFs, designated *S1*, *S2* and *S3*, encode Tat (Dorn et al., 1990; Noiman et al., 1990) and Rev (Rosin-Arbesfeld et al., 1993; Stephens et al., 1990), respectively, but the *S2* product has yet to be identified. The Tat protein was shown to activate in *trans* the viral long terminal repeat (LTR) (Sherman et al., 1988; Dorn et al., 1990; Stephens et al., 1990). This activity was dependent on the presence of the Tat-responsive domain (*TAR*) located immediately downstream from the cap site in the viral LTR (Sherman et al., 1988; 1989; Dorn and Derse, 1988; Carvallo and Derse, 1991), which can form a stem-loop structure (Hoffman et al., 1993). Such a structure was first identified in human lentiviral LTRs (reviewed in Cullen, 1992). In addition, sequences within the U3 were also found essential for EIAV Tat activity (Sherman et al., 1989). The Tat protein of EIAV shows significant sequence similarity to the Tat proteins of other primate lentiviruses (Noiman et al.,

Correspondence to: Dr. A. Gazit, Department of Human Microbiology, Sackler School of Medicine, Tel Aviv University, Tel Aviv 69978, Israel. Tel. (972-3) 640-9869; Fax (972-3) 642-2275; e-mail: micro1@ccsg.tau.ac.il

Abbreviations: aa, amino acid(s); bp, base pair(s); Cf2Th, canine thymus cells; CAT, chloramphenicol acetyltransferase; *cat*, gene encoding CAT; ECL, enhanced chemiluminescence; EIAV, equine infectious anemia virus; HIV, human immunodeficiency virus; IPTG, isopropyl-β-D-thiogalactopyranoside; LTR, long terminal repeat; NMR, nuclear magnetic resonance; ORF, open reading frame; PBS, phosphate-buffered saline (0.01 M Na·phosphate/0.14 M NaCl pH 7.3); PCR, polymerase chain reaction; PolIk, Klenow (large) fragment of *E. coli* DNA polymerase I; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide-gel electrophoresis; *TAR*, Tat-responsive element; Tat, Trans-activator protein of HIV or EIAV.

SSDI 0378-1119(94)00550-8

1990; Carroll et al., 1991). However, in contrast to primate Tat proteins (Kuppuswamy et al., 1989), EIAV Tat contains only three functional domains: the conserved core region, the basic region and the C-terminal region; thus, it lacks most of the Cys-rich domain and its N terminus is dispensable for *trans*-activation (Noiman et al., 1991; Carroll et al., 1991). Since vanishingly small amounts of Tat are present in infected cells, structure-function studies would be greatly facilitated by the production of biologically active Tat protein in bacteria. Although functional recombinant HIV Tat proteins have been described (Gentz et al., 1989; Slice et al., 1992; Graeble et al., 1993), the presence of the Cys-rich region has hampered efficient purification (Frankel et al., 1988). The present report describes the efficient production of a recombinant EIAV Tat protein in *E. coli*. This protein was shown to be functionally active when introduced into eukaryotic cells and to localize predominantly in the cytoplasm. The large quantity of pure recombinant, functionally active, Tat protein of EIAV will enable future structure-function studies.

