# Analysis of Human Retroviral Regulatory Proteins Tax and Tat

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## Introduction

The human retroviruses HTLV-I (human T-cell leukemia virus) and HIV-1 (human immunodeficiency virus) code, in addition to a complete set of replicatory genes (*gag*, *pol*, and *env*), for several regulatory genes including the *trans*-activators *tax* and *tat*, respectively, both of which lead to high expression of viral gene products. The molecular mechanism underlying these events is, however, different for the two proteins.

The Tax protein interacts with a Tax acceptor region (TAR) located in the HTLV-I long erminal repeat (LTR) upstream of the initiation site of RNA synthesis. The TAR harbors three 21-base-pair (bp) repeats, designated Tax response elements (TREs), which contain DNA sequence motifs recognized by a family of proteins such as AP-1, Jun, and cAMP response-element binding protein (CREB). The regions between the three 21-bp repeats comprise 27 and 79 nucleotides and may contribute to Tax function. Tax also trans-activates other cellular genes, presumably by an indirect mechanism. Previous analyses have indicated that Tax binds to DNA in a nonspecific fashion [1] and that several cellular proteins with molecular weights of 32, 36–42, 50, and 110 kDa bind to a single 21-bp repeat on a DNA affinity column [2].

The Tat protein of HIV-1 interacts with a Tat acceptor region (TAR; nucleotides +1 to +80) downstream of the RNA polymerase initiation site. Therefore, it is distinct from all known transcriptional activators. The Tat protein does not bind to this DNA but to the corresponding RNA instead, which can form a hairpin loop structure. Whether the Tat protein binds to this RNA directly or binding is mediated by cellular factors has been the subject of controversy. The Tat protein consists of a characteristic cysteine-rich domain and a highly basic domain with a cluster of six arginines and two lysines [3].

## Materials and Methods

**Prokaryotic Expression of Tat.** For construction of a *Tat*-expressing vector, pEx31B, a derivate of pPLC24 [4] carrying a polylinker between the *Bam* HI and *Hind*III sites, was used. In this vector, the first coding exon of *Tat* was cloned by ligating the *Taq*I-*Ava*II fragment of clone BH10 (nucleotides 5392 to 5706) [5] into the *Bgl* II site of the polylinker. For this purpose, the ends of the fragment were converted to *Bgl*II-sites using linkers (Biolabs, Beverly, USA). In order to express the MS2-Tat fusion protein, the $\lambda$PL promoter of pEX31B is induced by temperature shift of the growing bacteria at an $OD_{600}$ of 1–1.5 from 28° to 42 °C for 2.5 h.

**Cells and Antisera.** C81-66-45 cells [6], Jurkat cells, and Tat-expressing Jurkat cells [7] were cultured in RPMI-1640 with 10% fetal calf serum (FCS). Preparation

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of CREB-specific peptide antiserum (W39 peptide [8]) was performed essentially as described previously [1]. Antiserum against MS2-Tat was raised in rabbits against gel-eluted MS2-Tat fusion protein (Fig. 2A). Antiserum against the carboxy terminus of Tat was raised against a synthetic carboxy-terminal Tat peptide with the sequence Ser-Gln-Pro-Arg-Gly-Asp-Pro-Thr-Gly-Pro-Lys-Glu containing an additional cysteine residue at its amino terminus. The peptide was coupled to keyhole limpet hemocyanin (KLH) via the cysteine, as described previously [1]. Antiserum against β-Gal-Tat was raised in rabbits against a purified β-Gal-Tat fusion protein.

**Indirect Immunoprecipitation.** Indirect immunoprecipitation was carried out as described previously [1]. Metabolic labeling of C81-66-45 cells with [$^{35}$S]methionine was performed as described [1]. HIV-1/H9 cells were labeled metabolically with [$^{35}$S]cysteine (500 μCi ml$^{-1}$ per $10^7$ cells) for 90 min using cysteine-free medium and dialyzed calf serum.

