ISAS - INTERNATIONAL SCHOOL
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TRANSCRIPTIONAL AND NON TRANSCRIPTIONAL PROPERTIES OF THE TAT PROTEIN OF HUMAN IMMUNODEFICIENCY VIRUS TYPE 1

Mudit Tyagi

Thesis submitted for the degree of

Doctor Philosophiae

Supervisor

Prof. Mauro Giacca

Academic Year 1998/1999

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CONTENTS

Synopsis of the Thesis

Human immunodeficiency virus type 1 (HIV-1) is the causative agent of AIDS. In addition to the Gag, Pol and Env proteins, which are common to all retroviruses, the HIV-1 genome also encodes six regulatory proteins, among which Tat plays an essential role in the viral life cycle. This protein is a very powerful transcriptional activator of HIV-1 with unusual molecular properties, including the capacity of being released by infected cells. The results reported in this thesis have been obtained by addressing the study of some transcriptional and non transcriptional features of Tat.

In **Chapter I**, a brief molecular overview of HIV-1 replication is presented, with an emphasis on the regulation of viral transcription by the Tat protein. This small protein directly activates transcription from the viral Long Terminal Repeat (LTR) promoter by interacting with the TAR RNA sequence present at the 5' end of the primary viral transcript. Tat exerts its functions by promoting the recruitment of more processive RNA polymerase II complexes to the LTR and by increasing the rate of transcriptional initiation.

In **Chapter II**, the molecular properties of over 16 different mutants of Tat are described, all of which studied as recombinant proteins fused to GST. In particular, it is shown that amino acid substitutions of the cysteines in the cystein-rich domain, of the arginins in the basic domain, deletion of the N-terminal 21 amino acids and a single point mutations at histidine 13 strongly impair protein function. In the same chapter it is also shown that, through its basic domain, Tat specifically interacts with the CBP/p300 histone acetyltransferases (HAT) proteins, and that it recruits these proteins to the viral promoter to induce remodeling of the chromatin scaffold. These data provide a molecular explanation for the long-standing observation that Tat increases the rate of transcription initiation. Moreover, this is the first evidence suggesting that an RNA targeted transcription factor could act by recruiting a HAT activity to a promoter.

In **Chapter III**, I address the study of the molecular mechanisms involved in Tat intercellular trafficking. The results obtained provide biochemical and genetic evidence that Tat entry into the cells is mediated by cell surface proteoglycan molecules containing heparan sulfate (HS) as the glucosaminoglycan moiety. Cell lines genetically deficient in HS proteoglycans biosynthesis were unable to support the Tat entry. Consistently, the enzymatic removal of HS residues from the cell surface, or cell treatment with soluble heparin, specifically impaired Tat internalization. Interestingly, HS proteoglycans are not required for the release of Tat outside of the expressing cells.

Chapter IV, focuses on the possible role of Tat phosphorylation. It is demonstrated that Tat is an *in vitro* substrate for the nuclear DNA-dependent protein kinase (DNA-PK) which phosphorylates Tat at two specific serine residues. Amino acid substitution at these sites results in reduced transactivation activity of the mutated Tat protein.

Chapter I

GENERAL INTRODUCTION

The retroviral life cycle

All viruses belonging to the Retroviridae family are characterized by an RNA genome that is converted to a cDNA intermediate during the viral life cycle. The RNA genome of retroviruses is relatively small (approximately 10 kb) and contains three essential genes common to all members of the family, *gag*, *pol* and *env*. These genes encode structural proteins and enzymatic functions that are unique to this family of viruses. Retroviral particles are enveloped by a lipid membrane and have a diameter of approximately 100 nm. The *env*-encoded glycoproteins that mediate adsorption to susceptible cells are present at the surface of the viral particle. The virion core consists of several proteins encoded by the *gag* gene and contains the essential *pol* enzymes, protease, reverse transcriptase and integrase. Two copies of full-length genomic RNA are co-packed into a virion core in the form of a dimer.

Retroviral infection of a cell is initiated by the interaction between envelope glycoproteins of the viral particle and host cell receptors. The membrane of the virion fuses with the cellular membrane and subsequently the virion core is released into the cytoplasm. The viral RNA is copied into a double-stranded DNA by viral reverse transcriptase enzyme within this core structure. The core migrates to the nucleus and its DNA is integrated in the host genome at a random position by the viral integrase enzyme. The integrated provirus consequently serves as a template for the production of a messenger RNA by the transcription machinery of the host cell. This mRNA, which encodes viral proteins and functions as genomic RNA, is eventually packed into the virion. Virus particles assemble at the cell membrane and are released by budding, after which they mature by processing of the Gag and Gag-Pol precursor proteins by a viral-encoded protease enzyme.

Human Immunodeficiency Virus type 1 and AIDS

Seven genera of Retroviridae family have been defined on the basis of genome structure and nucleotide sequence relationships. The human immunodeficiency virus type 1 (HIV-1) belongs to the lentivirus genus (for a recent review on HIV-1 classification, see ref. 177). Since the isolation and identification of HIV-1 as the etiologic agent of the acquired immunodeficiency syndrome (AIDS) in the early '80s (22, 108), a remarkable body of knowledge has accumulated about the mechanisms of retroviral infections and evolution of the retroviral diseases. This knowledge has in turn

led to the development of effective therapeutic strategies that have rendered AIDS a more manageable pathology. Nevertheless, the very pathogenetic mechanisms leading from asymptomatic HIV-1 infection to full-blown AIDS are still elusive and the AIDS epidemic has become a major health emergency throughout the world.

The viral genome consists of two RNA molecules of approximately 9.5 kb each, encapsidated in the virion particle. Like all retroviruses, upon infection of susceptible cells, the viral genomic RNA is reverse-transcribed into DNA and subsequently integrated randomly into the host cell genome. The integrated viral genome, or provirus, is flanked by the two long terminal repeat (LTR) sequences that are generated during the process of reverse transcription. The genome of HIV-1 (Fig. 1.1) comprises three major genes, coding for the structural proteins Gag, Pol and Env; two regulatory genes, encoding Tat and Rev proteins; and several other auxiliary genes for Vip, Vpr, Vpu and Nef proteins. For a comprehensive review of the structure and function of these genes, see refs. 282, 290.

The 5' LTR acts as a strongly inducible promoter, originating a single genomic transcript which terminates at the 3' LTR. By multiple alternative splicing mechanisms, more than 30 different RNA molecules arise from the full length genomic transcript (273). In addition to this complex splicing pattern, all the viral proteins are synthesized by extensive use of differential processing events, such as alternative reading frames, ribosomal frame shift, bicistronic RNAs, and proteolytic cleavage of precursors into mature proteins.

Active viral replication in HIV-1 infected patients occurs throughout all the clinical stages of the disease (89, 234, 245) and increasing viral load in plasma has been demonstrated to be a reliable marker for disease progression (17, 45, 214). Most of HIV-1 in plasma is believed to be produced by short lived infected lymphocytes with an estimated half life of just a few days (141, 241, 311). Inhibition of new rounds of infection by combined antiretroviral therapy results in a dramatic decrease in viral load in plasma and lymphoid districts (141, 311). However, this rapid drop is followed by a lower rate of decrease, which has been ascribed to the chronic persistence of latently infected cells (55, 241). In fact, HIV-1 is able to establish a latent infection in resting memory CD4+ lymphocytes, where it can be reactivated by external stimuli acting on the infected cell (101). These lymphocytes may remain for many months in a non-dividing state and thus represent a reservoir for HIV-1 that decays very slowly (212).

The persistence of replication competent provirus in a form susceptible to reactivation is a substantial barrier to viral eradication using the current clinical antiretroviral protocols. Conventional antiretroviral therapy have no effect while the targeted steps in the viral life cycle are suspended, and latently infected cells may escape immune recognition and clearance while viral antigens are not expressed (reviewed in ref. 128).

All the above observations reinforce the notion that transcriptional activation of the HIV-1 provirus represents a key step in disease progression. Therefore, elucidation of the mechanisms that regulate viral gene expression is essential for understanding pathogenesis of the disease.

Regulation of gene expression

Viruses are obligatory parasites and are optimally adapted to use the host cell machinery for the production of new viral particles. This is also true for HIV-1. The HIV-1 LTR promoter contains *cis*-acting regulatory signals that are recognized by the host cell transcription machinery. Thus, HIV-1 transcription is executed by the same enzyme system that expresses cellular genes, which consists of RNA polymerase II (RNA pol II) holoenzyme as the main player. Most of the basic factors required for transcription by RNA pol II have been identified in reconstituted *in vitro* transcription reactions with purified protein fractions, which represents a minimal transcription system. Transcription *in vivo* is much more complex and requires a large number of cofactors in addition to those described below. In brief, at RNA pol II promoters, assembly of the pre-initiation complex at the TATA-box is started by an interaction of the TATA-box binding protein (TBP) with DNA. TBP associates with TBP-associated factors (TAFs) to form the TFIID complex (41). Subsequently, transcription factor TFIIB binds the TFIID-DNA complex and recruits the RNA pol II holoenzyme with its associated TFIIF complex (40). The TFIIE and TFIIH transcription factors also bind to the initiation complex and mediate the melting of the DNA duplex with the help of histone acetyltransferases, around the start site of transcription (144). This open complex allows for the synthesis of the first phosphodiester bond. The TFIIH enzyme is thought to be involved in the subsequent process of promoter clearance, either through its helicase activity (125) or by the phosphorylation of the C-terminal domain (CTD) of RNA pol II by TFIIH-associated CAK kinase (4). Promoter clearance marks the switch from transcription initiation to elongation, and most of the basal transcription factors

leave the elongating RNA pol II complex. General transcription elongation factors, such as P-TEFb, promote efficient transcription and prevent the RNA pol II complex from arresting (154, 255). Transcription termination is coupled to 3' end processing, which involves cleavage of the transcript and poly(A) addition (130). Recent reports indicate that transcription by RNA pol II and pre-mRNA processing are linked in a number of ways, since essential subunits for mRNA capping, splicing and polyadenylation associate with the RNA pol II enzyme during transcription (reviewed in 225).

The HIV-1 promoter

The HIV-1 promoter is located mainly in the U3 region of the LTR and contains a large number of *cis*-acting sequences that control basal and activated transcription. Part of this region also encodes the Nef protein that is essential for viral replication in primary cell types. Transcription starts at the U3/R border, such that the R-U5 sequences forms the 5' end of the viral genomic RNA. The downstream R-U5 region also contains elements that are important for HIV-1 transcription. In particular, one transcriptional motif is active as part of the viral transcript; the extreme 5' end of the messenger RNA folds to a hairpin structure termed TAR (*trans*-acting response) region, that forms the binding site for the viral *trans*-activator of transcription (Tat) protein. The HIV-1 core promoter that maps to sequences -80 to -1 (relative to the start site of transcription), contains a TATA box (position –28 to –24) and three binding sites for the SP1 transcription factor (positions –78 to –45). The TATA box serves as an assembly site for the transcription initiation complex and positions the RNA pol II complex to start transcription at the U3/R border.

The structural organization of the LTR of HIV-1 does not differ from that of the typical eukaryotic promoter. Activation of viral transcription is achieved through the recognition of the 5' LTR promoter by human transacting factors and their interaction with the basal transcriptional machinery. What is remarkable, is the number of cellular factors which bind to *cis*-acting elements present on the LTR and that altogether contribute to the fine modulation of the HIV-1 promoter (for a review, see ref. 114).

From a functional standpoint, the LTR can be divided in three main regions (Fig. 1.2). The basal promoter is composed of three tandem binding sites for the ubiquitously expressed SP1 transcription factor (134, 161), which are located upstream of the TATA

box sequences (111), and an initiator element at the transcription start site, whose functional significance is still controversial (162, 205, 206). These three elements are essential for minimal promoter activity. The enhancer region contains two binding sites for cellular factor NF-κB and is responsible for the strong inducibility of the LTR promoter by a variety of stimuli triggering cell activation and proliferation (84, 219, 288). The region extending upstream of the enhancer exerts an overall negative effect on viral transcription, as demonstrated by deletion studies and hence termed as the negative regulatory element (NRE) (258). Several binding sites for human nuclear proteins were mapped within the NRE, including among the others LEF (172), NFAT (276), c-Myb (71), and AP-1 (106). A sequence of the LTR located in the 3' portion of the NRE contains the hexanucleotide CAC(A/G)TG, which is the consensus *cis*-acting element recognized by proteins of the E-box binding family (69). Members of this family of transcription factors include c-Myc (218), Max (29), Mad (15), TFEB (46), and USF (270). Numerous cooperative interactions among the above mentioned factors have been described. In order to understand their precise role in the HIV-1 life cycle, an investigation of viral replication cycle in different cell types is required.

Transcription factor USF (Upstream Stimulatory Factor), was initially characterized for its ability to bind the upstream element of the Adenovirus major late promoter and activating viral transcription. After purification and cloning, USF turned out to be composed of two equimolarly represented proteins of 43 and 44 kDa, coded by two distinct genes and both were able to bind the consensus CACPuTG E-box. USF has all the structural characteristics of its family members. DNA binding is mediated by a basic region, while the helix-loop-helix and leucine zipper structures at the C-terminal domain of the protein allow dimerization. USF^{43} can form homo and heterodimers with its 44 kDa partner, although the formation of the heterodimer appears to be favored (99). *In vivo* footprinting studies carried out in our laboratory revealed that in infected cells the E-box of the HIV-1 LTR is engaged in protein binding (75). Moreover, USF is the major binding activity detected by *in vitro* binding assays using crude nuclear extracts from several cell types including T-lymphocytes and monocytes (74). The results from our laboratory (81) demonstrated that binding of recombinant USF to its binding site in HIV-1 LTR promoter leads to several folds activation of the LTR which is dependent on the presence of the intact E-box. Depletion of exogenous USF by binding site decoys causes a specific downregulation of viral transcription. These results imply that USF is a positive regulator of LTR-mediated transcriptional activation.

Tat, TAR, and HIV-1 promoter activation

Efficient transactivation of the HIV-1 LTR requires the virally-encoded transactivator protein Tat, which is a 14 kDa protein arising from multi-spliced transcripts and is found to be essential for viral replication (for a comprehensive review, see ref. 163). The two-exon gene of Tat is located in the 3' portion of the viral genome and in most primary isolates codes for a 101 aa-long polypeptide, although certain laboratory strains (derived from the HXB2 clone) produce an apparently fully functional Tat protein of only 86 aa. Tat is a unique *trans*-activator in that it binds to an RNA *cis*-element, termed *trans*-acting response (TAR) element (258), which is located on the 5' untranslated region of all viral RNAs (position +1 to +59 downstream of the transcription start site) (25). TAR RNA folds in a complex three-dimensional hairpin like structure, which is able to recruit Tat to the promoter (Fig. 1.3). The key recognition determinant of the TAR hairpin motif is a three nucleotide bulge present in the stem (82, 133, 260). A large number of cellular proteins are known to recognize these sequences (reviewed in 163). The TAR element is essential for viral viability, both in terms of conservation of the bulge sequence and maintenance of base complementarity in the stem region (175).

In addition to the importance of TAR for the recruitment of Tat protein for transcriptional activation of the provirus, which is discussed below, several old studies also indicated a possible role of this element in the regulation of translation of the HIV-1 mRNAs (31, 32, 65, 237, 275, 302). Specifically, the stable TAR RNA structure was shown to inhibit efficient mRNA translation. This translational block was overcome in the presence of Tat.

Finally, both the 5' and 3' TAR hairpins have also been shown to contribute to optimal packaging of the viral transcripts into the virions (70, 300).

Functional domains of Tat

The Tat proteinof HIV-1 is encoded by two exons and consists of 86 or 101 amino acids depending on the viral isolate. Similar to several other transcriptional activators, Tat has a modular structure consisting of distinct structural and functional domains (Fig. 1.4). Functionally, Tat can be divided into two functional domains, a

transactivation domain and an RNA-binding domain.

The transactivation domain consists of the first 48 amino acids of the protein and can be subdivided into three regions. The N-terminal region of 21 residues contain several acidic amino acids and proline residues and is termed the acidic domain. This region is the least conserved part of the transactivation domain and is predicted to form an amphipathic α-helical structure. Next, the cysteine-rich region (residues 22 to 37) contains 7 highly conserved cysteine residues, of which 6 are critical for Tat functions (267). Residues from 38 to 48 constitute the core region, which is the most conserved and is an essential part of the activation domain. The core domain is known to provide specificity to Tat-TAR interaction (56).

The RNA-binding domain consists of residues from 49 to 58 and includes a stretch of positively charged amino acids that interact with the TAR RNA hairpin that is present at the 5' end of all viral transcripts (309). This motif is also required for the nuclear localization of the protein (176), but can be functionally replaced by a heterologous nucleic acid binding domain (116).

Though the first 58 amino acids are found to be sufficient for the major activities of Tat, the C-terminal part of Tat (residues 59 to 86/101), that is partially encoded by the second exon, contributes marginally to Tat transactivation, and may also contribute to RNA-binding (56). The second coding exon of Tat encodes the C-terminal 14 or 29 amino acid residues depending on the viral isolates. The strong conservation of the second Tat coding exon suggests an important function in the viral life cycle. Several studies proposed specific roles for the second coding exon of Tat, which include transactivation of chromosomally integrated LTR promoters (158), interaction with PKR (213) and downregulation of MHC class I expression (149).

A typical feature of Tat protein is the presence of several cysteine residues in the activation domain. The importance of these cysteine residues has already been demonstrated by several mutational studies (179, 256, 261). Though the primary sequence of this part of the transactivation domain does not predict a classical zinc-finger motif, the interaction of zinc with Tat has been suggested by some studies (104, 176). However, this interaction was found to inhibit Tat functions and it was proposed that the essential cysteine residues of Tat are involved in intramolecular disulfide bridge formation (176).

Due to poor solubility properties of Tat and the presence of readily oxidizing cysteine residues in the activation domain, the biophysical studies of Tat protein were found to be very difficult and so far have revealed little structural detail (23). The core domain and the C-terminal domain of Tat contain stable secondary structure and are surrounded by flexible loops of the cysteine-rich and basic domains.