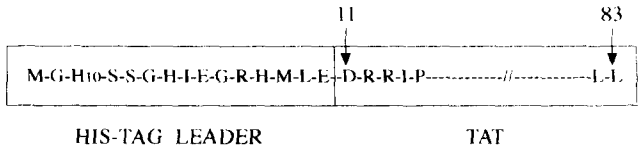

Fig. 1. Schematic presentation of the His-Tat fusion protein. The *E. coli* Tat expression plasmid pET/TAT(s) and the negative control pET/TAT(as), were constructed employing PCR (30 cycles at 94°C for 1 min, annealing at 60°C for 1 min and polymerization at 72°C for 1 min). To amplify the Tat coding sequence, 10 ng of the *tat* cDNA p105 (Noiman et al., 1991) were used as a template. The oligodeoxynucleotides 5′-GGCCGTCGACGGATCCCCGGGACAGCAGAGGAGAACTTA and 5′-GGCCGTCGACGGATCCTGTTCTTACTTATAACAAATATTG served as the sense and anti-sense primers, respectively. *Sal*I-*Bam*HI restriction sites were added to their 5′ ends. Following cleavage of the PCR product with *Sal*I and incomplete filling-in with dCTP and dTTP using PolIk, the PCR *tat* product (aa 15 to 83) was cloned into the *Bam*HI-cleaved pET16b (Novagen, Madison, WI, USA) following incomplete filling-in with dATP and dGTP. Correct orientation of the ligated insert was determined using PCR, in the presence of a sense primer derived from the upstream domain of the pET-16b cloning region and the anti-sense primer detailed above. Fidelity of the PCR product was assured by nt sequence analysis of the recombinant plasmids. The His-Tat fusion protein is shown. It should be noticed that the filling-in and the ligation reaction restored the four aa (11–15) of the Tat ORF, thus enabling the synthesis of aa 11 to 83 of the Tat ORF within the fused His-Tat protein.

EXPERIMENTAL AND DISCUSSION

(a) Expression of bacterially synthesized EIAV Tat protein

The pET system (Novagen), which utilizes the strong T7 transcription and translation signals, was employed (Studier et al., 1990). The 5′ leader sequence of the pET vector used in this study encodes a stretch of several His residues, which is followed by a protease cleavage site. Thus, this system enables affinity chromatography purification and protease-release of the Tat protein.

The 8-kDa Tat protein is encoded by at least three alternatively spliced transcripts (Noiman et al., 1990; 1991; Dorn et al., 1990; Stephens et al., 1990; Schiltz et al., 1992). Although the synthesis of Tat was shown to initiate at a non-AUG start codon residing within the first exon of *tat* transcripts (Dorn et al., 1990; Stephens et al., 1990; Noiman et al., 1991; Carroll and Derse, 1993), the coding region required for activity was localized to residues 38 to 83 of the *tat* ORF which are encoded by the second exon of the *tat* transcripts (Noiman et al., 1991; Derse et al., 1991). In order to express the EIAV Tat protein in pET bacterial expression vector, the *tat* coding region (Fig. 1) was amplified by PCR and the PCR product was inserted in-frame downstream from the Tag leader sequence in pET16b (Novagen), thus resulting in a plasmid designated pET/TAT(s) which would express a fused His-Tat protein. As a negative control, the *tat* ORF was inserted in the opposite orientation, resulting in a construct designated pET/TAT(as). Following transformation of *E. coli*, protein expression was induced by IPTG. To optimize production of the fusion Tat protein in the bacterial cells, aliquots were removed at 0, 90, 150 and 240 min after induction, and proteins were analyzed by SDS-PAGE (Fig. 2). It can be seen that 4 h following induction, the pET/TAT(s) plasmid expressed an approx. 12-kDa protein. This protein was not induced following transfection with the pET/TAT(as). To confirm that the induced protein was encoded by the *tat* ORF of EIAV, Western analysis was performed using an anti Tat serum, prepared against an *S1*-derived oligopeptide (Noiman et al, 1991) (Fig. 2). The size of the 12-kDa band, specifically detected by anti-Tat serum, is consistent with that expected of the fusion protein. Pre-immune serum or serum preadsorbed with the immunizing oligopeptide, did not recognize the Tat-specific band (data not shown). A minor band of approx. 9.5 kDa, which was stained with varying intensity in different preparations (data not shown) is probably a breakdown product of the fusion protein (Weiss et al., 1992).

In order to produce greater amounts of the Tat protein, 100 ml of exponentially growing transformed *E. coli* cells were induced with IPTG. Whole-cell lysates were prepared and the His-Tat fusion protein was purified by affinity chromatography. As demonstrated in Fig. 3, the His-Tat fusion protein could be purified almost to homogeneity by this one step.