**Immunoaffinity Purification of Tax and CREB Proteins.** The Tax protein was isolated from [$^{35}$S]methionine-labeled C81-66-45 cells according to a previously published procedure [1], the only difference being that the immunoaffinity column was eluted with 6 *M* guanidine HCl containing β-mercaptoethanol 0.1% and bovine serum albumin (BSA) 0.1 mg/ml. The eluate was subsequently dialyzed against 50 m*M* Tris HCl, pH 7.5, 2 m*M* EDTA, 8% glycerol, 2 m*M* dithiothreitol (DTT). For storage, proteins were adjusted to 30% glycerol and 10 m*M* DTT and BSA was supplemented to 0.1 mg/ml. The immunoaffinity column consisted of peptide-specific immunoglobulin against the carboxy terminus of the Tax protein [1]. The CREB protein was purified from $2 \times 10^8$ C81-66-45 cells by immunoaffinity chromatography using a CREB-specific peptide antibody (W39 peptide [8]) and processed as described for the purified Tax protein. Purification of CREB-specific immunoglobulins and the immunoaffinity column set up were essentially as described previously [1].

**Nuclear Extract Preparation and DNA Mobility Shift Assay.** Nuclear extracts from C81-66-45 cells were prepared and depleted from the Tax protein as described previously [2]. DNA mobility shift analysis were performed as described [2], and binding reactions with nuclear extracts contained 0.5 μg poly[d(I-C)] for precompetition of nonspecific binding as previously described [2]. Shift assays in the presence of specific immunoglobulin were performed as follows: nuclear extract or purified protein was preincubated with excess of specific antibody for 15 min before gel shift buffer and, in the case of nuclear extracts, poly[d(I-C)] was added. The binding reaction was initiated by addition of the $^{32}$P-labeled DNA probe.

**In Vitro Transcription.** The TAR RNA (+1 to 200) was transcribed in vitro from a SP6-TAR construct. For radioactive labeling, [α$^{32}$P]UTP was added during the RNA synthesis.

**Protein Preparation and RNA Mobility Shift Assay.** Nuclear extracts ($5 \times 10^8$ cells) were prepared to a final concentration of 0.5–1 μg/μl as described previously [2], except that the extraction buffer contained 500 m*M* NaCl. MS2-Tat protein (0.5 μg/μl) was isolated according to [9]. The MS2-Tat protein partially precipitates during renaturation. Both the soluble and insoluble material were tested for RNA binding. RNA mobility shift assays were performed as described previously [2] in the presence of RNasin and poly(I-C) instead of poly[d(I-C)] for nonspecific competition.

## Results and Discussion

### *Analysis of Tax-TAR DNA Interaction*

The Tax protein was isolated by immunoaffinity chromatography from metabolically labeled C81-66-45 cells, an HTLV-I immortalized human T lymphocyte line which produces Tax but no other viral proteins [1] (Fig. 1 A). Alternatively, nuclear extracts were prepared from C81-66-45 cells as described previously [2] and the Tax protein removed from the extract by use of the identical Tax-specific immunoaffinity column. Presence or absence of Tax protein form the total nuclear extract and the Tax-depleted extract was confirmed by indirect immunoprecipitation analysis using extracts from [$^{35}$S]methionine-labeled cells and Tax-specific antiserum (Fig. 1 A).

To analyze TRE DNA-protein interactions, a gel electrophoresis DNA mobility shift assay was used. A DNA oligonucleotide of 26 bp corresponding to the 5′-TRE, spanning nucleotides − 251 to − 231 of the HTLV-I LTR, was synthesized and radioactively labeled. This $^{32}$P-labeled oligonucleotide was analyzed for DNA-protein interaction with purified proteins and nuclear extracts by separating DNA-protein complexes from unbound oligonucleotide on nondenaturing polyacrylamide gels (Fig. 1 B). The affinity-purified Tax protein (IA-Tax) was unable to bind directly to the TRE DNA and both the Tax-containing and the Tax-depleted nuclear extracts gave rise to identical DNA-protein complexes, irrespective of the presence or absence of Tax protein. The HTLV-I LTR is responsive to cyclic AMP and all three of the 21-bp TREs contain the sequence motif TGACG, which is homologous to the half-site binding sequences of the CREB/ATF binding site TGACGTCA and the Jun/AP-1 binding site TGACTCA.