Mechanisms of Tat transactivation

In the presence of Tat, LTR transcription increases to several hundred folds over its basal levels. How Tat binding to TAR RNA mechanistically affects this amplification has been an arena of active investigation. Several possible steps at which Tat can function include: i) anti-termination of RNA pol II-directed transcription; ii) increased processivity of the RNA pol II complex; iii) augmented formation of transcriptionally competent complexes at the promoter; iv) clearance or transition of initiation complex into elongation complex from the promoter. Out of these four possible functions, clear evidences have been accumulated in the last years that Tat is mostly active at the level of transcriptional initiation by augmenting the rate at which the cellular RNA polymerase II starts transcription, and at the level of transcriptional elongation by increasing the processivity of the RNA polymerase II (64).

Tat and transcriptional elongation.

A number of recent converging observations have clearly indicated that Tat promotes the conversion of the transcriptional complexes formed at the LTR promoter into an elongation competent state. The finding that Tat affects promoter clearance is fully compatible with biochemical evidences that Tat associates with a kinase activity that can phosphorylate the carboxy-terminal domain (CTD) of the large subunit of cellular RNA polymerase (pol) II. This kinase has been originally named TAK (138). It was demonstrated that the requirement of CTD in transcription by RNA pol II is promoter dependent (115). The HIV-1 LTR promoter, unlike for instance the HTLV-1 promoter, was found to be dependent on the integrity of CTD domain for activated transcription (53). The CTD of the mammalian large RNA pol II subunit contains 52 repeats of the Tyr-Ser-Pro-Thr-Ser-Pro-Ser motif. Phosphorylation of the serine, threonine and tyrosine residues in these repeats is correlated with the elongation capacity of the

enzyme (67). It has been shown *In vivo* that paused RNA pol II have predominantly hypophosphorylated CTDs while processively elongating polymerase possess hyperphosphorylated CTDs (228). Thus, one perspective on the mechanism of HIV-1 LTR activation is that Tat recruits TAK to the promoter, which in turn hyperphosphorylates the CTD of RNA pol II docked at the promoter leading to clearance and stable elongation complexes of RNA polymerases. In this respect, several studies using Tat-dependent cell free transcription systems have clearly shown that CTD was extensively phosphorylated in the presence of Tat (170, 171).

It was further shown that the kinase inhibitor staurosporine was able to reduce CTD phosphorylation and Tat-mediated activation of transcription in *in vitro* assays. Interestingly, Tat transactivation of the HIV-1 promoter through TAR was also found to be sensitive to the treatment with purine nucleoside analogue 5,6-dichloro-1-α-D-ribofuranosylbenzimidazole (DRB) (204). In particular, the formation of long transcripts by elongation competent RNA pol II complex was inhibited. DRB, like staurosporine, inhibits the kinase that phosphorylates the RNA pol II CTD. In addition, several other kinase inhibitors were shown to inhibit Tat-induced transactivation (200) . All these studies suggested the involvement of some kinase activity in the transactivation capacity of Tat.

More than half a dozen kinases can phosphorylate RNA pol II-CTD, including DNA-PK (68, 154) - see below. Out of those, in the Tat-induced CTD phosphorylation, the involvement of two protein kinases has already been well established. These are the cyclin-dependent kinases CDK7 and CDK9 that belong to the TFIIH and P-TEFb kinase complexes respectively (Fig. 1.5).

By using Tat column chromatography purification of HeLa nuclear extracts, a protein fraction was isolated with CTD kinase activity. This fraction was shown to contain several TFIIH components and to mediate Tat transactivation *in vitro* (113). The TFIIH complex is involved in an early steps of transcription, which include the formation of initiation complex and/or promoter clearance (125). TFIIH consists of a number of proteins, some of which have enzymatic activity. In addition to two helicase subunits, TFIIH contains a kinase activity, CDK7, that is able to phosphorylate the CTD of RNA pol II (83). Several studies have demonstrated a direct and specific interaction of Tat with CDK7-containing complexes (63, 113, 235). Interestingly, all studies that indicated a role for TFIIH in Tat-induced transactivation describe an effect on transcription

elongation, a step of transcription that has so far not been formally associated with TFIIH activity.

The P-TEFb complex was first identified in *Drosophila* nuclear extract and shown to act as a positive transcription elongation factor in *in vitro* studies (207). This multi-subunit complex contains a kinase activity that is able to phosphorylate the CTD of RNA pol II in a DRB-sensitive manner. Cloning of the kinase subunit of *Drosophila* P-TEFb revealed homology to human PITALRE, a member of the family of cyclin-dependent kinases (333). A separate study also suggested the involvement of PITALRE in Tat-induced transactivation and identified PITALRE as the catalytic subunit of TAK (Tat-associated kinase) (124). In addition, the phosphorylation of the RNA pol II CTD by TAK/CDK9 was also found to be sensitive to DRB. A further link between TAK, Tat and TAR was established by Wei *et al* (310), who demonstrated that cyclin T_1, the cyclin component of the P-TEFb complex, can form a stable ternary complex with Tat and TAR RNA. This complex was shown to enhance the affinity and specificity of the Tat-TAR interaction. cyclin T_1 is believed to interact with Tat through metal ions stabilized by essential cysteine residues found in both proteins (27, 110). One very recent study has confirmed the direct role of P-TEFb in Tat-induced hyperphosphorylation of RNA pol II CTD during transcriptional elongation (154).

The combined results from the above mentioned biochemical and genetic studies imply that Tat functions at a post-initiation step of transcription. These findings also foster the notion that TFIIH and P-TEFb are utilized in a sequential manner to promote phosphorylation and hyperphosphorylation of the RNA pol II CTD, respectively (154).

Tat and transcriptional initiation.

In cell free settings, Tat has a well-characterized and firmly established role in promoting elongation by RNA pol II at a step(s) after the formation of an initiation-competent complex at the promoter. To correlate these *in vitro* findings with *in vivo* conditions, one has to keep several points into considerations. First, *in vitro* transcription conditions do not effectively permit multiple rounds of initiation. Hence, these results do not formally exclude the possibility that Tat does not independently increase initiation events or affects initiation in conjunction with elongation. Second, *in vivo* the HIV-1 LTR exists as an integrated nucleosomally organized DNA segment

(277, 318), while in cell-free studies nucleosomal-free LTR templates are generally utilized.

The proviral LTR in HIV-1 infected cells is strongly silenced by chromatin structure. In particular, the HIV-1 LTR sequence, acting as a very strong promoter when analyzed as naked DNA *in vitro* (235), is almost silent when integrated into the cellular genome in the absence of stimulation. This striking difference is critical to understand the functions of Tat. In fact, Tat activity *in vitro* is generally modest with respect to its *in vivo* activity, and detectable only when the basal level activity of the promoter is down-regulated (203).

Some of the results reported in this thesis (Chapter 2) demonstrate that the strong activating effect of Tat on transcription initiation can be ascribed to its ability to relieve the inhibition imposed on the LTR promoter by the chromatin scaffold.

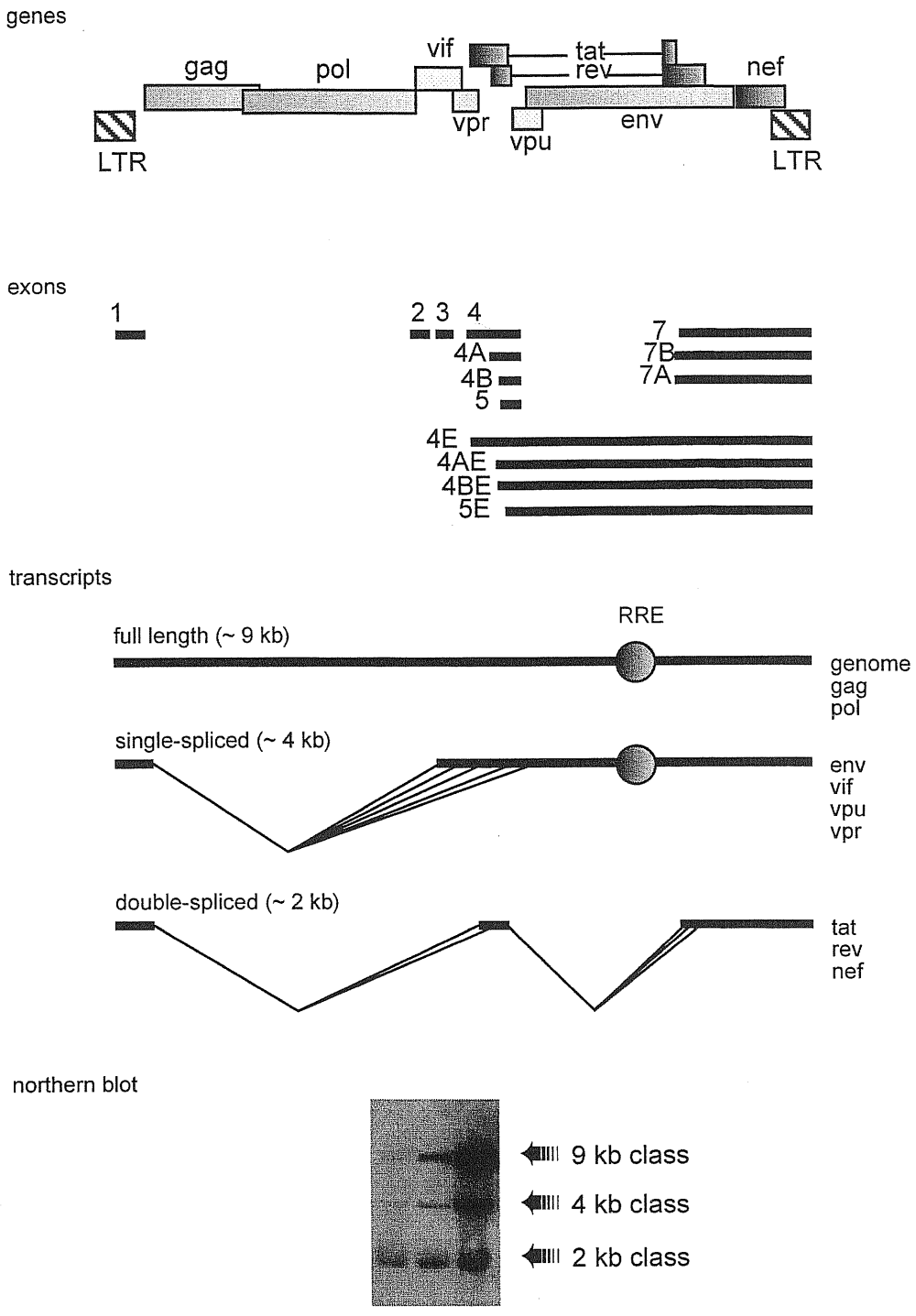


Figure 1.1
Genomic organization and transcripts of HIV-1

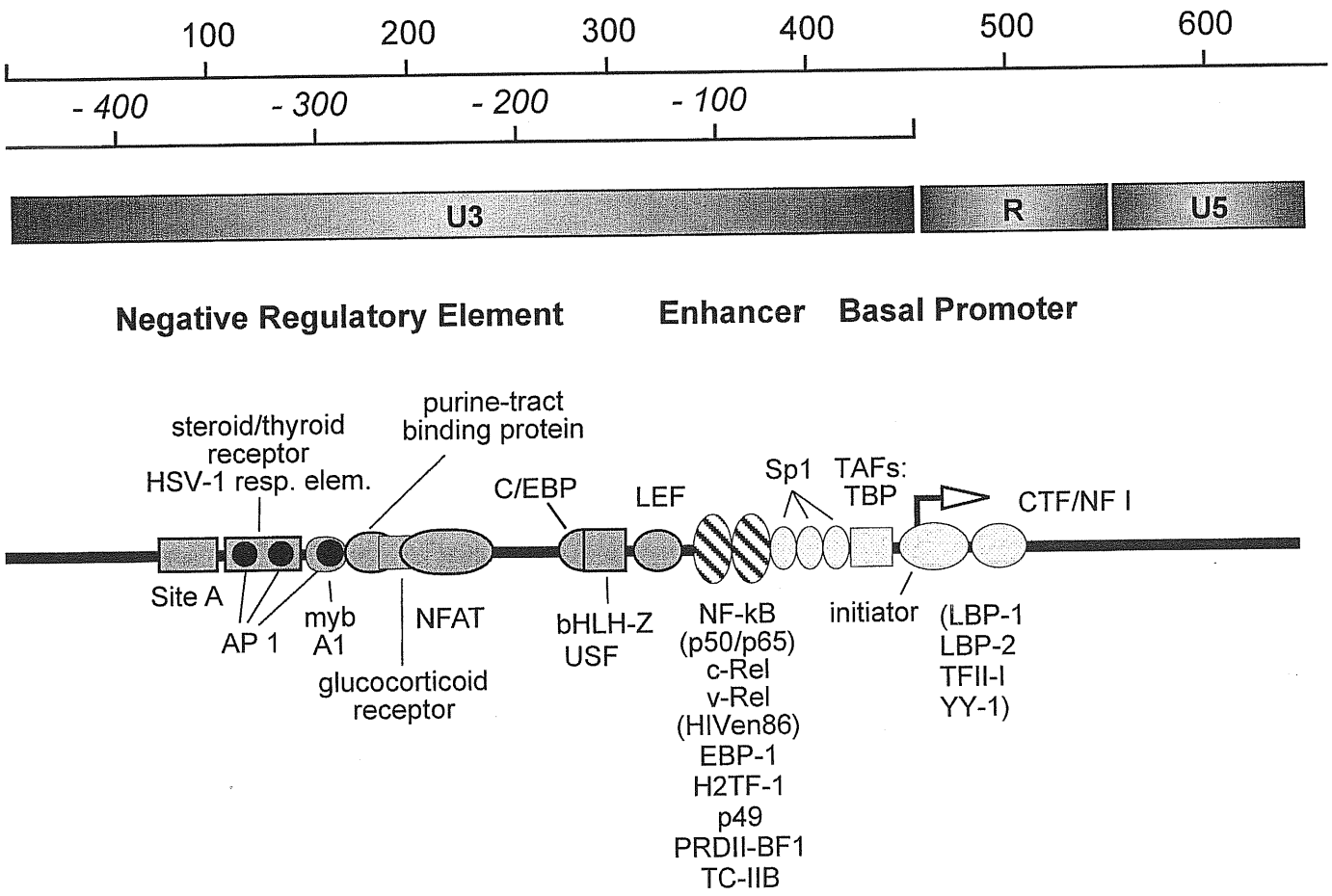


Figure 1.2
Transcriptional domains and protein binding sites at the HIV-1 long terminal repeat

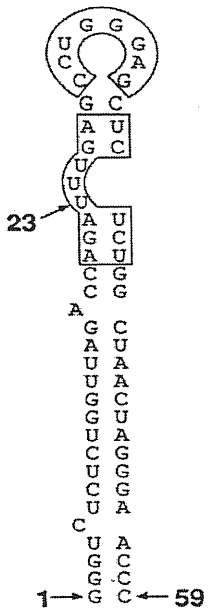


Figure 1.3
Sequence and structure of HIV-1 TAR RNA

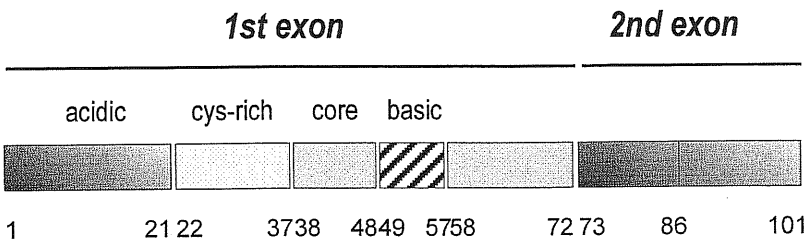


Figure 1.4
Structural domains of HIV-1 Tat protein

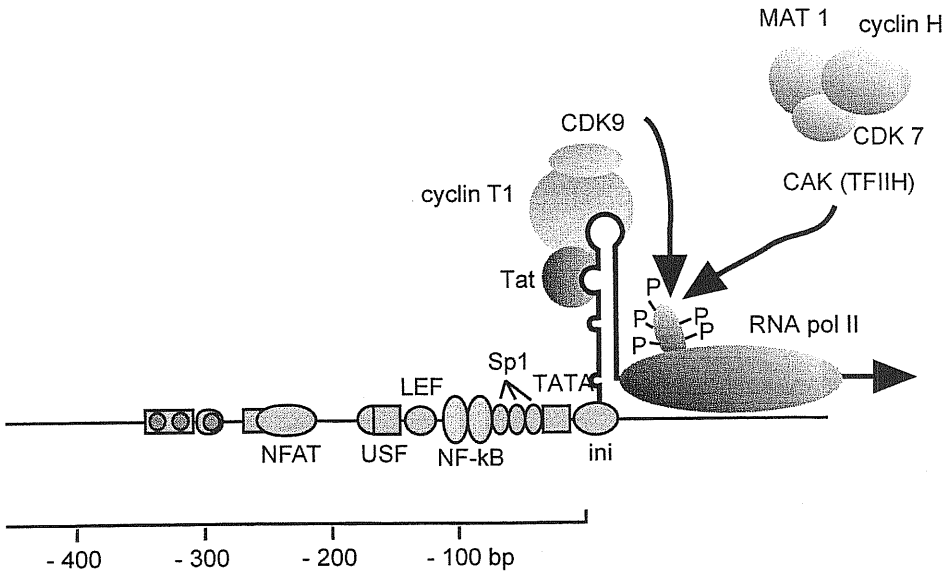


Figure 1.5
Increase of RNA pol II processivity by Tat-associated kinases

HAT	Histones modified	Ref.
hGCN5	H3, H4	Brownell, J.E. et al. (1996) Cell 84, 843-851
P/CAF	H3, H4	Yang, X-J. et al. (1996) Nature 382, 319-324
p300/CBP	H2A, H2B, H3, H4	Bannister, A.J. and Kouzarides, T. (1996) Nature 384, 641-643 Ogryzko, V.V. et al. (1996) Nature 382, 319-324
TAFII250	H3, H4	Mizzen, C.A. et al. (1996) Cell 87, 1261-1270
SRC1	H3, H4	Spencer, T.E. et al. (1997) Nature 389, 194-198
ACTR	H3, H4	Chen, H. et al. (1997) Cell 90, 569-580