Since cleavage of the His.Tag leader using Factor Xa was inefficient, preparative cleavage of the purified His-

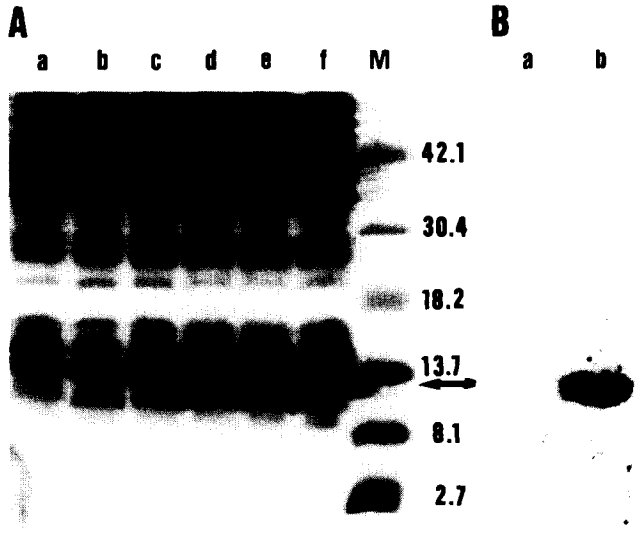

Fig. 2. Induction of EIAV Tat protein in *E. coli*. (**A**) BL21(DE3) cells (Novagen) were transfected with pET/TAT(s) (lanes b to f) or with pET/TAT(as) (lane a) as a negative control. Following transfection, protein synthesis was induced in mid logarithmic cultures of *E. coli*, by the addition of 4 mM IPTG, according to the recommendation of the manufacturer. Following incubation for various periods of time (lane b, 0 min; lane c, 30 min; lane d, 90 min; lane e, 150 min; lanes a and f, 240 min), aliquots of 5×10^9 cells were harvested, lysed and subjected to electrophoresis on 0.1% SDS-15% PAGE. Proteins were stained with Coomassie brilliant blue and compared to prestained molecular mass protein standards (3–43 kDa, BRL; lane M). (**B**) Crude lysates of uninduced (lane a) or IPTG-induced (lane b) cells were electrophoresed and proteins were electrophoretically transferred to nitrocellulose membranes using a Hoefer electroblotting apparatus. The blots were treated with rabbit polyclonal antibody raised against a peptide corresponding to aa 49–63 of the *tat* ORF (Noiman et al., 1991). Proteins were visualized with the enhanced chemiluminescence (ECL) Western blotting system (Amersham). Arrows indicate the Tat protein.