To determine whether to CREB protein is involved in the complex formed by nuclear extracts and TRE oligonucleotide, CREB-specific immunoglobulin was included in the gel shift assay. This resulted in an additional CREB-specific gel shift complex (marked by the arrowhead in Fig. 1 B). As expected, Tax-specific immunoglobulin did not affect the DNA-protein complex. To confirm both direct binding of CREB protein to the TRE DNA oligonucleotide and the specificity of the additional intermediate formed with CREB-specific immunoglobulins, CREB was purified by immunoaffinity chromatography as described in Materials and Methods. The affinity-purified CREB protein (IA-CREB) gave rise to a specific gel shift complex when analyzed with the TRE DNA oligonucleotide. This had the same migration properties as the complex formed with total nuclear extract proteins and was shifted up when CREB-specific immunoglobulins were added to the binding reaction (Fig. 1 B). The specificity of the DNA-protein complex formation has been confirmed by specific and non-specific competition analyses and mutation of the CREB binding motif in the 21-bp TRE ([2], and data no shown).

### *Analysis of Tat-TAR RNA Interaction*

In order to analyze the effect of the Tat protein on TAR RNA, the Tat protein was expressed as a recombinant protein in *Escherichia coli*. The first of the two coding exons, comprising 72 out of 86 amino acids, was expressed as MS2-Tat fusion protein, which is under the control of a $\lambda$PL promoter and inducible by temperature shift [4] (Fig. 2 A). The MS2-Tat protein was sliced out of the gel, electroeluted, and used as antigen (Fig. 2 A) for the production of polyvalent antibodies. The serum precipitates the authentic Tat protein from HIV-1-infected H9 cells labeled metabolically with [$^{35}$S]cysteine for 90 min (Fig. 2 B). The result shows two bands, one corresponding to both exons and one to one exon only. The presence of the Rev