Figure 1.6
Known nuclear histone acetyltransferases

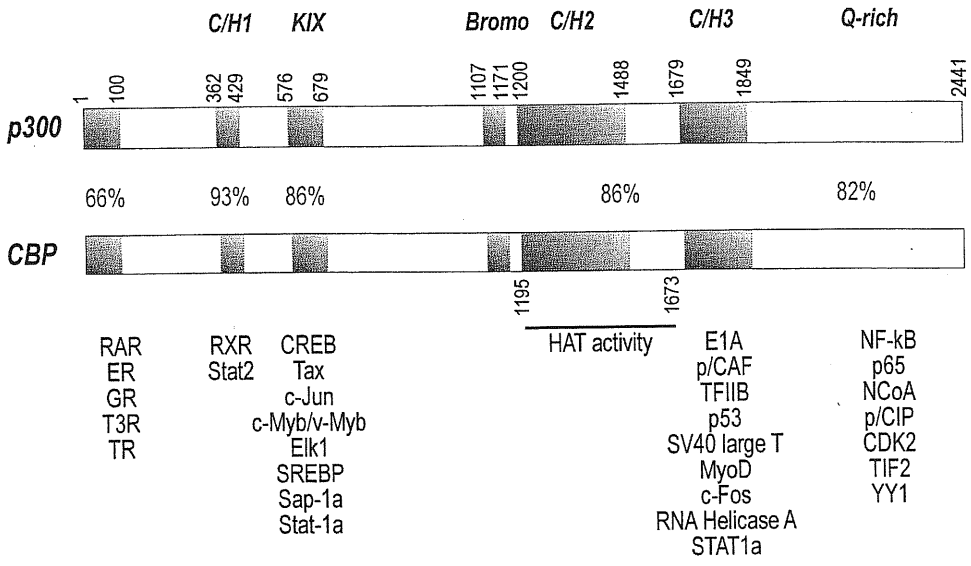


Figure 1.7
Structure of p300 and CBP, degree of homology, and interacting factors

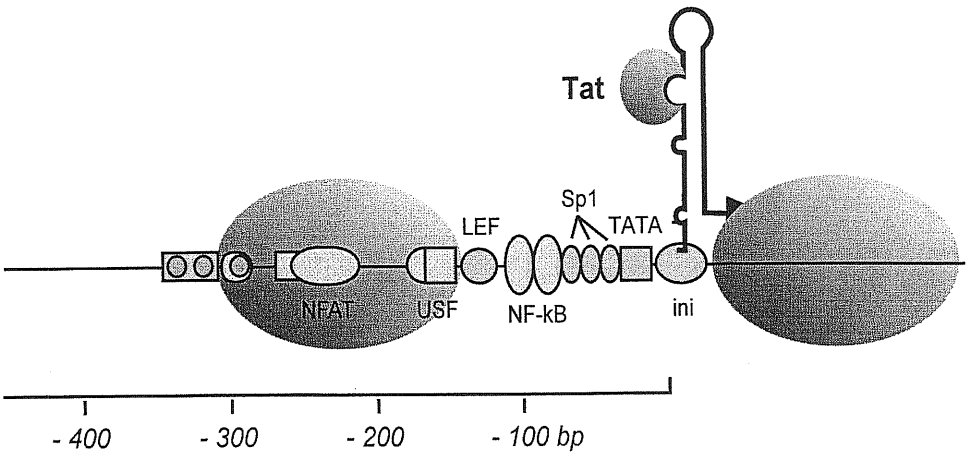


Figure 1.8
Nucleosomes positioned on the integrated HIV-1 LTR

Chapter II

HIV-1 TAT PROTEIN RECRUITS p300/CBP HATs TO THE LTR PROMOTER FOR TRANSACTIVATION

II - INTRODUCTION

Chromatin modulation and HIV-1 LTR transactivation

In the cell nucleus, the eukaryotic genome is packaged into a highly condensed chromatin structure. The fundamental subunit of chromatin is the nucleosome core formed by the wrapping of DNA around an octamer of core histone proteins. Nucleosomes negatively regulate gene expression by restricting access to DNA-binding factors and by impeding elongation by RNA polymerase II (236, 292, 296, 320).

A central question in eukaryotic transcription is how the transcriptional machinery is able to gain access to specific loci tightly packed in chromatin. One way to gain access to histone-packaged promoters is through the acetylation of histone tails. Acetylation of histones is associated with transcriptionally active chromatin and is thought to weaken the histone-DNA interactions, thereby relieving the repressive effects of the chromatin scaffold (279, 305). The molecular mechanism by which acetylation of histones contributes to transcriptional activation are not fully understood. The neutralization of the positively charged lysine residues within histone tails by acetylation was reported to decrease the affinity of the histone H4 for DNA (146) and alter the conformation of nucleosomes *in vitro* (226). Crystal structure studies suggest that histone tails may also be involved in contacts between adjacent nucleosomes and thus play a role in the formation of higher order chromatin structure (194). Currently, several transcription co-factors, including p300, CBP, PCAF, and TAF250, have been shown to possess histone acetyltransferase (HAT) activities (reviewed in 28). Interestingly, they do not share a common domain responsible for their HAT activity and show different histone substrate specificity (Fig. 1.6). In particular, p300 and CBP are the only human nuclear HATs so far identified that are able to acetylate all the four core histones (Fig. 1.6).

P300 and the closely related CBP proteins are two large polypeptides with a molecular weight of approximately 300 kDa. They are very conserved from Drosophila to mammals and share a high degree of homology at the amino acid level, especially in the domains important for their enzymatic and protein-recognition properties (Fig. 1.7). P300/CBP were originally identified as transcriptional coactivators (52, 87). In fact, although not able to directly bind to a specific DNA sequence, they act as bridging units between a variety of cellular and viral transcription factors and the general

transcriptional machinery (123, 157). Because of their similarity and the fact that both bind the same cellular targets, they are often referred to as p300/CBP. However, recent results suggest that p300 and CBP might play rather different functions in the cell in spite of their shared partner proteins (168, 327). In addition to their HAT activity, p300/CBP are involved in a variety of crucial cellular functions, ranging from signaling pathways to cell growth, differentiation and apoptosis, to embryonic development (for a comprehensive review, see refs. 123, 168).

In cells infected with HIV type 1 (HIV-1), like any other cellular gene, the HIV-1 provirus is also incorporated into a highly compacted chromatin structure (298, 299) (Fig. 1.8). In particular, the viral long terminal repeat (LTR), which acts as a very strong promoter when analyzed as naked DNA *in vitro* (see, for example, ref. 235), is almost silent when integrated into the cellular genome in the absence of stimulation (159, 247). Nuclease accessibility studies of the proviral chromatin structure indicated that the LTR, independently of the integration site, is incorporated into two distinct nucleosomes, separated by a nuclease-hypersensitivity region containing the enhancer and basal promoter elements (280, 298, 299). Access to these protein-packaged LTRs integrated into chrosomes is likely to be a rate limiting step in Tat-mediated transcriptional activation (158). Through genomic footprinting studies in our laboratory, we have shown that in the silent LTR, several critical protein-DNA interactions are still preserved in this region (73, 74).

Although, a large body of evidence is available that elucidates the functions of Tat in transcriptional processivity, some important questions are yet to be answered. In fact, it remains to be explained how Tat relieves the block in transcriptional initiation imposed on the LTR by chromatin. When transcription is activated, the chromatin associated with sequences immediately downstream of the transcription start site becomes accessible to nucleases (299). In particular, remodeling of the chromatin structure can be induced by Tat, but not by other stimuli acting through the upstream enhancer sequence (88). Chromatin remodeling associated with activation of transcription is generally accomplished by reversible acetylation of lysine residues in the amino-terminal domains of core histones H2A, H2B, H3 and H4. This modification weakens histone-DNA interactions, thereby relieving the repressive effects of the chromatin scaffold (for reviews, see refs. 279, 305). A growing body of evidence indicates that the presence of nucleosomes on the viral promoter is likely to play an important role in the

regulation of HIV-1 transcription (88, 297). Consistently, the silent integrated LTR can also be strongly activated by drugs inducing sustained high levels of histone acetylation in latently infected cell lines (183, 184, 297).

Altogether, these observations strongly suggest that histone acetylation at the LTR promoter plays a key role in the activation of HIV transcription. We therefore have explored the possibility that the function of Tat in transcriptional initiation could be ascribed to the recruitment of HAT proteins to the viral promoter. Some of the results presented in this thesis demonstrate that Tat associates both *in vitro* and *in vivo* with p300 and with the closely related CREB-binding protein (CBP) HATs and targets them to the integrated LTR promoter. Overexpression of p300 both in human and in rodent cells increases Tat-mediated transactivation of the integrated LTR promoter.

II - RESULTS

Construction and purification of recombinant wt Tat and mutants

To perform a number of experiments presented in this thesis, I took advantage of the properties of several wild type (wt) recombinant GST-Tat proteins expressed in E. coli and purified to homogeneity on agarose-glutathione beads. In addition, I also constructed several mutated derivatives of Tat. A comprehensive scheme of all the 16 proteins obtained is presented in Figure 2.1. These constructs were assayed for a number of biological properties in the course of my work as well as during the work of other colleagues in the laboratory. The main results of these studies are summarized as follows.

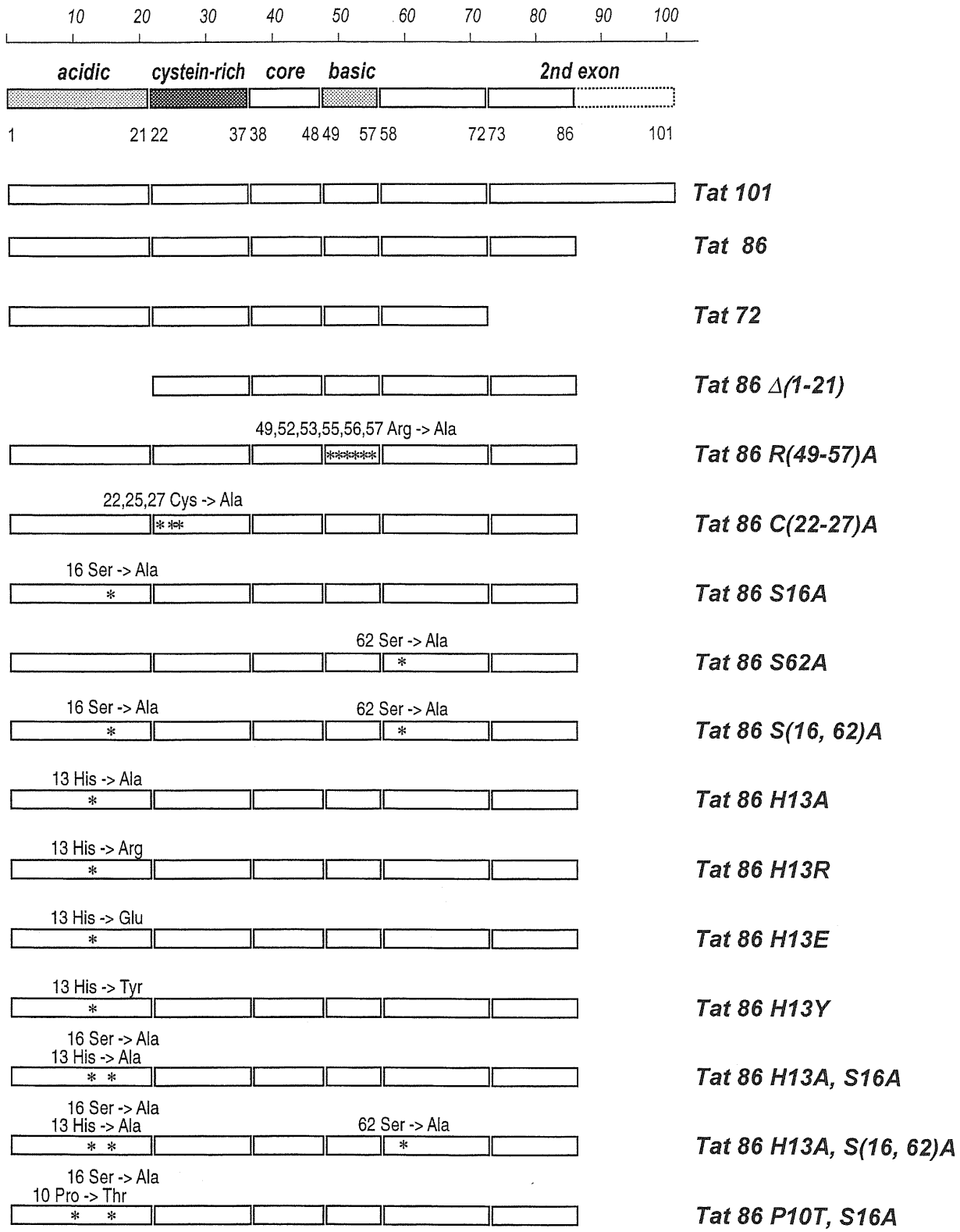
Wild type Tat protein of 101 amino acids (present in most clinical strains), of 86 amino acids (HXB2 clone), and one exon Tat (Tat 72) have comparable activities as far as transactivation is concerned. This conclusion is achieved by both transfection studies using mammalian expression vectors and by addition of the recombinant proteins to the cell culture medium of HL3T1 cells in the presence of 100 μM chloroquine followed by CAT assay as described (see Experimental procedures and below). The mutants containing deletion of the first 21 amino acids (Tat 86 Δ (1-21), mutations of 6 arginines in the basic domain (Tat 86 R(49-57)A), and mutations of cysteins at positions 22, 25 and 27 (Tat 86 C(22-27)A) were completely inactive for transactivation in both types of assays (Fig. 2.2).

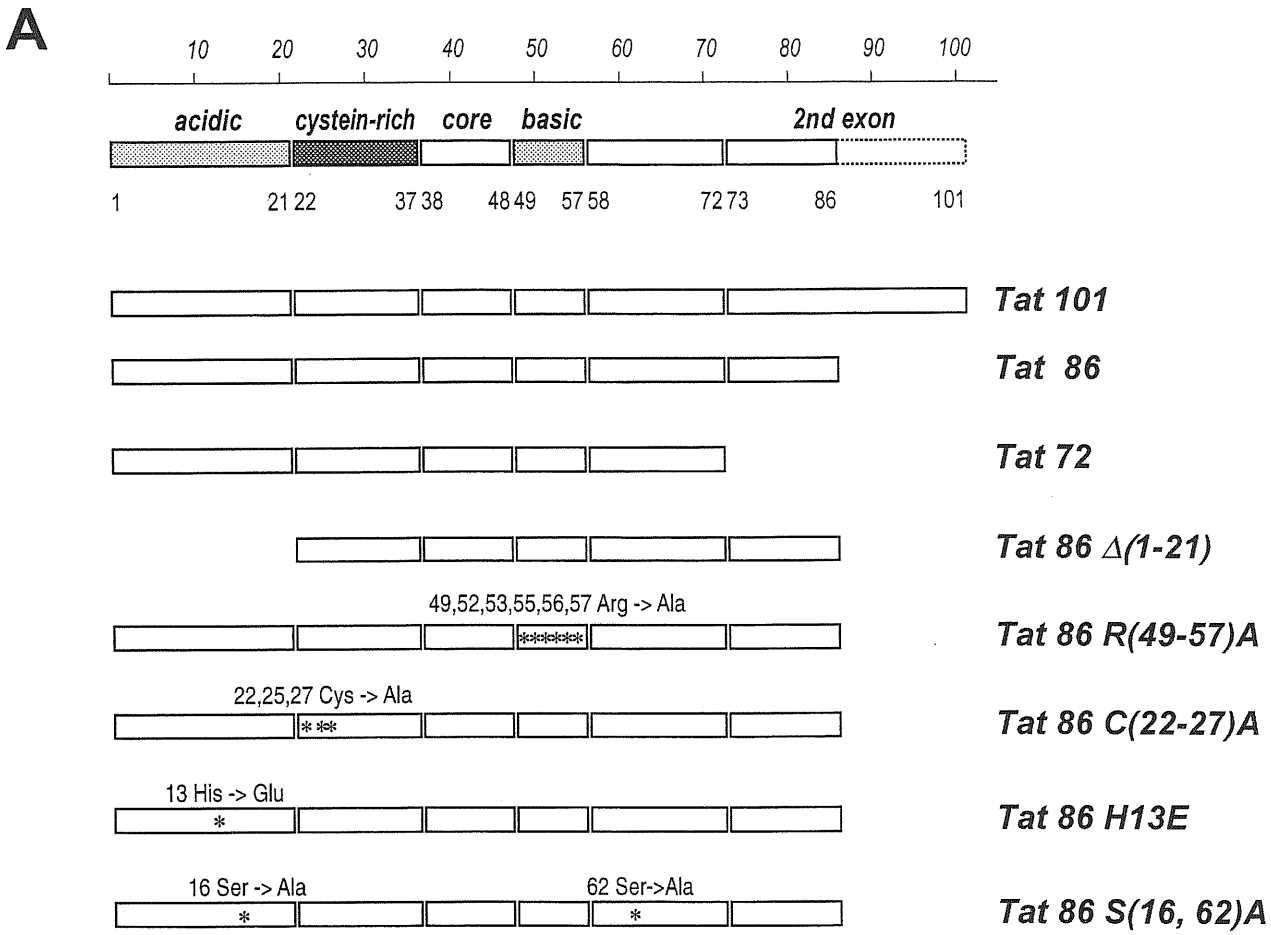
All the recombinant proteins are fully proficient both for cellular internalization and for TAR RNA binding, with the exception of mutant Tat 86 R(49-57)A, which is deficient for both activities (not shown).

Other specific properties of some of these mutants will be described in the following specific chapters.