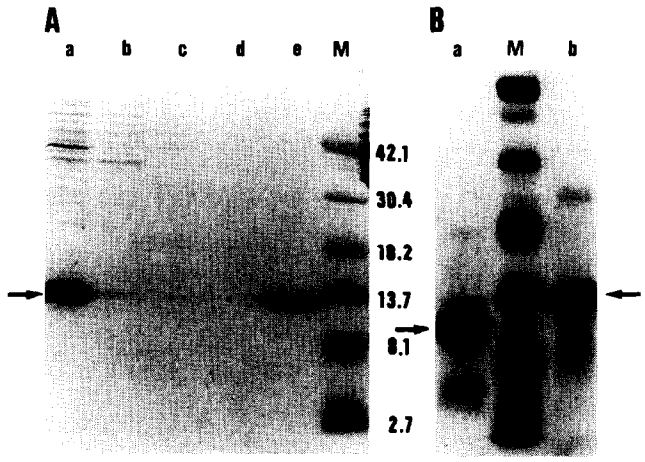

Fig. 3. Affinity purification of the bacterially-synthesized EIAV Tat protein. (**A**) 3 h following transformation of pET/TAT(s) into BL21(DE3) cells, cells were induced with IPTG and 5 h later, 10^{11} cells were harvested and processed essentially as recommended by the supplier, with slight modifications as follows: after pelleting, cells were resuspended in column binding buffer (5 mM imidazole/0.5 M NaCl/6 M urea/20 mM Tris·HCl pH 7.9) and disrupted by sonication. Cell debris was removed by centrifugation at $39\,000 \times g$ for 20 min and the cleared material was filtered through 0.45-μm filters (Millipore). The material (4 ml total volume) was then loaded onto the Novagen's $NiSO_4$-containing His-Bind™ resin. Following two washes (15 ml each), the first one with binding buffer and the second one with slightly increased (20 mM) imidazole concentration, the His-Tat fusion protein was eluted with 15 ml elution buffer (1 M imidazole/0.5 M NaCl/6 M urea/20 mM Tris·HCl, pH 7.9). From each purification step, 30 ml samples were examined on 0.1% SDS-15% PAGE and proteins were revealed by staining with Coomassie brilliant blue. Lane a, initial column load; lane b, column flow-through; lane c, first wash; lane d, second wash; lane e, eluent; lane M – molecular mass protein marker (BRL). The eluent, containing the Tat protein, was freed of urea and imidazole through a PD10 column (Pharmacia) by elution with PBS containing 0.5 mM $MgCl_2$ and 1 mM $CaCl_2$. (**B**) Following CNBr digestion (100 μg CNBr/ml in 0.1 M HCl for 24 h) and lyophilization, the material was redissolved in binding buffer and after adjusting the pH to 7.9 it again was loaded onto Novagen's $NiSO_4$-containing His-Bind™ resin. The column was washed with binding buffer and the flow-through contained the cleaved Tat, whereas the remaining His-tail and the uncleaved His-Tat were still bound to the column. The Tat protein was then desalted as described in **A**. Lane a, cleaved Tat protein; lane b, fused His-Tat protein; M, molecular mass protein markers. The arrows indicate the Tat protein.

tagged Tat was achieved using CNBr. Since the EIAV Tat contains no internal Met residue, cleavage with CNBr took place at a Met residue within the upstream vector sequence (see Fig. 1). From 100 ml of liquid culture (40 mg total protein), approx. 20 mg of highly purified Tat protein were obtained.

(b) Biological activity of the recombinant Tat protein

Although many eukaryotic proteins can be synthesized in bacteria in high amounts, they are often not suitable for biochemical and functional analyses due to incorrect processing or lack of post-translational modifications. In order to investigate the activity of the bacterially-synthesized EIAV Tat protein, we employed the 'scrape-loading' approach (Gentz et al., 1989) to efficiently introduce the purified Tat protein into a canine thymus cell line containing the pEIAV LTR-CAT, in which the *cat* gene was under the control of the EIAV LTR (Sherman et al., 1988). CAT activity was assayed after 48 h, as previously described (Noiman et al., 1991). It can be seen (Fig. 4) that transient transfection of canine thymus cells with pEIAV LTR-CAT plasmid, resulted in a very low basal CAT activity. Addition of purified recombinant Tat to these cells stimulated *cat* expression in a dose-dependent manner up to 24-fold. A synthetic EIAV Tat protein (Willbold et al., 1993) exhibited similar *trans*-activation profiles (data not shown).

(c) Intracellular localization of the EIAV Tat protein

The basic region of HIV-1 Tat contains a nucleolar localization signal which targets Tat to its correct intracellular location (Hauber et al., 1989; Ruben et al., 1989; Subramanian et al., 1990). In order to determine the location of the EIAV Tat, the purified bacterially synthesized