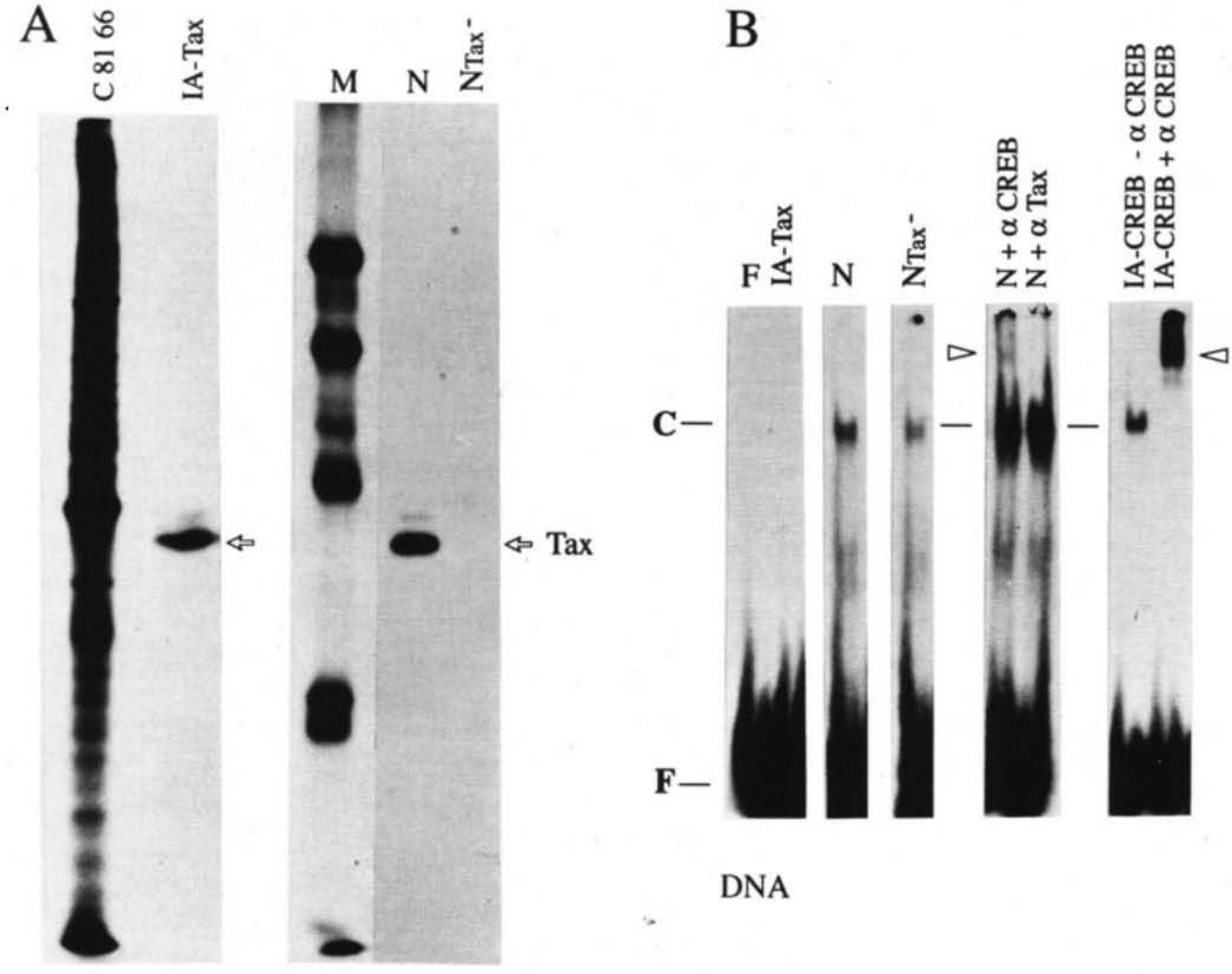


**Fig. 1.** **A** *Left*, Immunoaffinity column purification of the 40-kDa, Tax protein from [$^{35}$S]methionine-labeled C81-66-45 cells. Aliquots of the total cellular lysate and the purified Tax protein, designated *C8166* and *IA-Tax* respectively, were analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography. *Right*, Indirect immunoprecipitation analysis of nuclear extracts. A Tax-specific immunoaffinity column was used to deplete nuclear extracts from [$^{35}$S]methionine-labeled C81-66-45 cells of Tax protein. The total nuclear extract and the Tax-depleted extract, designated *N* and $N_{Tax-}$ respectively, were treated for indirect immunoprecipitation with Tax-specific antiserum. The precipitates were analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography. *M* indicates $^{14}$C-labeled marker proteins, from top to bottom: 92, 68, 53, 45 and 30 kDa. **B** DNA mobility shift analysis, using a $^{32}$P-labeled synthetic DNA oligonucleotide of 26 bp which contains the 5′ 21-bp enhancer motif of the HTLV-I LTR and purified proteins or nuclear extracts. *IA-Tax*, affinity-purified Tax protein; *N*, Tax-containing nuclear extracts $N_{Tax-}$, Tax-depleted nuclear extracts; $N+\alpha CREB$, $N+\alpha Tax$, Tax-containing extracts in the presence of affinity-purified immunoglobulin specific for the cyclic AMP-responsive element binding protein (CREB) and the Tax protein respectively; $IA\text{-}CREB-/+\alpha CREB$, immunoaffinity purified CREB protein in the absence or presence of CREB-specific immunoglobulins; *F*, uncomplexed DNA oligonucleotide; *C*, specific DNA-protein complexes; *Arrowheads*, CREB-DNA complexes shifted by CREB-specific immunoglobulins

protein in the infected cells partially inhibits splicing and thereby leads to synthesis of the smaller Tat protein. An antiserum against a β-Gal-Tat fusion protein, patient serum, and carboxy-terminal synthetic peptide antiserum are shown as controls. The last one allows precipitation of the complete Tat protein, not of the protein corresponding only to the first coding exon (Fig. 2 B).

To study the RNA-Tat interaction, an *in vitro* transcribed RNA corresponding to the TAR RNA region was synthesized. It was labeled radioactively and used for