In the course of this study, it was noticed that the mutation of the histidine residue present at position 13 within the amino-terminal region of Tat strongly impairs transcriptional activation. Mutations were introduced at this amino acid by site-directed mutagenesis to alanine (neutral), glutamic acid (acidic), arginine (basic) or tyrosine (aromatic) amino acid residues. The corresponding proteins were obtained as GST-fusions and purified by agarose-glutathione beads (Fig. 2.3). All the four proteins were

Figure 2.1
Schematic representation of Tat mutants





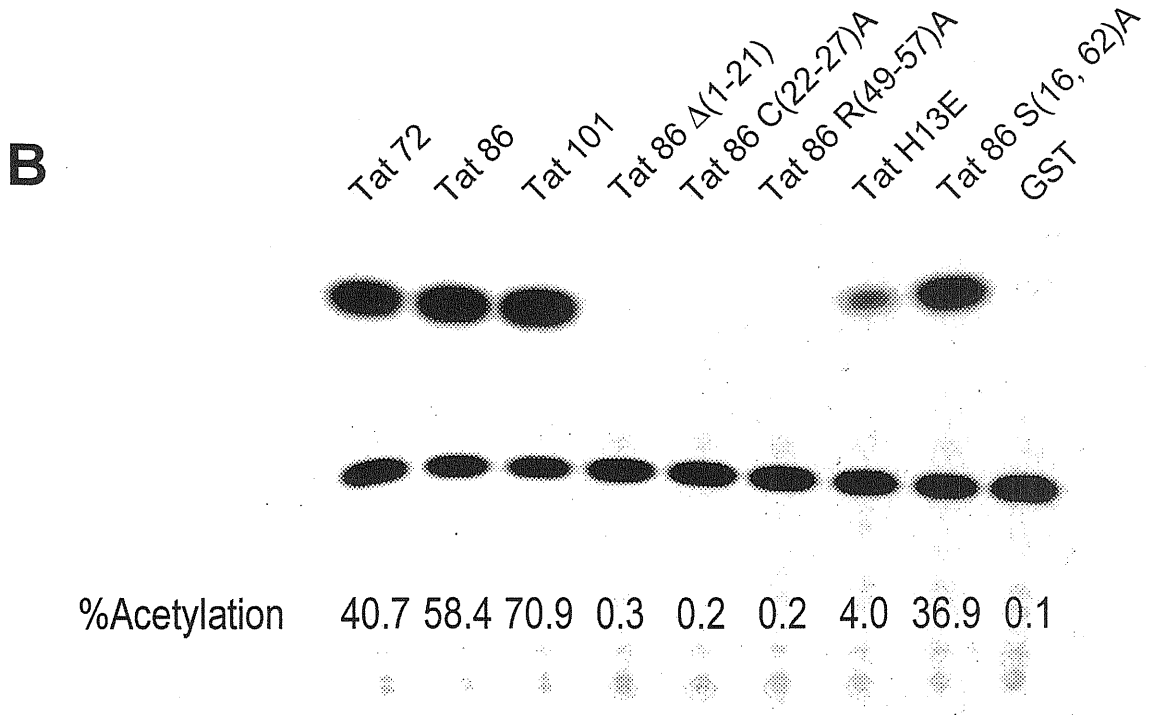


Figure 2.2
Transcriptional activation of the LTR by selected recombinant Tat mutants

A. Schematic representation of the domain structure of the different types of wild type HIV-1 Tat proteins (consisting 101, 86 or 72 amino acids) and of the Tat mutants, the deletions and point mutatuions are indicated.

B. LTR-transactivation activity of the various mutants was assayed in HL3T1 cells. GST-Tat constructs were supplied to the cells in the cell-culture medium along with chloroquine and their transactivation activity was determined by CAT assay, see the Experimental Procedures for details.

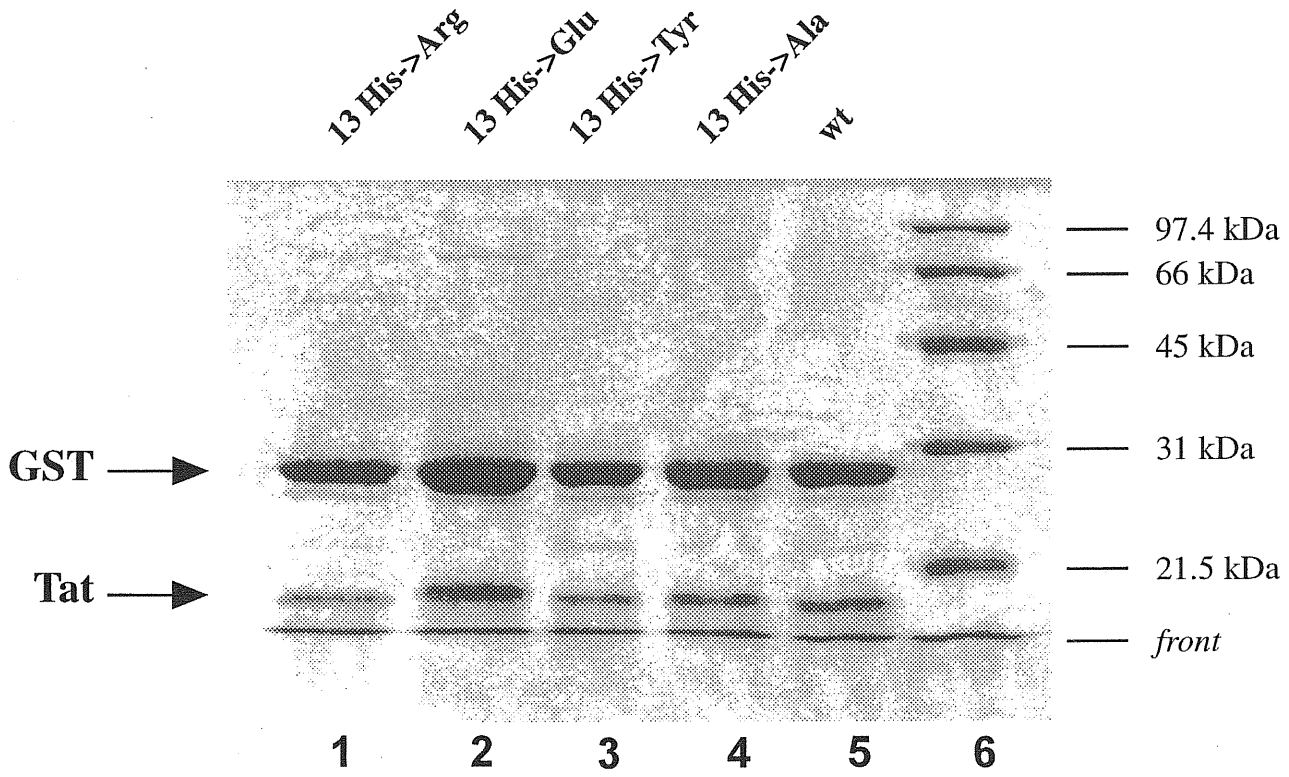


Figure 2.3
Production of recombinant Tat 13 mutants

Aaffinity purified GST-fusion proteins were digested with thrombin, as discribed in Experimental Procedures. The proteins were resolved on a 10 % SDS-PAGE along with protein molecular weight markers.

Lanes 1, 2, 3 and 4, The thrombin-cleaved GST-fusion proteins of 13His-> Arg, Glu, Tyr and Ala respectively.

Lane 5, Wild type GST-Tat protein after thrombin digestion.

Lane 6, Molecular mass standards.

not impaired in their capacity of binding to the TAR RNA sequence. In gel-retardation assays with radio-labeled TAR RNA, specific retarded bands were generated by these mutants as well as by the wt Tat 86 protein (Fig. 2.4). This result was expected, since several studies have shown that the TAR-interacting domain of Tat resides in its basic portion (aa 49-57).

The H13 mutants were analyzed by CAT assay using a reporter cell line (HL3T1) carrying an integrated LTR-CAT reporter and exploiting the property of recombinant GST-Tat to enter the cells and being delivered to the nucleus in the presence of 100 μM chloroquine (73, 81). As compared to wt Tat 86, all four mutants resulted impaired in LTR transactivation (Fig. 2.5). The poor transactivating ability was not related to impaired access to the cells, since by immunofluorescence assays using protein specific antibodies, I could show that these proteins were equally able to enter the cells (not shown). Thus, these results suggest that the H13 mutation might have produced some conformational changes in the protein or is involved in the binding to some cellular factor(s) required for Tat transactivation. In particular, the N-terminal domain of Tat (first 21 amino acids) has long been recognized to be essential for transcriptional activation (252); however, no specific role has yet been attributed to this domain, except for the recently described interaction with the NFAT1 transcription factor (196). Interestingly, mutation at this residue was also detected in the defective integrated provirus of the latently infected U1 monocytic cell line (90).

Tat associates with a HAT activity *in vivo* and *in vitro*

To study he properties of Tat in promoting transcriptional initiation at the HIV promoter, we investigated the ability of HIV-1 Tat protein to interact with HAT enzymes. HL3T1 cells, a HeLa-derivative cell line carrying an integrated HIV-1 LTR-CAT construct, were treated with recombinant wild type Tat (Fig. 2.6 A, left), by exploiting the property of the protein to enter intact cultured cells (73, 105). Five hours after protein delivery, cell lysates were prepared, immunoprecipitated with an anti-Tat antibody and analyzed for the ability to acetylate purified histones in the presence of radiolabeled acetyl-CoA. Using this procedure, we recovered HAT activity from the lysates of cells treated with wild type Tat but not from cells treated with GST. In order to confirm the specificity of the interaction between Tat and HAT protein(s), we also expressed wild type Tat in HL3T1 cells by transfection of an eukaryotic expression vector containing the wild type

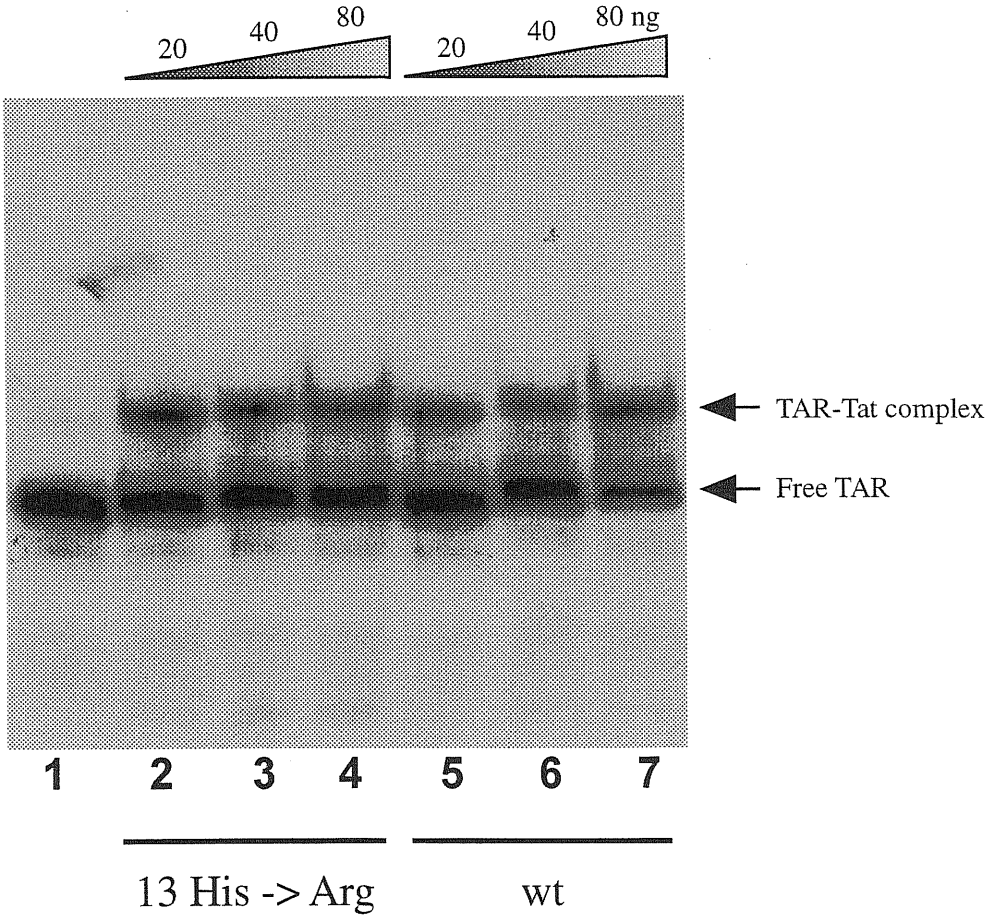


Figure 2.4
Tat 13 His->Arg binds to TAR

The radio (32P) labeled TAR RNA probe was mixed with increasing amounts of one of the 13 His mutants, namely 13His->Arg mutant (2-4), along with wild type Tat as positive control (5-7) and incubated at 25 degree for 20 minutes. RNA-protein complexes were resolved on 5% polyacrylamide gel in TBE.

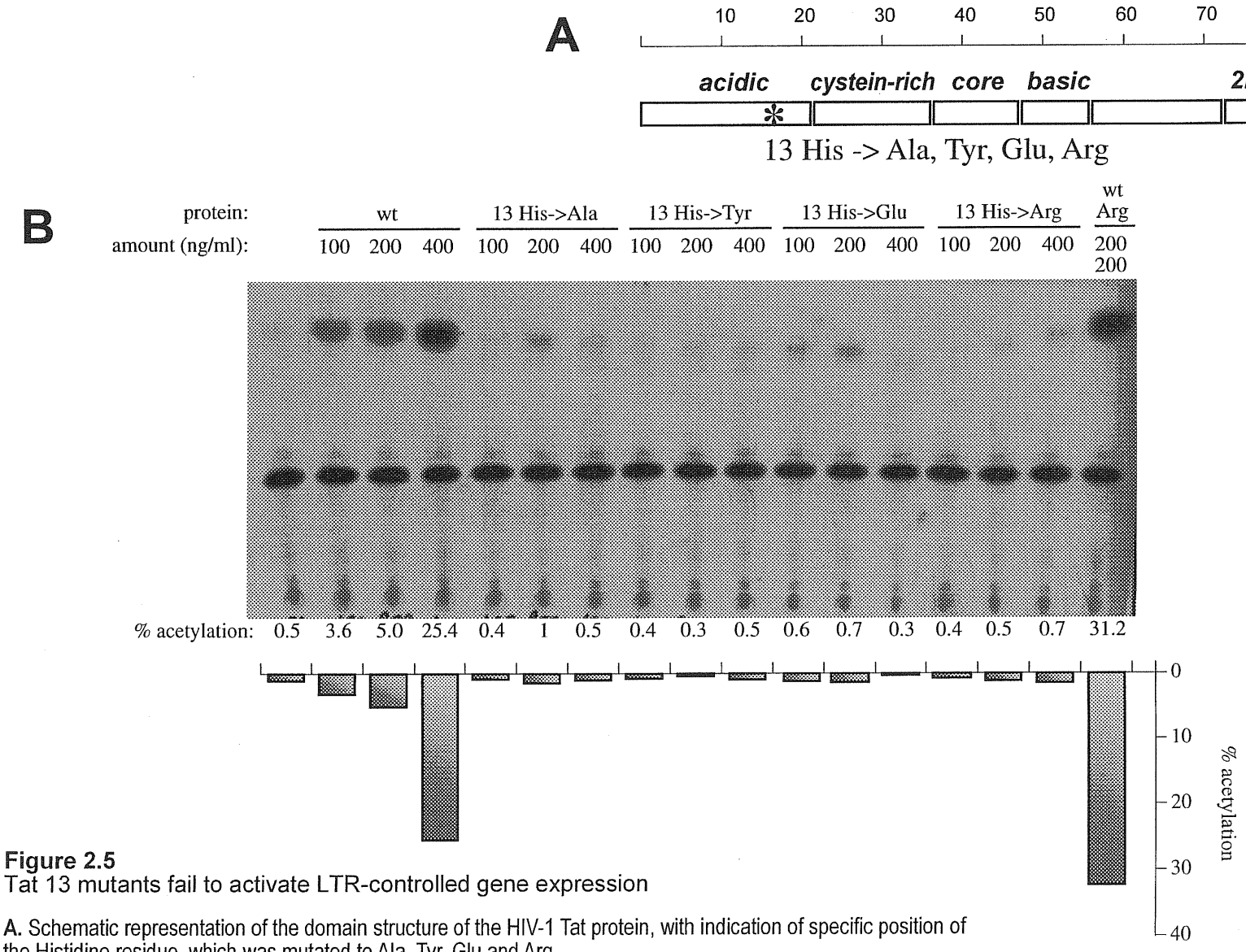


Figure 2.5
Tat 13 mutants fail to activate LTR-controlled gene expression

A. Schematic representation of the domain structure of the HIV-1 Tat protein, with indication of specific position of the Histidine residue, which was mutated to Ala, Tyr, Glu and Arg.

B. Increasing amounts of Tat mutants (as indicated) were tested for their LTR transactivation activity relative to wild type Tat, by CAT assay. Proteins were supplied to the culture medium of HL3T1 cells as GST-fusion.