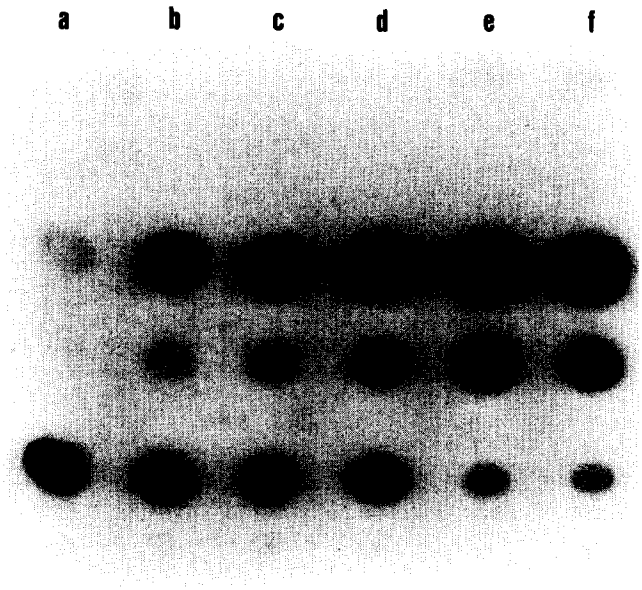

Fig. 4. *Trans*-activation assay of the bacterially synthesized purified His-Tat protein. Semi-confluent canine thymus cells (Cf2Th) (ATCC CRL 1430) were transfected with 5 μg of the reporter plasmid pEIAV LTR-CAT. 48 h later, medium was removed and various amounts of the purified His-Tat in PBS was added: lane a, 0; lane b, 200 ng; lane c, 500 ng; lane d, 1 μg; lane e, 10 μg; lane f, 25 μg. Cells were immediately scraped with a rubber policeman, and resuspended in medium containing 10% fetal calf serum (Gentz et al., 1989). After 48 h incubation, CAT assay was performed as previously described (Sherman et al., 1988). Percent conversions representing the amount of [^{14}C]chloramphenicol converted to acetylated derivatives, are as follows: a, 4%; b, 28%; c, 37%; d, 50%; e, 97%; f, 97%.

EIAV Tat, was iodinated and then scrape-loaded into canine cells. Autoradiography showed that the EIAV Tat was located predominantly in the cytoplasm (Fig. 5a). The same cytoplasmic distribution was demonstrated by introducing a synthetic EIAV Tat protein (Willbold et al., 1993) into canine cells (data not shown). In contrast, the

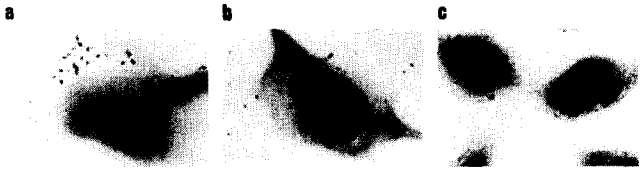

Fig. 5. Subcellular localization of the bacterially-synthesized Tat protein. The bacterially synthesized CNBr-cleaved Tat protein of EIAV (**a**) and a synthetic Tat of HIV1 (**b**), were iodinated employing the chloramine T procedure (McConahey and Dixon, 1980), and introduced by scrape-loading into canine cells which were then grown on Culture Chamber Slides (Nunc). A mock control is shown in panel **c**. After incubation at 37°C for 24 h, cells were washed twice with PBS and then fixed with 2% paraformaldehyde for 10 min at room temperature (RT). Following dehydration steps in 70%, 85% and 100% ethanol (10 min at RT each), slides were air dried and cells were covered with NTB3 emulsion (Kodak). After 9 days of exposure at 4°C, slides were developed using Kodak D-19. Cells were then stained by 0.1% hematoxylene for 5 min and examined under light microscope.

HIV Tat was located both in the nucleus as well as in the cytoplasm (Fig. 5b). The possibility that the cytoplasmic location of the EIAV Tat was due to inefficient renaturation of the protein is unlikely, based on NMR spectroscopy studies which determined the three dimensional structure of both the recombinant, as well as the synthetic EIAV Tat proteins (Sticht et al., 1993; Willbold et al., 1993; 1994), thus suggesting complete renaturation of the recombinant Tat. The distinct patterns of distribution of the EIAV and HIV Tat proteins are consistent with those observed in D17 cells, a canine osteosarcoma cell line (Carroll et al., 1992). The cytoplasmic location of the EIAV Tat protein suggests that the association of the Tat activation domain and a cellular factor may occur in the cytoplasm prior to nuclear import and Tat-*TAR* interaction (Carroll et al., 1992). In this regard it is of interest that the mutation $Arg^{52} \rightarrow Leu$ within the nuclear localization signal of the HIV Tat protein, which drastically impaired its nuclear import, still preserved most of the *trans*-activation capacity (Sadie et al., 1990), suggesting that even low levels of nuclear Tat are sufficient for *trans*-activation to occur.