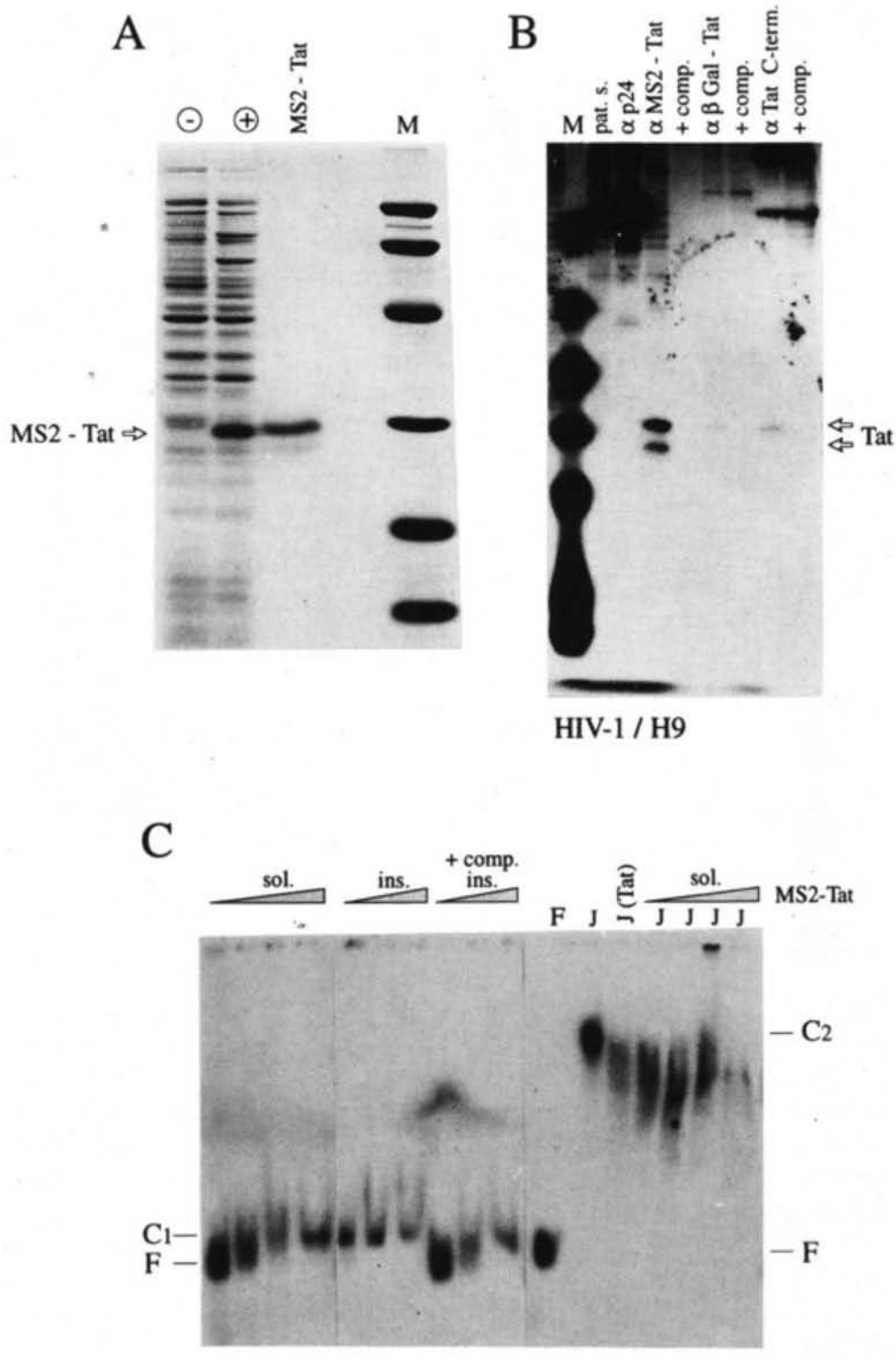


**Fig. 2.** **A** Expression of MS2-Tat fusion protein in *E. coli.* −, and +, bacterial lysates without and with induction of MS2-Tat (see *arrow*); *MS2-Tat*, isolated protein used as antigen; *M*, marker proteins, from top to bottom: 92.5, 66.2, 45, 31, 21.5, and 14.4 kDa. **B** Indirect immunoprecipitation of Tat protein from [$^{35}$S]cysteine metabolically labeled HIV-1-infected H9 cells ($5 \times 10^6$). The sera used were: patient serum (*pat.s.*), antiserum ($\alpha$) against p24, MS2-Tat, $\beta$-Gal-Tat, and a carboxy-terminal Tat peptide serum (*C-term*); *+ comp.*, competition with the respective antigen. *M*, $^{14}$C-labeled marker proteins, from top to bottom: 43, 25.7, 18.4, 14.3, 6.2 and 3 kDa. **C** RNA-protein interaction was analyzed by gel shift analysis using increasing concentrations of soluble (*sol.*) and insoluble (*ins.*) MS2-Tat proteins (ranging from about 1 to 10 µg); *+ comp.*, competition with poly(I-C) (2 µg); *J*, *J*(Tat), nuclear extracts of Jurkat or Tat-expressing Jurkat cells (1 µg each); *F*, free RNA; $C_1, C_2$, protein-RNA complexes