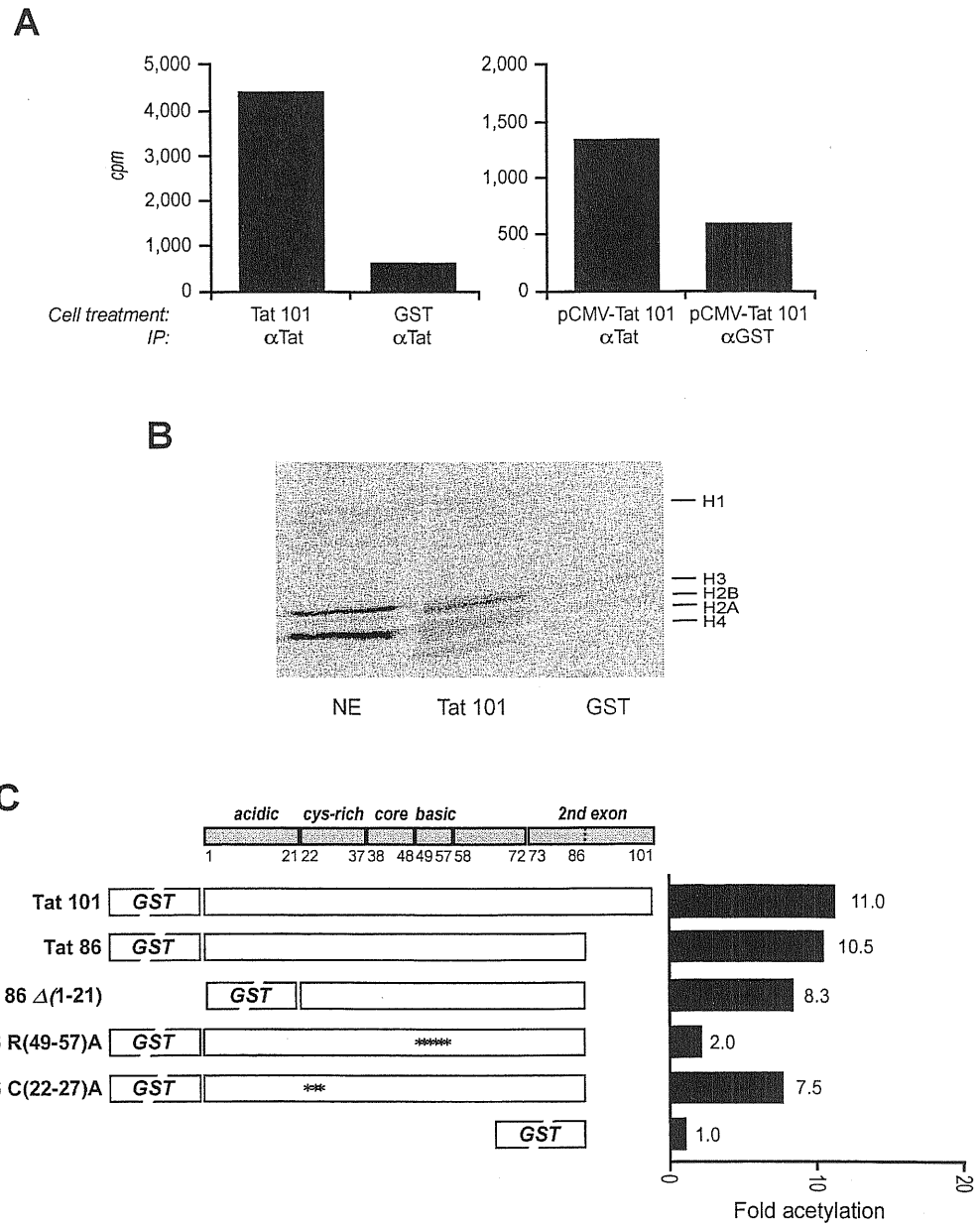


Figure 2.6
Association of Tat with HAT activity

A. Tat co-immunoprecipitates with a HAT activity. Whole cell extracts from HL3T1 cells treated with GST-Tat 101 or GST (left side) or transfected with a Tat expressing vector (right side) were immunoprecipitated with the indicated antibodies. Immunoprecipitates were analyzed for HAT activity by liquid scintillation counting.

B. GST-Tat pulls down a HAT activity specific for all the 4 core histones. A Dignam nuclear extract from HeLa cells was incubated with GST-Tat 101 or GST on agarose beads. Bound proteins were assayed for HAT activity; acetylated histones were resolved by SDS-PAGE and detected by autoradiography. NE: pattern of histone acetylation by the nuclear extract before pull down.

C. The arginine-rich domains of Tat is involved in the association with HAT activity. GST pull down experiments were performed as in B with the indicated proteins. The results are expressed as fold acetylation with respect to GST.

Tat cDNA (Fig. 2.6 A, right). Consistently, immunoprecipitation with an anti-Tat antibody specifically recovered HAT activity, whereas background activity was only detected in the immunoprecipitate obtained by the use of an anti-GST antibody. It must be noted that the HAT activity co-immunoprecipitated with recombinant Tat is several fold stronger than that associated with plasmid-expressed Tat. As verified by western blotting (not shown), this is most likely because of the higher concentration of Tat in the cell nucleus resulting from direct protein delivery as opposed to transfection of the expression vector.

We next determined the specific pattern of histone acetylation of the Tat-associated HAT. Agarose bead-immobilized wt Tat or GST was used in pull-down experiments on nuclear extract (Fig. 2.6 B). We assayed the HAT activity of the proteins thus recovered and that of an aliquot of nuclear extract before treatment. All four core histones, H3, H2B, H2A and H4, were acetylated by the Tat-associated HAT, the band of H3 being the most intense. Interestingly, the pattern of histone acetylation observed in total nuclear extracts is substantially different from that associated with Tat, suggesting that the latter HAT activity is not the predominant one present in the nuclear extract.

To determine the domains of Tat which are important for association with HAT activity, GST pull-down experiments were performed using wt Tat 101, wt Tat 86 and some mutated derivatives of the latter, Tat 86 Δ(1-21), Tat 86 R(49-57)A and Tat 86 C(22-27)A. As shown in Figure 2.6 C, both wild type Tat 101 and 86 Tat proteins bound the HAT activity present in the nuclear extract with similar efficiencies. The affinity was only slightly decreased in mutants Tat 86 Δ(1-21) and Tat 86 C(22-27)A. On the contrary, amino acid substitutions of the arginines in the arginine-rich domain almost completely abolished association of Tat with the HAT activity.

Tat binds p300 *in vitro*

Does Tat directly interact with HATs or is the binding mediated by another cellular component present in the complex? To answer this question, we assayed the ability of immobilized GST-Tat to bind to *in vitro*-translated ^{35}S-p300. We found that specifically p300 but not another protein such as hTAF32 (Fig. 2.7 A) or luciferase (not shown) binds to Tat. Thus, probably there is a direct interaction between the two proteins, albeit we cannot exclude the possibility of an unknown component of the translation

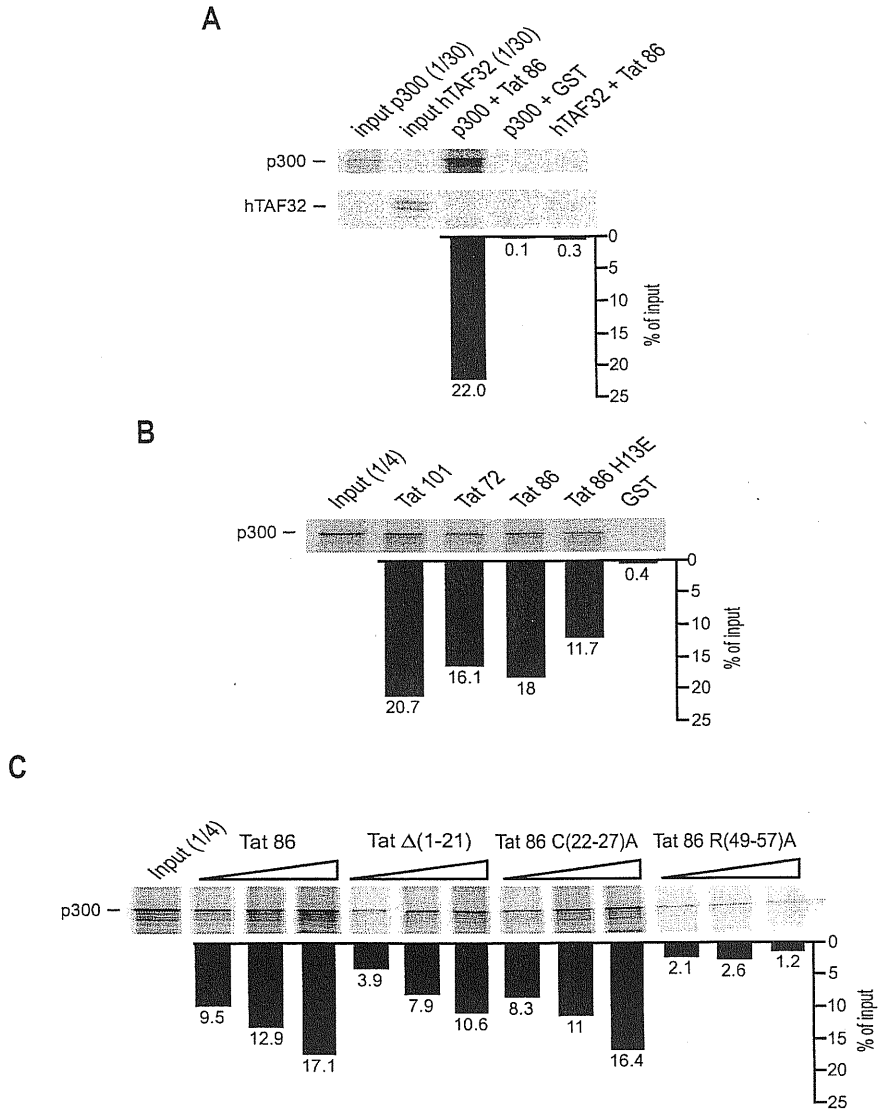


Figure 2.7
Interaction of Tat with p300 in vitro

The indicated GST fusion proteins (5 µg) on agarose beads were incubated with [^{35}S]-labeled p300 or hTAF32 (negative control). Bound proteins are expressed as percentages of the input radiolabeled protein.

A. GST-Tat binds specifically to p300.

B. Effects of truncations at the C-terminus of Tat.

C. Binding of p300 to Tat mutants. Immobilized GST fusion proteins (1.5, 3 and 6 µg) were incubated with [^{35}S]-labeled p300. Samples were processed as in panel A.

lysate to mediate indirect binding.

We mapped the sites of interaction of Tat with p300 using a series of mutated Tat derivatives (Fig. 2.7 B and 2.7 C). The transcriptionally active proteins (Tat 101, Tat 86 and Tat 72), as well as the protein mutated in the cysteine-rich domain efficiently bound to p300 (Fig. 2.7 B). On the contrary and in good agreement with the results obtained studying the association of Tat with HAT activity (Fig. 2.6 C), the interaction of Tat with p300 strongly depended on the integrity of the arginine-rich domain (Fig. 2.7 C). Deletion of the N-terminal 21 amino acids (one of the regions which are essential for the interaction of Tat with cellular co-factors) lowered the affinity of Tat for p300 (Fig. 2.7 C). The same was observed in the Tat protein bearing a single point mutation in this domain (histidine at position 13 to glutamine; Fig. 2.7 B).

The suppression of Tat-p300 interaction *in vitro*, resulting from mutations of the 6 arginines in the basic domain, suggests a direct role of the basic domain in the complex formation. Nevertheless, it cannot be excluded that such a modification could drastically alter the tertiary structure of the protein.

Tat binds p300 and CBP *in vivo*

The results reported above clearly demonstrate *in vitro* interaction of Tat with p300, which is capable of acetylating all four core histones. Several HAT enzymes have so far been identified and characterized in terms of molecular structure and substrate specificity (for reviews, see references 36, 321). Among the known nuclear HATs, p300 and CBP, two structurally very similar HATs are the only ones, which are capable of acetylating all four core histones (87, 195, 230). We therefore assessed the association of Tat with p300 and CBP *in vivo* by co-immunoprecipitation studies using lysates of cells treated with Tat (Fig. 2.8). Immunoprecipitation with an anti-CBP or an anti-p300 antibody resulted in the co-immunoprecipitation of CBP or p300 as well as Tat in Tat- but not GST-treated cells. Accordingly, immunoprecipitation with an anti-Tat antibody recovered Tat as well as CBP and p300. None of the three proteins was immunoprecipitated by an unrelated control antibody. These results prove that the interaction between Tat and p300/CBP also occurs within cell.

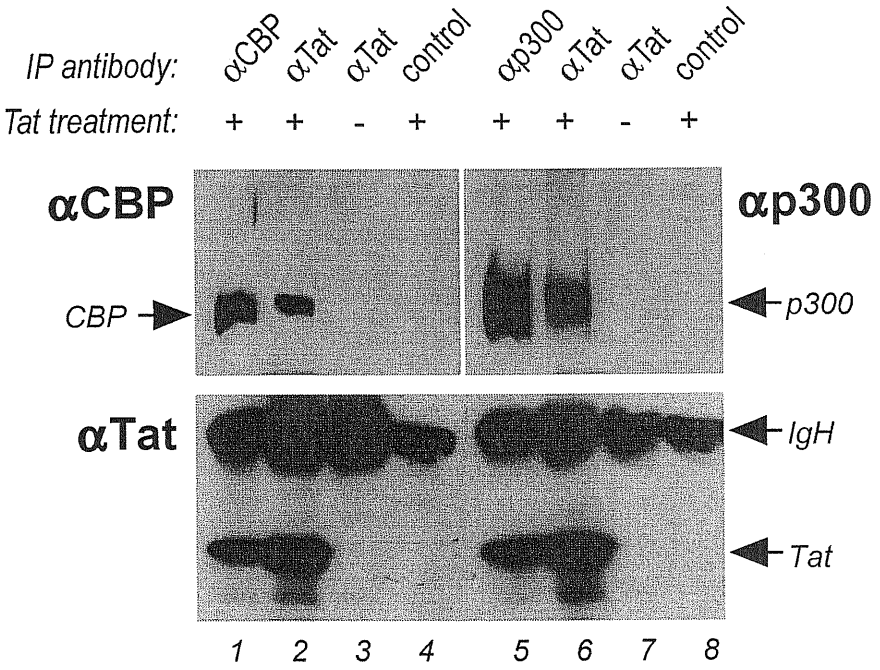


Figure 2.8
Association of Tat with p300 and CBP *in vivo*

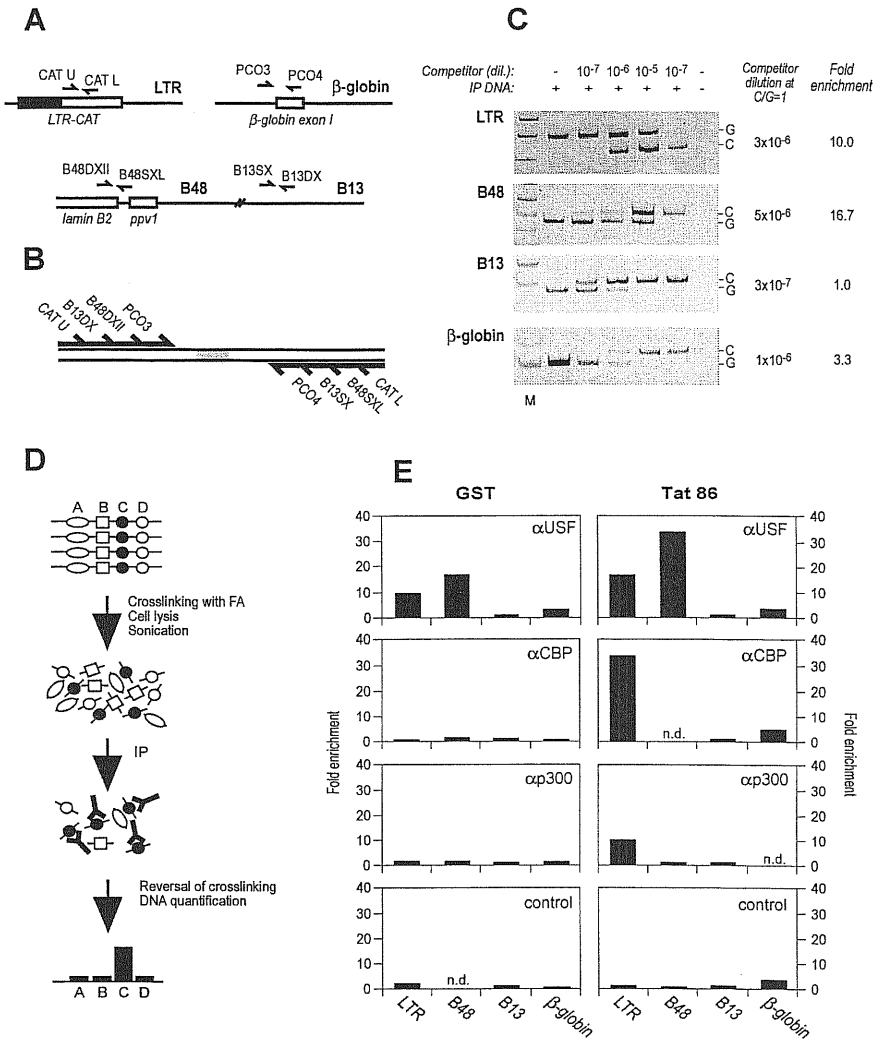
Whole cell lysates prepared from HL3T1 cells either untreated (lanes 3 and 7) or treated with GST-Tat added to the culture medium in the presence of chloroquine (lanes 1, 2, 4-6 and 8) were immunoprecipitated with the indicated antibodies. Bound proteins were resolved by SDS-PAGE (5% acrylamide upper part, 10% lower part), and transferred to a nitrocellulose membrane which was subsequently cut in three parts. The lower portion of the filter was reacted with an anti-Tat antibody, while the upper two parts with anti-CBP (upper left panel) or anti-p300 (upper right panel) antibodies. The position of the p300, Tat and CBP proteins are indicated. IgH: immunoglobulin heavy chain.

Tat recruits p300 and CBP to the LTR

An essential issue of our research study was to understand whether the interaction of Tat with p300/CBP takes place specifically on the integrated promoter. We analyzed the chromosomal events involved in Tat transactivation by a novel procedure for quantitative chromatin immunoprecipitation (Fig. 2.9). This technique is based on the cross-linking of protein-DNA and protein-protein complexes within the cell by formaldehyde treatment (233), followed by chromatin sonication, immunoprecipitation with specific antibodies and precise quantification of the immunoprecipitated DNA segments by competitive PCR (Fig. 2.9 D). This procedure quantitatively assesses the *in vivo* direct or indirect binding of a given protein to a defined chromosomal region. Four different genomic sites were investigated in HL3T1 cells: The HIV-1 LTR, two regions of the lamin B2 gene domain (B48, close to a human origin of DNA replication (118), and B13, ~7 kb away from the origin), and one region in the β-globin gene (Fig. 2.9 A). As a competitor, we used a single DNA fragment containing all the four primer pairs arranged to generate PCR products of different length from the ones obtained from genomic DNA (Fig. 2.9 B). The competitive PCR quantifications were carried out by the addition of a increasing amount of the multi-competitor to a fixed volume of immunoprecipitated DNA, followed by PCR amplification of aliquots of the mixture with the appropriate primer pairs.