ACKNOWLEDGEMENTS

We thank Dr. Rainer Frank of the Zentrum fur Molekularbiologie Heidelberg, Germany, for generously providing the synthetic HIV Tat protein. This study was supported by a project grant from the United States-Israel Binational Foundation, and is in partial fulfillment of the requirements for the Ph.D. degree of R.R.-A. from the Sackler School of Medicine at Tel Aviv University.

REFERENCES

Carroll, R., Peterlin, B.M. and Derse, D.: Inhibition of human immunodeficiency virus type 1 Tat activity by coexpression of heterologous *trans*-activators. J. Virol. 66 (1992) 2000–2007.

Carroll, R. and Derse, D.: Translation of equine infectious anemia virus bicistronic *tat-rev* mRNA requires leaky ribosome scanning of the *tat* CTG initiation codon. J. Virol. 67 (1993) 1433–1440.

Carroll, R., Martarano, L. and Derse, D.: Identification of lentivirus Tat domains through generation of equine infectious anemia virus/human immunodeficiency virus type 1 *tat* gene chimeras. J. Virol. 65 (1991) 3460–3467.

Carvalho, M. and Derse, D.: Mutational analysis of the equine infectious anemia virus Tat-responsive element. J. Virol. 65 (1991) 3468–3474.

Cullen, B.R.: Mechanism of action of regulatory proteins encoded by complex retroviruses. Microbiol. Rev. 56 (1992) 375–394.

Derse, D., Carvalho, M., Carroll, R. and Peterlin, B.M.: A minimal lentivirus Tat. J. Virol. 65 (1991) 7012–7015.

Dorn, L. and Derse, C.: *Cis*- and *trans*-acting regulation of gene expression of equine infectious anemia virus. J. Virol. 62 (1988) 3522–3526.

Dorn, L., DaSilva, L., Martarano, L. and Derse, D.: Equine infectious

anemia virus tat: Insights into the structure, function and evolution of lentivirus *trans*-activator proteins. J. Virol. 64 (1990) 1616–1624.

Frankel, A.D., Bredt, D.S. and Pabo, C.O.:Tat protein from immunodeficiency virus forms a metal-linked dimer. Science 240 (1988) 70–73.

Gentz, R., Chen, C.-H. and Rosen, C.A.: Bioassay for *trans*-activation using purified human immunodeficiency virus *tat*-encoded protein; *trans*-activation requires mRNA synthesis. Proc. Natl. Acad. Sci. USA 86 (1989) 821–824.

Graeble, M.A., Churcher, M.J., Lowe, A.D., Gait, M.J. and Karn, J.: Human immunodeficiency virus type 1 transactivator protein, Tat, stimulates transcription read-through of distal terminator sequences in vitro. Proc. Natl. Acad. Sci. USA 90 (1993) 6184–6188.

Hauber, J., Malim, M.H. and Cullen, B.R.: Mutational analysis of the conserved basic domain of human immunodeficiency virus Tat protein. J. Virol. 63 (1989) 1181–1187.

Hoffman, D.W., Colvin, R.A., Garcia-Blanco, M.A. and White, S.W.: Structural features of the *trans*-activation response RNA element of equine infectious anemia virus. Biochemistry 32 (1993) 1096–1104.

Kuppuswamy, M., Subramanian, T., Srinivasan, A. and Chinnadurai, G.: Multiple functional domains of Tat, the *trans*-activator of HIV-1, defined mutational analysis. Nucleic Acids Res. 17 (1989) 3551–3561.

McConahey, P.J. and Dixon, F.J.: Radioiodination of proteins by the use of the chloramine-T method. Methods Enzymol. 70 (1980) 210–213.

Montelaro, R.C., Ball, J.M. and Rushlow, E.E.: Equine retroviruses. In: Levy, J.A. (Ed.), The Retroviridae, Part 2. Plenum Press, New York, NY, 1992, pp. 257–360.

Noiman, S., Gazit, A., Tori, O., Sherman, L., Miki, T., Tronick, S.R. and Yaniv, A.: Identification of sequences encoding the equine infectious anemia virus *tat* gene. Virology 176 (1990) 280–288.