gel shift analysis using purified Tat protein. This was recovered from gel slices, electroeluted, denatured by guanidine HCl, and renatured by dilution, which leads to some soluble Tat protein and some insoluble precipitates. Increasing concentrations of both proteins were analyzed in RNA mobility shift assays and gave rise to shift effects (Fig. 2C). The interaction was competed with the non-specific competitor poly(I-C), and resulted in some resistance of the RNA-Tat complex (Fig. 2C). The MS2-Tat-RNA interaction was not due to the MS2 moiety, which was excluded by using MS2-Orf as control (not shown). The TAR RNA also binds to cellular factors, as evidenced by using nuclear extracts from Jurkat cells which give rise to a gel shift band (Fig. 2C, right). This band was shifted down when the extract from Tat-expressing Jurkat cells was used. Also, addition of increasing amounts of purified soluble Tat protein gave rise to an increased migration velocity, suggesting some effect of the Tat protein on cellular proteins binding to the TAR RNA.

In summary, our data indicate that the Tax protein interacts with its TAR DNA element in concert with other factors, one of them being the CREB protein. The exact mechanism needs to be elucidated. The Tat protein, in contrast, binds to the TAR RNA, not DNA. Again, several host factors can bind to this region as well. The amount of Tat protein required for RNA shifts indicates that its binding is extremely inefficient, which may be due to the high degree of insolubility of the Tat protein or the need for additional host cell factors.

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## References

1. Beimling P, Moelling K (1989) Isolation and characterization of the Tax protein of HTLV-I. Oncogene 4:511–516
2. Beimling P, Moelling K (1990) Tax-independent binding of multiple cellular factors to Tax-response element DNA of HTLV-I. Oncogene 5:361–368
3. Cullen BR (1990) The HIV-1 Tat protein: an RNA sequence-specific processivity factor. Cell 63:655–657
4. Remaut E, Stanssens P, Fiers W (1983) Inducible high level synthesis of mature human fibroblast interferon in *Escherichia coli*. Nucleic Acids Res 11:4677–4688
5. Ratner L, Haseltine W, Patarca R, Livak KJ, Starcich B, Josephs SF, Doran ER, Rafalski JA, Whitehorn EA, Baumeister K, Ivanoff L, Petteway Jr SR, Pearson ML, Lautenberger JA, Papas TS, Ghrayeb J, Chang NT, Gallo RC, Wong-Staal F (1985) Complete nucleotide sequence of the AIDS virus, HTLV-III. Nature 313:277–284
6. Salahuddin SZ, Markham PD, Wong-Staal F, Franchini G, Kalyanaraman VS, Gallo RC (1983) Restricted expression of human T-cell leukemia virus (HTLV) in transformed human umbilical cord blood lymphocytes. Virology 129:51–64
7. Dayton AI, Sodroski JG, Rosen CA, Goh WC, Haseltine WA (1986) The trans-activator gene of the human T-cell lymphotropic virus type III is required for replication. Cell 44:941–947
8. Gonzalez GA, Yamamoto UK, Fischer WH, Karr D, Menzel P, Biggs III W, Vale WW, Montminy RR (1989) A cluster of phosphorylation sites on the cyclic AMP-regulated nuclear factor CREB predicted by its sequence. Nature 337:749–752
9. Hager DA, Burgess RR (1980) Elution of proteins from sodium dodecyl sulfate-polyacrylamide gels, removal of sodium dodecyl sulfate, and renaturation of enzymatic activity: results with sigma subunit of *Escherichia coli* RNA polymerase, wheat germ DNA topoisomerase and other enzymes. Anal Biochem 109:76–86