Analysis of protein interactions at the selected regions was performed in HL3T1 cells after treatment with Tat or GST. In either case, immunoprecipitation with an antibody against cellular transcription factor USF resulted in the enrichment for the DNA segments encompassing the LTR and the B48 region (Fig. 2.9 E; the actual competitive PCR results for the quantification of anti-USF immunoprecipitates in GST-treated cells are shown in Fig. 2.9 C). This finding is consistent with our previous results showing that the LTR and B48 regions are targets for USF, and that both sequences are actually bound by the protein *in vivo* (1, 66). Strikingly, in the absence of Tat both the anti-CBP and the anti-p300 antibodies failed to immunoprecipitate the LTR DNA segment, as well as the other segments. After Tat treatment, a remarkable enrichment for this genomic region (10-fold for p300 and 33-fold for CBP) was observed (Fig. 2.9 E). These data demonstrate that Tat-mediated activation of the integrated LTR *in vivo* is concomitant with the recruitment of p300 and CBP specifically to the promoter region.

Figure 2.9
Recruitment of p300 and CBP to the LTR upon Tat-mediated transcriptional activation *in vivo*



A. Human chromosomal regions analyzed by quantitative chromatin immunoprecipitation. LTR-CAT, β-globin gene exon I, lamin B2 gene B13 and B48 DNA segments were studied. For each of these regions, two primers were selected (small converging arrows). The boxes schematically indicate the location of relevant genomic elements (LTR-CAT cassette, β-globin exon I, lamin B2 gene 3' end and ppv1 gene) with respect to primer localization.

B. Multicompetitor DNA for competitive PCR. The multicompetitor DNA fragment contains all primer recognition sites arranged in order to generate PCR products of different but comparable size to those obtained from amplification of genomic DNA.

C. Quantification of the sample obtained from GST-treated HL3T1 cells immunoprecipitated with anti-USF antibody (Fig. 2.9E, upper left graph). Quantification of immunoprecipitated DNA was obtained by mixing a fixed amount of immunoprecipitated DNA with the indicated scalar amounts of competitor DNA, followed by PCR amplification with each primer pair. DNA quantification was obtained from the ratio between the amplification products for genomic (G) and competitor DNAs (C). M: molecular weight markers

D. Flow chart of the quantitative chromatin immunoprecipitation assay. A, B, C, and D indicate four genomic DNA segments which directly or indirectly are cross linked to different proteins in vivo by treatment with formaldehyde (FA). When immunoprecipitation (IP) of sonicated chromatin is performed with an antibody reacting with the protein cross linked to C, the immunoprecipitate will be enriched for this DNA segment.

E. Results of quantitative chromatin immunoprecipitations of the four analyzed regions after treatment of HL3T1 cells with GST (left side) or GST-Tat 86 (right side) and in the presence of chloroquine, using the indicated antibodies (control: antibody against the HA epitope). Results are expressed as fold enrichment with respect to B13 region. Antibody against USF immunoprecipitates cross linked B48 and LTR-CAT regions but not B13 and β-globin; the effect is augmented by Tat treatment. Antibodies against CBP and p300 immunoprecipitate only cross linked LTR-CAT DNA after Tat treatment. Control antibody failed to immunoprecipitate any of the four DNA regions after GST as well as Tat treatment. The graph reports the results obtained in a representative experiment. At least three independent experiments have been performed for each antibody and each DNA region, obtaining consistent results. n.d.: not done

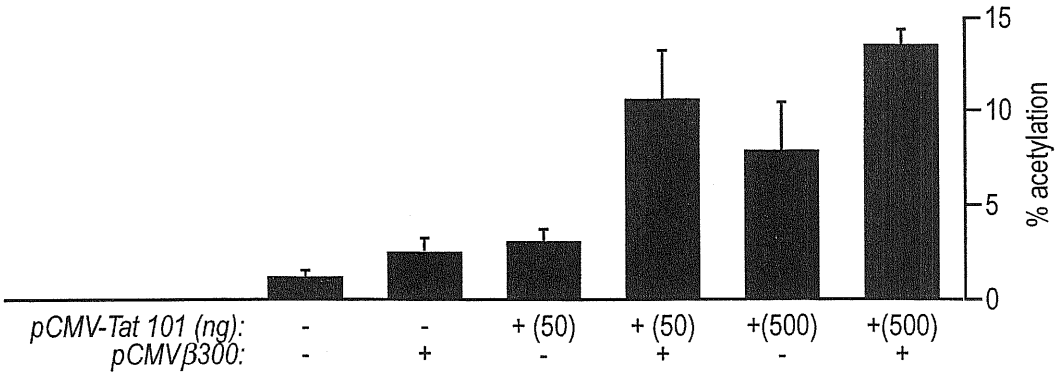


Figure 2.10
Effects of p300 expression on Tat activity

Hamster CHO cells were transfected with a plasmid containing an HIV-1 LTR-CAT cassette and the neo-resistance gene, and stable transfectants were obtained by G418 selection. CAT assays were performed in the absence or 48 hrs after transfection of the indicated amounts of pCMV-Tat 101 and of pCMVbp300 (10 μg where indicated) expression vectors. The results represent the average of at least three independent transfections.

Over-expression of p300 enhances Tat transactivation Expression of p300 and CBP in human HeLa cells is constitutive and relatively high, as detected by Western blotting on total cell lysates. Consequently, co-transfection of p300 in HL3T1 cells had only a modest, although reproducible, positive effect on Tat-mediated transactivation of the LTR (~2-fold increase over Tat alone; data not shown). Conversely, the synergistic effect of p300 and Tat could be better observed in a hamster CHO cell line. This cell line was obtained by transfection of an LTR-CAT cassette and selection for stable integration. As in other rodent cells, Tat activity in these cells is poor when sub-optimal amounts of Tat are transfected (Fig. 2.10, 50 ng of Tat per plate). This is in agreement with the well established notion that rodent cells do not support efficient Tat transactivation (10, 135). In these conditions, transfection of pCMVβp300 significantly enhanced Tat-mediated activation of the LTR, while p300 alone had a very modest effect on the basal level of transcription from the integrated LTR. When Tat concentration was increased (500 ng of transfected expression plasmid), the potentiation effects of p300 still could be observed although in a less pronounced manner.

II - DISCUSSION

Tat and HAT

Most eukaryotic DNA exists as chromatin in a nucleosomal format, packaged with histones and non-histone proteins. In intact nuclei, gene transcription is a multistep process that involves, minimally, the binding of upstream activators, the assembly of general transcription factors into an active complex at the promoter, and the initiation and elongation of transcription by RNA polymerase (173). How these steps are accomplished on naked DNA templates has been well studied in cell-free systems. The corresponding steps in the settings of chromatinized DNA are not well understood. There is, however, a consensus that nucleosomes structurally hinder access of transcription factors to their cognate sites on DNA. Thus, many transcription factors bind with a 2-log reduced affinity for nucleosomal DNA as compared to naked DNA (2). Similarly, there is also an evidence that polymerase elongation is more efficient on naked as compared to nucleosome-organized DNA (155).

Viruses are obligatory parasites of host cells. Unlike some nuclear DNA viruses or cytoplasmic RNA viruses, all retroviruses integrate obligatorily into host chromosomes during the replicative life cycle. Reasonably, then, regulatory mechanisms for productive retroviral infections must conserve functions evolved to accommodate the structural organizations inherent to eukaryotic DNA. Indeed, retrovirus integration studies have shown a strong intracellular preference by integrase for nucleosomal rather than nucleosome-free DNA (248, 249).

HIV-1 is one of the better studied paradigms for retroviral transcription. Work from various laboratories (239, 298, 299, 318) has shown that chromatin structure is a major contributory factor in the regulation of HIV-1 provirus expression. Hence, it has been suggested that transcription of the same HIV-1 DNA template is controlled by distinct mechanisms depending on its presence in an integrated versus an unintegrated format (158). Additionally, it is known that HIV-1 LTR in its integrated form is organized into nucleosomal and nucleosomal-free structures (299). Disruption of one or more of the LTR-associated nucleosomes by diverse stimuli such as tumor necrosis factor-α (297) has been correlated with activation of HIV-1 transcription. Binding of upstream factors

such as NF-κB and Sp1 to cognate sites has also been linked to remodeling of positioned nucleosomes in the HIV-1 LTR (239, 318). At the same time, a genetic analysis of latent HIV-1 infections has found Tat expression to be the rate-limiting event for activation of proviral transcription (3). Collectively, these observations suggest that remodeling of chromatin is important for the transcription of proviruses and that, for the integrated HIV-1, a Tat associated function might trigger the first step towards the opening up of the nucleosome-associated template.

If a Tat-associated activity is rate limiting for the transcription of the integrated provirus, then it is reasonable that this activity could influence the accessibility of chromatin-associated LTR. Indeed, a number of Tat-binding cellular proteins that influence transcriptional activation of the viral LTR have been described. These include TBP (167, 301), TAK (53, 138, 325), TBP-1 (224, 231), TAP (77, 329), TAF55 (50), Tip60 (166), Sp 1 (62, 159), TFIIH (113, 235) and RNA pol II (209, 322). However, based on the existing knowledge, none of these factors or activities would be expected to fulfil a role in chromatin remodeling. Thus, the idea that a Tat-associated factor remodels chromatin, led us to think about the factors involved in two most logical pathways, namely proteins of the SWI/SNF/NURF/RSC (reviewed in 291) and/or proteins with histone acetyltransferase activity (reviewed in 129). Because inhibitors of histone de-acetylation, such as sodium butyrate (183, 184), trapoxin and trychostatin (297), were recently shown to activate the integrated HIV-1 LTR, we were thus directed to explore the existence of a Tat-associated histone acetyltransferase. Some of the results described in this thesis confirmed the above notion and suggests an additional function for HIV-1 Tat, which is to relieve chromatin inhibition on transcription by recruiting p300 and CBP histone acetyltransferase to the HIV-1 LTR promoter.

Among the factors associated with the basal transcription complex, p300 and the related CREB binding protein (CBP) have emerged as coactivators for a broad group of cellular transcription factors (reviewed in 86). Both molecules are large nuclear phosphoproteins that respond to changes in cell growth (86) and are involved in the regulation of diverse signal transduction pathways (147, 221, 289). Proteins p300 and CBP are two evolutionary conserved and highly homologous proteins, acting as molecular bridges between transcription factors and components of the basal transcriptional machinery (87, 195). In the last few years, a growing number of cellular transcription factors have been identified for their capacity to interact with p300/CBP,

including among others, nuclear hormone receptors, CREB, c-Jun/v-Jun, Sap 1a, MyoD, c-Fos, and NF-κB p65 (for a recent review, see ref. 123) and also with other transcriptional coactivators, such as PCAF (326) and ACTR (49). Given the pivotal role of p300/CBP in the control of gene expression, it is not surprising that several viruses encode proteins targeting these two factors. The adenovirus E1A, the HTLV-I Tax, and the SV40 large T proteins (14, 87, 121) are among these viral products.

Tat specifically associates with p300 and CBP *in vitro* and within the cells. Moreover, by *in vivo* quantitative chromatin cross-linking experiments, we also showed that the interactions of Tat with p300 and CBP actually occurs at the LTR. This reinforces the notion that the two HAT proteins become components of the protein complex inducing promoter activation during transcription initiation. Our results are also supported by an independent observation that showed that the p300/CBP-interacting adenoviral E1A protein is able to repress Tat-induced HIV-1 gene expression in an NF-κB - independent manner (148). Given the numerous factors demonstrated to be bound by these large adaptor proteins, it remains to be formally elucidated whether Tat contacts and associates with p300/CBP directly or via another cellular component.

Tat acts as an extremely powerful transcriptional activator targeting the LTR promoter through various and distinct pathways. Besides the association with HAT proteins, Tat interacts with transcription factors binding to the LTR (e.g. Sp1 (159), associates with components of the basal transcriptional machinery (e.g. TBP (167), induces NF-κB which in turn binds to the LTR (73), and recruits protein kinases phosphorylating the carboxyl-terminus of RNA polymerase II (160). Furthermore, Tat also affects LTR promoter activity through several indirect pathways deriving from its non transcriptional functions within the cell and at the cell membrane, ranging from the regulation of apoptosis to the induction of cytokine gene expression. Given these pleiotropic functions, it is not surprising that the synergistic effects of Tat and p300 in transient transfection experiments could be observed only in conditions in which the levels of Tat protein were limiting. Most likely, these are the same conditions that physiologically occur at the integrated LTR in non activated cells.

Proteins p300 and CBP interact with cellular transcription factors and possess intrinsic HAT activity (230). The findings described here suggest that these coactivators can serve as adapters also for RNA binding proteins and contribute to transcriptional regulation via targeted acetylation of chromatin. These findings serves to clarify several

previous observations and suggest a molecular mechanism to explain how Tat provides a rate-limiting switch for the activation of integrated proviruses (3). Possibly, a small amount of Tat protein, synthesized from the multispliced RNA transcripts produced early during infection, serves as the initial trigger that de-repress the chromatinized LTR promoter. This would explain how Tat participates in initiating access and formation of a RNA pol II complex at the integrated promoter and is consistent with the preferential importance of Tat-associated HAT in the activation of integrated but not unintegrated HIV-1 templates. Second, based on the knowledge that nucleosomes negatively influence polymerase elongation (155), an RNA polymerase-associated HAT activity would also address a processivity defect previously described for the transcription of integrated HIV-1 templates (97, 163). The finding that Tat is associated with RNA pol II (62, 112, 170), also fosters the possibility that Tat could physically recruit and transfer (97) a HAT activity to an the elongating polymerase. Should this be the case, it would be analogous to the recruitment of HAT to RNA pol II complexes by other cellular RNA-binding proteins (169, 222).

The basic domain of Tat is responsible for binding to p300/CBP as well as to TAR. How these two interactions could physically occur will clearly need more investigation. The suggestion that Tat could forms dimers (104), would be consistent with simultaneously binding of two molecules with p300/CBP and TAR RNA. Alternatively, binding to different partners could be result of subsequent events in the process leading to the recruitment of the preinitiation complex at the promoter. In this respect, it also appears plausible that chromatin remodeling and the increase in polymerase processivity (both of which are mediated by Tat at the HIV-1 LTR) are two highly coordinated processes also occurring both at cellular and viral promoters. Not surprisingly, additional non-histone targets of acetylation by p300 are other components of the basal transcription machinery, including TFIIE and TFIIF (152). The former factor also can stimulate the TFIIH-dependent phosphorylation of the carboxyl-terminal domain of RNA polymerase II (145, 210). This provides another possible indirect link between Tat-mediated promoter activation and the increase in efficiency of transcriptional elongation.

Finally, these findings, besides explaining the function of Tat in transcriptional initiation from the LTR promoter, have some implications also for the understanding of the pathogenetic mechanisms of HIV disease. In fact, in HIV-infected patients a large number of cells harbor proviral DNA molecules that are transcriptionally inactive (38,

89, 281). The Tat-mediated recruitment of HATs to the viral promoter in these latently infected cells is likely to represent a critical step in viral reactivation.

Parallel to our results (208), studies from other laboratories have also confirmed the role of different HATs (CBP/P300, PCAF, $hTAF_{II}250$) on HIV-1 LTR promoter transactivation by Tat (24, 148, 312).

Chapter III

MECHANISM FOR INTERCELLULAR TRAFFICKING OF HIV-1 TAT PROTEIN

III - INTRODUCTION

The HIV-1 Tat gene product is unusual in that, although being a transcription factor, it can be released from the cells and has autocrine and paracrine activities (105). From the evolutionary stand point, it is unclear what could be the role of Tat release in the framework of HIV-1 infection in patients. At the experimental level, however, the protein can clearly enter cells that are neighbors to those actually producing the protein. If these cells harbor a latent provirus, Tat could activate viral gene expression and contribute to a burst of HIV-1 replication (242). While this function is highly speculative, nevertheless exogenous Tat can exert several pleiotropic functions also in cells not infected with HIV-1, as it will outlined below. These functions might prime cells to become permissive for infection and could have implications for both pathogenesis and treatment of HIV-1.

Depending on the cell types, exogenous Tat has been shown to activate the expression of several genes, including genes for extracellular matrix proteins (6, 286), cytokines including IL1, IL6, IL10, TNF-α, TNF-β, TGF-β, NF-kB, IL4 receptor, chemokine receptors, G6PD, metalloproteinase-9 (39, 73, 120, 150, 180, 181, 193, 220, 232, 244, 250, 272, 274, 294, 295, 314, 315, 330, 331). Other genes are as well downregulated by Tat, including the Mn^{2+}-dependent superoxide dismutase, the IL2 and IL2 receptor, the TNF receptor, the MHC-class I, the p53 tumor-suppressor, and the protein kinase R (PKR) genes (34, 102, 189, 246, 251,149). In this respect, it should be observed that the recent observation from our laboratory that Tat can activate the translocation of NF-kB (73), through the functions of the cellular kinase PKR (76), suggests that the regulation of the expression of some of these genes by Tat could be indirect and mediated by NF-kB. Consistently, none of the above mentioned genes has been certainly proved to directly interact either in the form of DNA or as mRNA with Tat.

Extracellular Tat has also been shown to exert global functions on cell growth and metabolism. According to the cellular systems and to the experimental conditions in which it was tested, Tat was shown either to promote apoptosis (188, 313) or induce uncontrolled cell growth and proliferation (6, 19, 44, 59, 60, 91, 193).

On specific cell types the protein also possess peculiar characteristics. In neuronal

cells Tat has neurotoxic activity, which could be related to the neurological manifestations that are invariably associated with HIV disease progression (142, 197, 198, 223, 244, 266, 286, 308). On lymphocytes, it inhibits antigen-induced cell activation and proliferation, and could be involved in the immunological anergy that accompanies HIV disease (131).