Noiman, S., Yaniv, A., Tsach, T., Miki, T., Tronick, S.R. and Gazit, A.: The Tat protein of equine infectious anemia virus is encoded by at least three types of transcripts. Virology 184 (1991) 521–530.

Rosin-Arbesfeld, R., Rivlin, M., Noiman, S., Mashiah, P., Yaniv, A., Miki, T., Tronick, S.R. and Gazit, A.: Structural and functional characterization of *rev*-like transcripts of equine infectious anemia virus. J. Virol. 67 (1993) 5640–5646.

Ruben, S., Perkin, A., Purcell, R., Joung, K., Sia, R., Burghoff, R., Haseltine, W.A. and Rosen, C.A.: Structural and functional characterization of human immunodeficiency virus Tat protein. J. Virol. 63 (1989) 1–8.

Sadie, M.R., Mukhopadhyaya, R., Benaissa, Z.N., Pavlakis, G.N. and Wong-Staal, F.: Conservative mutations in the putative metal-binding region of the human immonodeficiency virus Tat disrupt virus replication. AIDS Res. Hum. Retrovirus 6 (1990) 1257–1263.

Schiltz, R.L., Shih, D.S., Rasty, S., Montelaro, R. and Rushlow, K.E.: Equine infectious anemia virus gene expression: characterization of the RNA splicing pattern and the protein products encoded by open reading frames S1 and S2. J. Virol. 66 (1992) 3455–3465.

Sherman, L., Gazit, A., Yaniv, A., Kawakami, T., Dahlberg, J.E. and Tronick, S.R.: Localization of sequences responsible for *trans*-activation of the equine infectious anemia virus long terminal repeat. J. Virol. 62 (1988) 120–126.

Sherman, L., Yaniv, A., Lichtman-Pleban, H., Tronick, S.R. and Gazit, A.: Analysis of regulatory elements of the equine infectious virus and caprine arthritis-encephalitis virus long terminal repeats. J. Virol. 63 (1989) 4925–4931.

Slice, L.W., Codner, E., Antelman, D., Holly, M., Wegrzynski, B., Wang, J., Toome, V., Hsu, M.-C. and Nalin, C.M.: Characterization of recombinant HIV-1 Tat and its interaction with TAR RNA. Biochemistry 31 (1992) 12062–12068.

Stephens, R.M., Derse, D. and Rice, N.C.: Cloning and characterization of cDNAs encoding equine infectious anemia virus *tat* and putative *rev* proteins. J. Virol. 64 (1990) 3716–3725.

Sticht, H., Willbold, D., Bayer, P., Ejchart, A., Hermann, F., Rosin-Arbesfeld, R., Gazit, A., Yaniv, A., Frank, R. and Rosch, P.: Equine infectious anemia virus Tat is a predominantly helical protein. Eur. J. Biochem. 218 (1993) 973–976.

Studier, W., Rosenberg, A.H., Dunn, J.J. and Dubendorff, J.W.: Use of T7 RNA polymerase to direct expression of cloned genes. Methods Enzymol. 185 (1990) 60–89.

Subramanian, T., Kuppuswamy, M., Venkatesh, L., Srinivasan, A. and Chinnadurai, G.: Functional substitution of the basic domain of the HIV-1 *trans*-activator, Tat, with the basic domain of the functionally heterologous Rev. Virology 176 (1990) 178–183.

Weiss, S., Konig, B., Morikawa, Y. and Jones, I.: Recombinant HIV-1 nucleocapsid protein p15 produced as a fusion protein with glutathione *S*-transferase in *Escherichia coli* mediates dimerization and enhances reverse transcription of retroviral RNA. Gene 121 (1992) 203–212.

Willbold, D., Kruger, U., Frank, R., Rosin-Abersfeld, R., Gazit, A., Yaniv, A. and Rosch, P.: Sequence-specific resonance assignments of the H-NMR spectra of a synthetic biologically active EIAV Tat protein. Biochemistry 32 (1993) 8439–8445.

Willbold, D., Rosin-Abersfeld, R., Sticht, H., Frank, R. and Rosch, P.: Structure of the equine infectious anemia virus Tat protein. Science 264 (1994) 1584–1587.