In particular, picomolar concentrations of extracellular Tat can exert very strong inflammatory and angiogenic effects on Vascular cells, such as endothelial cells and KS-cells, the spindle cells of endothelial cell origin derived from Kaposi's sarcoma lesions of the AIDS patient, (AIDS-KS) (6, 92). This disease is very common and aggressive in HIV-1 infected individuals and has particular potential impact on AIDS-associated manifestations (268). Tat can also induce the angiogenesis in transgenic mice (59, 60, 304). Tat is expected to perform its angioproliferating activities by modifying the cellular levels of several cytokines, which in turn increases the levels of basic fibroblast growth factor (bFGF) and vascular endothelial growth factor VEGF), the two well established angiogenic factors (92, 107).

Recently, another link between Tat and a cytokine-based model of KS pathogenesis was made by demonstrating that HIV-1 Tat can directly affect the mitogenic Flk-1 / KDR / VEGFR-2, an VEGF-A receptor (9). The Flk-1 receptor is specific for endothelial cells (KS) and is a key molecule in mediating angiogenesis (42, 287). Moreover, Tat is also known to modulate the signal transduction pathways involving not only Flk-1 / KDR receptor, but also mitogen-activated protein kinases and components of focal adhesion in KS cells (109). On the other hand, interaction of Tat with integrin receptors modulate the angiogenic responses of these angiogenic factors, by mediating the adhesive interactions between endothelial cells and the extracellular matrix (ECM) proteins (21, 103).

Considering all the above mentioned effects, it is not clear at what extent they are mediated by the actual cellular uptake of exogenous Tat or are mediated by the interaction of Tat with cell surface receptors followed by signal transduction. In this respect, different Tat receptors have been identified. In endothelial cells, Tat has been shown to bind to the integrin receptors (20, 303) and to the vascular endothelial growth factor tyrosine kinase receptor Flk-1/KDR, in a manner analogous to vascular endothelial growth factor itself (9). In monocytes, subnanomolar concentrations of Tat

induce chemotaxis by interacting to and signaling thorugh the tyrosine kinase receptor for vascular endothelial growth factor encoded by *fms*-like tyrosine kinase gene (VEGFR-1/Flt-1) (216).

Since from the late Eighties it has been clearly demonstrated that exogenous HIV-1 Tat has the ability to enter the cell and translocate to the nucleus in an active form (105, 201). However, the precise mechanism of exogenous Tat uptake and intracellular transport to the nucleus are still unclear. The cellular entry of exogenous Tat and its HIV-1-LTR transactivation activity is inhibited by heparin (201, 263); this interaction involves the basic domain of Tat (265). Heparin is not physiologically present on the cell membranes. However, the heparan sulfate (HS) glycosaminoglycan (GAG), a close structural homologue of heparin, is the major constituent of the proteoglycans present on the surface of cells and in the extracellular matrix (26).

Proteoglycans constitute a diverse class of compounds which are produced by most animal cells. They consist of a protein core and one or more covalently attached polysaccharide GAG side chains. Each GAG moiety consists of repeating disaccharide units (156, 174). The glycosaminoglycan substitution depends on the ability of the protein to serve as an acceptor for xylosyltransferase, the enzyme that begins the synthesis of most types of glycosaminoglycans. Proteoglycans exist in all sizes and shapes, and their only common feature may be the presence of glycosaminoglycans.

Proteoglycans are present in intracellular secretion granules, in the extracellular matrix and on the cell surface. The widely distributed membrane associated proteoglycans mainly contain four types of GAG moieties, which include heparan sulfate (HS) and chondroitin sulfates A through C. These unbranched GAGs are defined by the repeating disaccharide units that compose their chains, by their specific site of sulfation and by their susceptibility to bacterial enzymes, known to cleave distinct GAG bonds (191). These chains undergo a series of modification reactions that include N-deacetylation, N-sulfation, O-sulfation and epimerization of glucurinic acid to iduronic acid. The extent of these reactions varies and give rise to an enormous structural heterogeneity of the GAG moieties. The high content of sulfates and the presence of hexuronic acids impart a large negative charge to GAGs.

Proteoglycans can be modified by more than one type of GAG and have a diversity of functions, including cell growth and differentiation. The versatility of proteoglycans and their capacities for multiple interactions with other molecules give them the ability to

function as a multipurpose "glue" in cellular interactions. They can bind together extracellular matrix components, mediate the binding of cells to the matrix and capture soluble molecules such as growth factors into the matrix and at cell surfaces. The proteoglycan-mediated entrapment of growth factors provide a mean of concentrating growth factor activities and directing them into a geometry appropriate for the architecture of the tissue (for reviews, see 93, 259, 262). In addition, cell surface proteoglycans are known to act as cellular receptors for bacteria, parasites and several animal viruses, including adeno-associated virus type 2 (AAV-2) (284) and also HIV-1 (238). In fact, the cell surface proteoglycans, especially those contain HS GAGs carbohydrate chains are rapidly internalised and degraded (323). Thus membrane proteoglycans may act directly to internalise ligands, that bind to the GAG chains by a receptor mediated endocytosis pathway (93). Such a mechanism of entry has already been described for other HS proteoglycan ligands, including basic fibroblast growth factor and lipoprotein lipase (257, 271).

The observations that i) Tat binds with high affinity to heparin (263, 306); ii) the angiogenic, mitogenic and chemotactic activities of Tat on cultured endothelial cells are modulated by heparin (6); iii) the ubiquitous distribution of heparan sulfate on the cell surface of most cell types that parallels the ability of Tat of entering the same cells, prompted us to investigate the role of cell membrane heparan sulfate and other proteoglycans on Tat internalization. Here we provide genetic and biochemical evidence that heparan sulfate proteoglycans act as major receptors for extracellular Tat uptake.

III - RESULTS

Cellular uptake of recombinant proteins fused to Tat

To explore the mechanisms of Tat transactivation of the HIV-1 LTR, in the last few years we have extensively taken advantage of the properties of a GST-Tat fusion protein to enter *in vitro* cultured cells when added to the culture medium (73, 76, 208). This protein contains the first 86 amino acids of Tat fused at the C-terminus of the GST protein (~34 kDa total). The kinetic analysis of HIV-1 LTR transactivation by this protein, and by a fusion protein additionally containing the green fluorescent protein (GFP) at the C-terminus (~60 kDa total), are shown in Figure 3.1 A. Transactivation of the LTR starts occurring at few hours after addition of 200 ng GST-Tat to the culture medium of HL3T1 cells, containing a integrated LTR-CAT reporter gene which is silent in the absence of stimulation (98) and reaches a maximum at 10-15 hours. The kinetics of LTR transactivation by the same amount of GST-Tat-GFP protein is delayed by few hours, but still reaches the same levels after 15-20 hours. These data are consistent with previous experiments in which we analyzed the levels of LTR-CAT mRNA expression after transactivation with exogenous GST-Tat, that peaked at 4-5 hours after addition of the protein to the culture medium (73). As shown in Figure 3.1 B, transactivation is already appreciable at a concentration of 50 ng per ml of medium for the GST-Tat protein (~1.5 μM) and tends to reach a plateau for concentration above 250 ng/ml. The same dose response curve is evident for the GST-Tat-GFP protein, considering the molar differences between the two preparations. Entry of GST-Tat into the cells was also directly appreciated by labeling the protein with ^{125}Iodine. As shown in Figure 3.1 C, the protein was found inside the cells already after 4 hours from the addition to the medium. By staining with an antibody specific for Tat, after 2 hours the protein started to be found inside the cells with a distribution compatible with its presence inside endosomal vesicles, as already described (105, 201). At four hours, the anti-Tat antibody stained the nuclei of most cells, consistent with the transactivating activity of the protein (Figure 3.1 E). Cell entry of the GST-Tat-GFP protein was also visualized by flow cytometric analysis of the treated cells (Figure 3.1 D). A time course study showed that increased cell fluorescence started to be detected at 2 hours after addition of the protein to the cell culture and increased in the first 24 hours. Cellular fluorescence was specifically due to Tat-mediated internalization of the recombinant

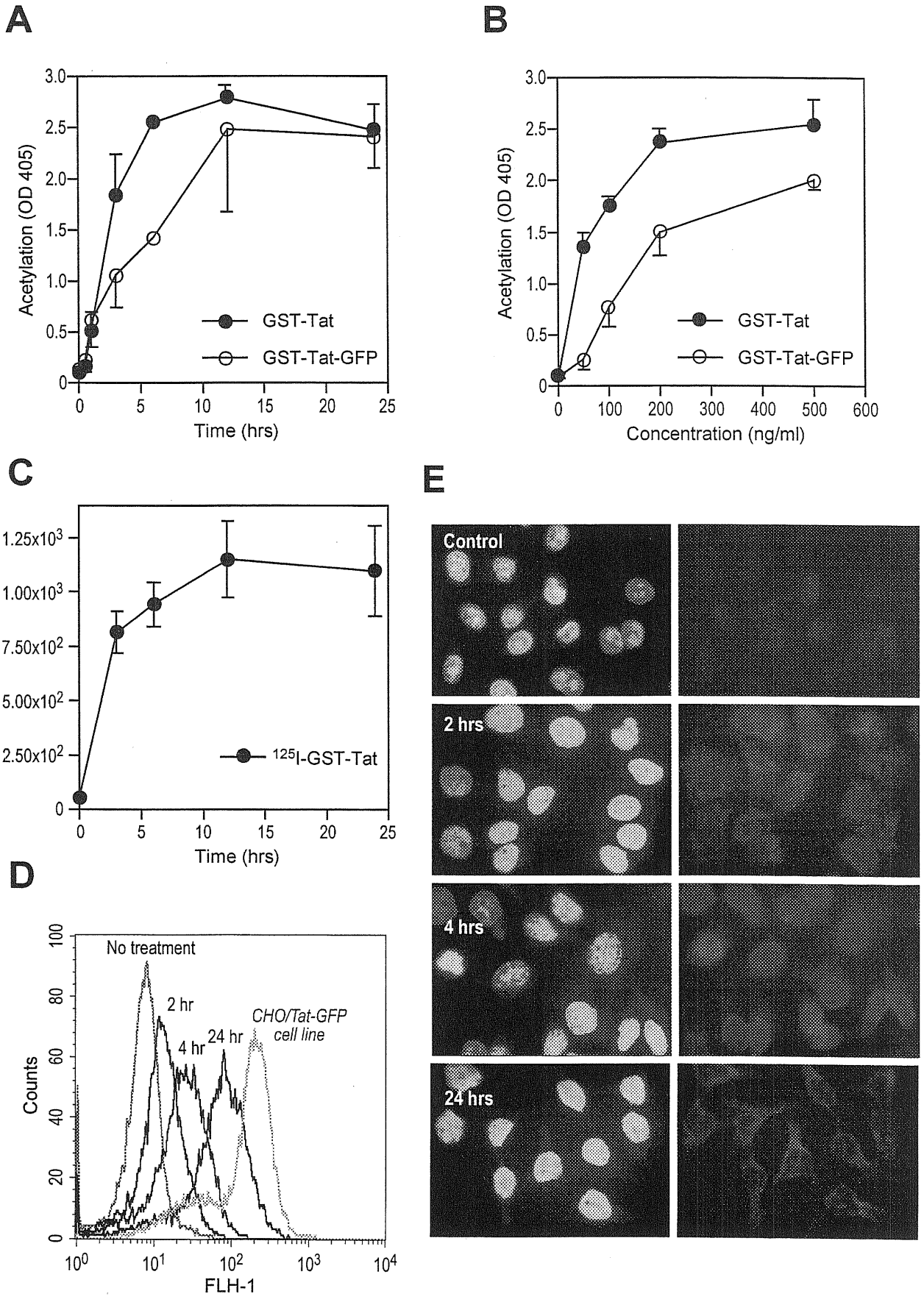


Figure 3.1 - see legend on following page

Figure 3.1 - legend
Kinetics of uptake of extracellular Tat fusion protein

A. *Time-dependent transactivation activity of exogenous recombinant GST-Tat and GST-Tat-GFP.* The recombinant proteins (200 ng/ml), purified as GST fusions, were added to the culture medium of HL3T1 cells, containing an integrated LTR-CAT construct. At the indicated time points, cells were extensively washed and fresh medium was added. CAT activity was determined by ELISA after additional 24 hrs.

B. *Dose-dependent transactivation activity of exogenous recombinant GST-Tat and GST-Tat-GFP.* Transactivation of the LTR-CAT construct of HL3T1 cells was analyzed after 28 hrs incubation with the indicated amounts of GST-Tat and GST-Tat-GFP.

C. *Internalization of radiolabeled GST-Tat.* HL3T1 cells were incubated in serum free medium containing 0.15% gelatin and 20 mM Hepes buffer, pH 7.5 in the presence of 20 ng/ml of ^{125}I-GST-Tat and 200 ng/ml of unlabeled GST-Tat as a carrier. After 16 hrs incubation, cells were washed three times, lysed, and radioactivity of the cell lysates was measured. Under these experimental conditions, up to 90% of the radioactivity remains associated to the cells after a wash with 2.0 M NaCl in sodium acetate pH 4.0, thus demonstrating the intracellular localization of cell-associated ^{125}I-GST-Tat [Rusnati, 1999 #455].

D. *Analysis of internalization of GST-Tat-GFP by flow cytometry.* HL3T1 cells were incubated for 2, 4, and 24 hrs with recombinant GST-Tat-GFP (1 μg/ml), washed extensively and then analyzed for intensity of fluorescence by flow cytometry. Cellular fluorescence was specifically due to Tat-mediated internalization of the recombinant protein, since a GST-GFP fusion was unable to modify cellular fluorescence even after prolonged treatment (not shown). The rightmost peak shows the fluorescence of a CHO K1 cell clone stably expressing the Tat-GFP fusion protein (see Figure 7A).

E. *Subcellular localization of internalized GST-Tat.* HL3T1 cells were treated with recombinant GST-Tat (1 μg/ml) for the indicated time intervals. After treatment, cells were extensively washed, fixed, and reacted with an anti-Tat antibody followed by recognition with a rhodamine-labeled second antibody (panels on the right side). In the same preparations, nuclei were also visualized by reactivity to Hoechst 33342 (panels on the left side). The two uppermost panels show cells treated for 24 hrs with a recombinant GST protein (1 μg/ml) as a control.

protein, since a GST-GFPfusion was unable to modify the fluorescence pattern of the cells even after prolonged treatment with high protein concentrations (data not shown).

Altogether, these data indicate that functional recombinant Tat is able to enter into the cells with a relatively rapid kinetics and can direct entry of larger protein cargos inside the cells. Flow cytometric analysis of cells treated with the GFPfusion constituted the basis for the further investigations presented in this work.

Inhibition of Tat internalization and LTR transactivation by soluble GAGs

Earlier studies from our (263-265) as well as from other groups (201, 306) have indicated that Tat specifically interacts with heparin and that heparin inhibits Tat internalization. These studies suggested that heparin could compete with cell surface GAGs for binding to Tat. To extend these observations, we analyzed the effects of heparin and other soluble GAGs on the internalization of recombinant GST-Tat-GFP (1 μg/ml) by hamster CHO K1 cells. These experiments were performed by the direct visualization of fluorescent cells that had internalized the protein, by flow cytometry at 12 hours after addition to the cell culture medium. The CHO K1 cell line was chosen since mutants genetically impaired in GAGs biosynthesis are available derived from these cells (see below) - the results obtained with these cells are identical to those observed also with human HeLa cells (not shown). As shown in Figure 3.2, out of the four GAGs tested for their inhibition ability, only heparin inhibited Tat entry in a dose dependent manner. At a concentration as low as 5 μg/ml, heparin almost completely prevented Tat entry. On the contrary, cell treatment with chondroitin sulfate B (dermatan sulfate), chondroitin sulfate A and chondroitin sulfate C did not demonstrate any significant inhibition under our experimental conditions. To demonstrate that the inhibition of Tat uptake by heparin is not the result of some effects of heparin on the cells, pre-incubation experiments were performed by cell incubation with the above mentioned concentrations of GAGs for one hour, followed by extensive washing and incubation with the same concentration of recombinant protein. Unlike the competition experiments, these pre-incubation studies demonstrated very little inhibitory effect of heparin on protein entry (less than 10% inhibition; data not shown).

One of the essential modifications of the GAG chains of cellular proteoglycans is sulfation, which provides a high negative charge density to these molecules. In the

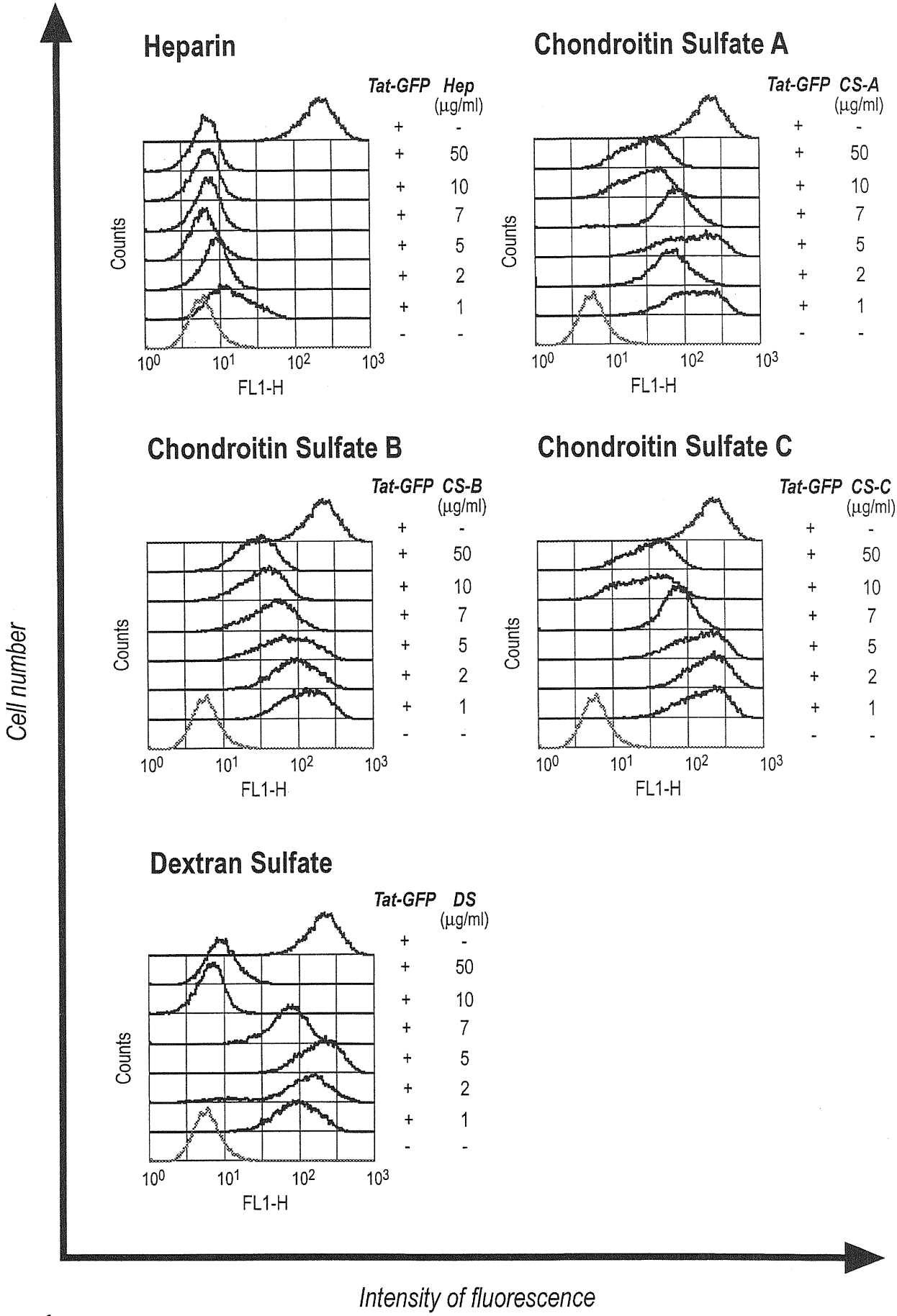


Figure 3.2
Internalization of GST-Tat-GFP in cells treated with soluble GAGs

CHO K1 cells were incubated with 1 μg/ml of recombinant GST-Tat-GFP in the presence of the indicated concentrations of heparin (Hep), chondroitin sulfate A, B, and C (CS-A, -B, and -C respectively) and dextran sulfate (DS). After 12 hrs, cells were extensively washed and analyzed by flow cytometry for the intensity of fluorescence.

past, we had observed that the high affinity Tat- heparin interaction *in vitro* is dependent on the extent of sulfation of the 2-O-, 6-O-, and N-positions of heparin (263), that imparts has higher charge density to this molecule than chondroitin sulfates (262). To understand if inhibition of Tat entry by heparin is structure specific or mediated ionic interactions, competitive inhibition studies were also performed using dextran sulfate (average molecule weight 5,000), another highly sulfated GAG analogue. While heparin was effective at concentrations as low at 2 μg/ml, much higher concentrations (> 10 μg/ml) were necessary for dextran sulfate to inhibit Tat internalization (Fig. 3.2), thus suggesting that Tat-heparin interaction is essentially determined by structure recognition in addition to ionic interactions.

The above described results were further confirmed by determining the ability of different GAGs to inhibit HIV-1 LTR promoter transactivation by the recombinant Tat-GFP protein. CHO K1 cells were transfected with a plasmid containing the reporter CAT gene under the control of the HIV-1 LTR and, after 48 hours, they were incubated with the Tat protein in the presence of different concentrations of GAGs. As shown in Figure 3.3, heparin showed a dose-dependent inhibition of LTR transactivation. Differently from the internalization studies of Figure 3.2, heparin at a concentration of 5 μg/ml inhibited CAT reporter gene expression only of 50%, while the protein could be not visualized in the cells at the same concentration. This observation is in agreement with the notion that only very limited amounts of Tat are sufficient to exert a still potent transactivation activity. Contrary to heparin, none of the chondroitin sulfates significantly inhibited LTR activation, even at much higher concentrations. Although much less efficient than heparin, dextran sulfate at a concentration of 100 μg/ml inhibited Tat transactivation, in agreement with the data obtained by flow cytometric analysis that indicated the reduced Tat entry inside the cells by this treatment.

Altogether, these observations reinforce the notion that Tat directly interacts with heparin, suggest that this interaction is both ionic and structural, and suggests a role for cell surface HS proteoglycans in Tat uptake by the cells.

Cell surface HS proteoglycans mediate cellular uptake of Tat

The observation that Tat entry is inhibited by GAGs structurally similar to cell membrane HS, prompted us to study Tat internalization after removal of different cell

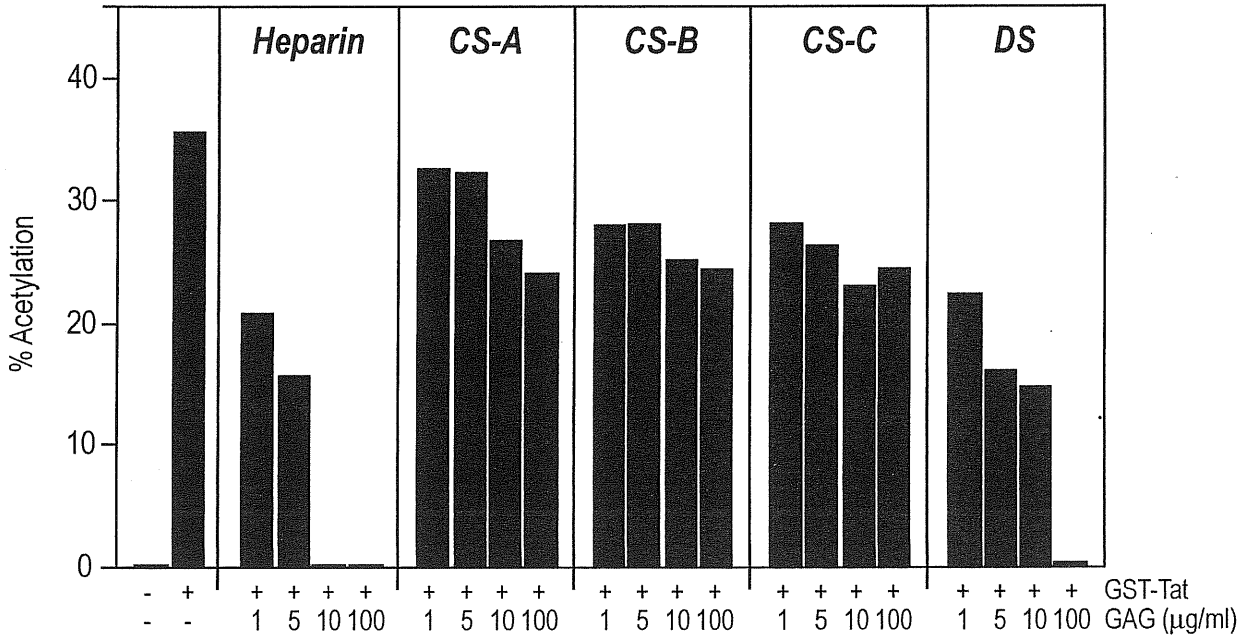


Figure 3.3
Inhibition of Tat transactivation by soluble GAGs

CHO K1 cells were transfected with an LTR-CAT plasmid and, after 48 hrs, treated with 1 µg/ml GST-Tat-GFP for 14 hrs, in the presence of the indicated concentrations of soluble GAGs. CAT assays were performed after additional 36 hrs.

surface GAGs. Exposed GAGs of CHO K1 cells were digested with the GAG lyases indicated in Figure 3.4, then cells were treated with the Tat-GFP protein and were analyzed by flow cytometry 5 hours later to assess the amount of intracellular protein. Heparinase I and heparinase III cleave distinct linkages found in HS. Chondroitinase ABC cleaves at a linkage found in all chondroitin sulfates, including determatan sulfate (chondroitin sulfate B), while chondroitinase AC cleaves only chondroiting sulfates A and C (190, 191). Cell treatment with 4 mIU of heparinase III almost completely inhibited Tat entry, again suggesting the requirement of HS for Tat internalization. Heparinase I, which is known to be active on heparin but at a much lesser extent on HS (78, 79) showed detectable inhibition of Tat internalization only at higher concentrations (16 and 30 mIU). On the contrary, treatments with chondroitinases AC and ABC were completely ineffective and Tat protein entry was equivalent to that of control cells.

These results further indicate that cell surface HS proteoglycans act as major receptors for Tat internalization.

Tat entry is impaired in mutant cell lines that are defective in proteoglycan biosynthesis.

To provide a genetic proof that cell surface proteoglycans mediate Tat entry, we analyzed different mutant cell lines defective in GAG biosynthesis, which were established by J.D. Esko and collaborators by mutagenizing parental CHO K1 cells (reviewed in 259). The pgs A-745 cell line does not produce detectable levels of proteoglycans since it lacks xylosyltransferase, an enzyme necessary for the initiation of GAG synthesis. Mutant pgs B-618 has a defect in the galactosyltransferase-I enzyme gene and makes about 15% of the amount of proteoglycans synthesis of wild type cells. Cell line pgs E-606 is partially deficient in HS N-sulfotransferase and produces an undersulfated form of HS proteoglycan. The cell line pgs D-677 has a single mutation that affects both N-acetyl-glucosaminyl-transferase and glucuronosyl-transferase activities that are necessary for the polymerization of HS disaccharide chains and does not synthesize any HS proteoglycan. Instead, this mutant cell line produces approximately three times more chondroitin sulfate than wild type cells. Finally, mutant cell line pgs C-605 has a defect in a saturable, 4-acetamido-4-isothiocyanostilbene-2,2'-disulfonic acid-sensitive transport system required for sulfate

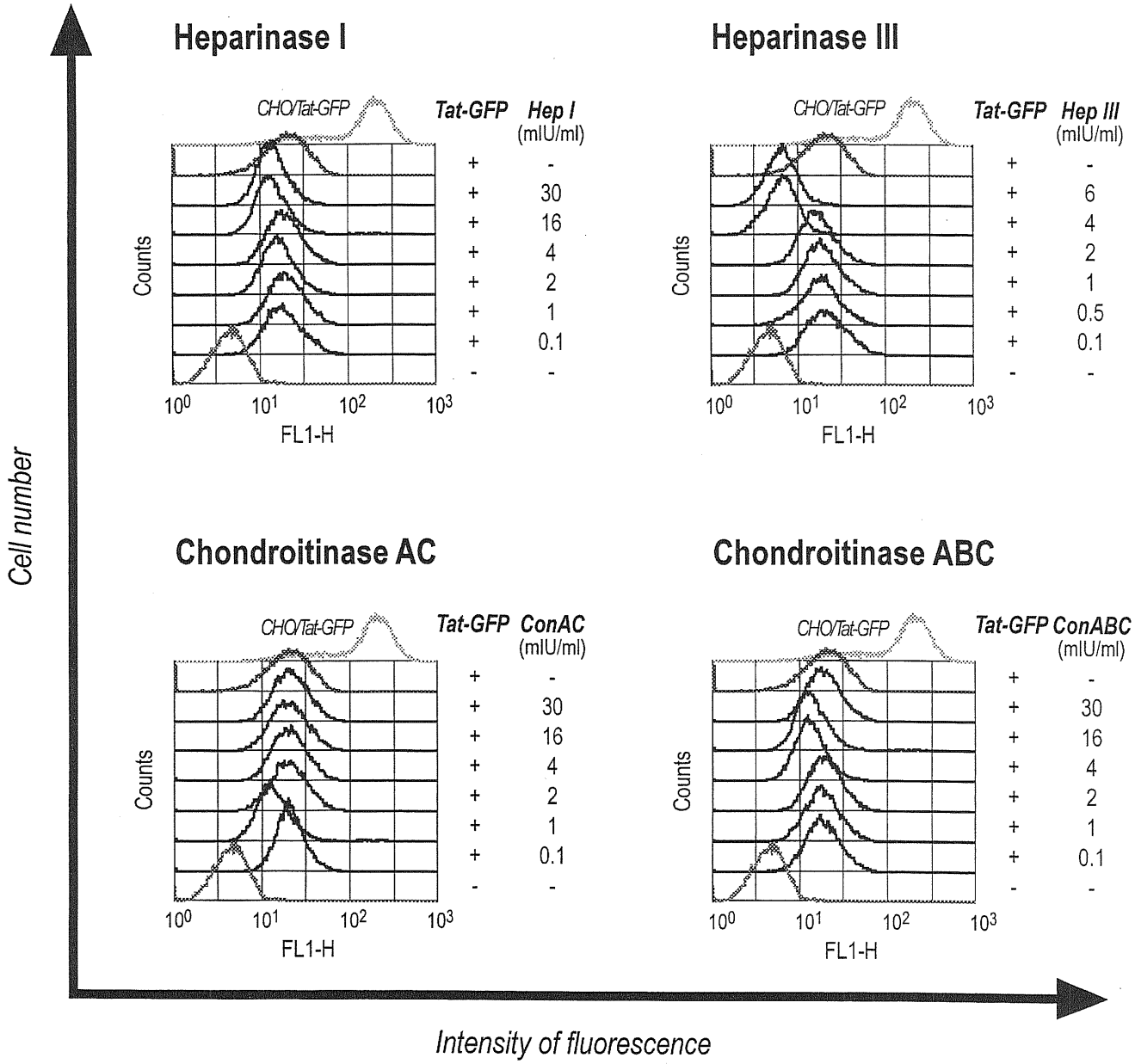


Figure 3.4
Effects of GAG lyases on the uptake of extracellular Tat

CHO K1 cells were incubated with the indicated GAG lyases for 40 min and then incubated with 1 μg/ml GST-Tat-GFP for 5 hours. After this time period, cells were extensively washed and analyzed by flow cytometry. Hep I: heparinase I; hep III: heparinase III; ConAC: chondroitinase AC; ConABC: chondroitinase ABC. In each panel, the uppermost FACS profile shows fluorescence of a CHO K1 cells clone stably expressing Tat-GPF.

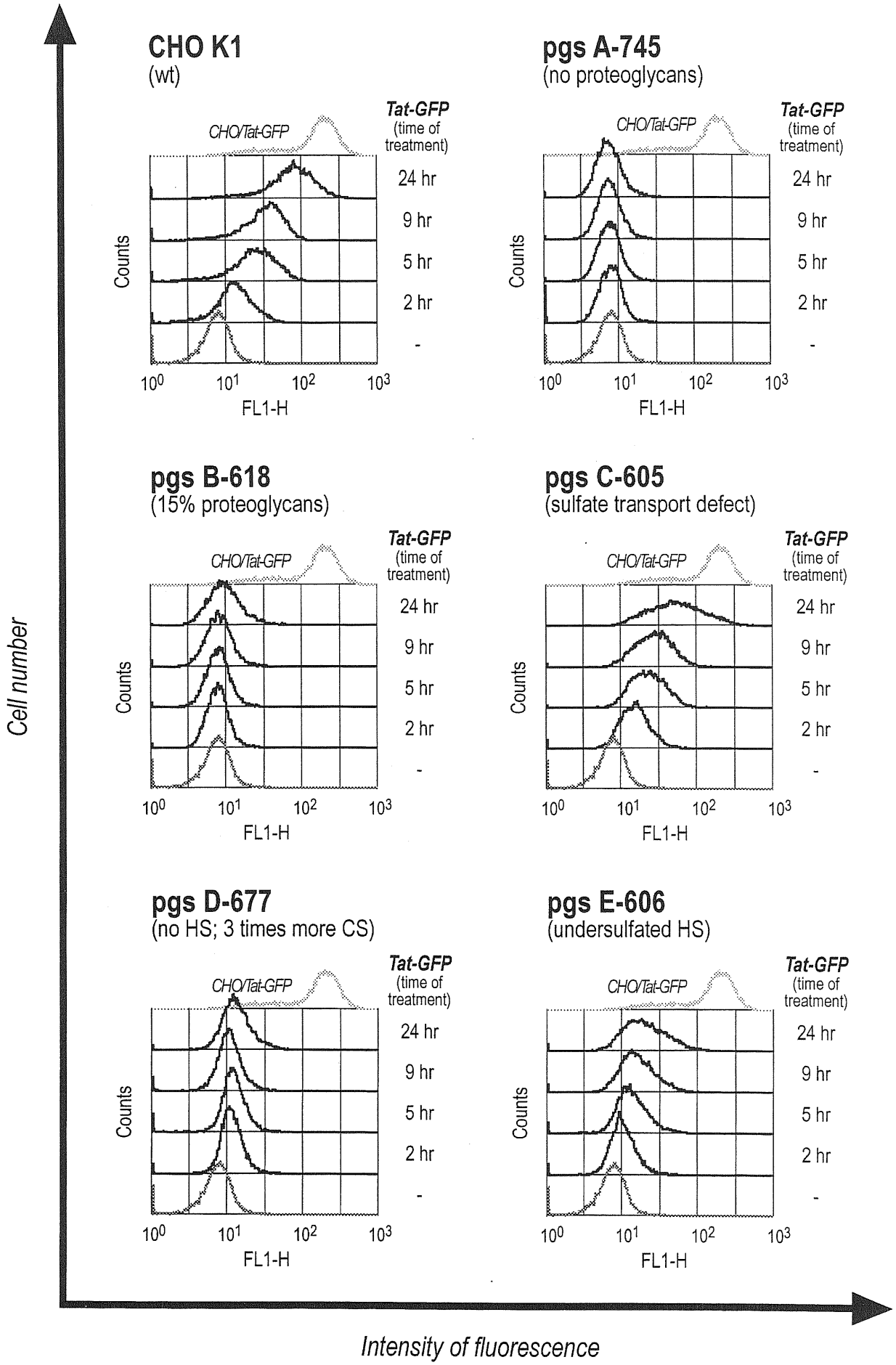


Figure 3.5
Tat internalization into cells genetically defective in GAG biosynthesis

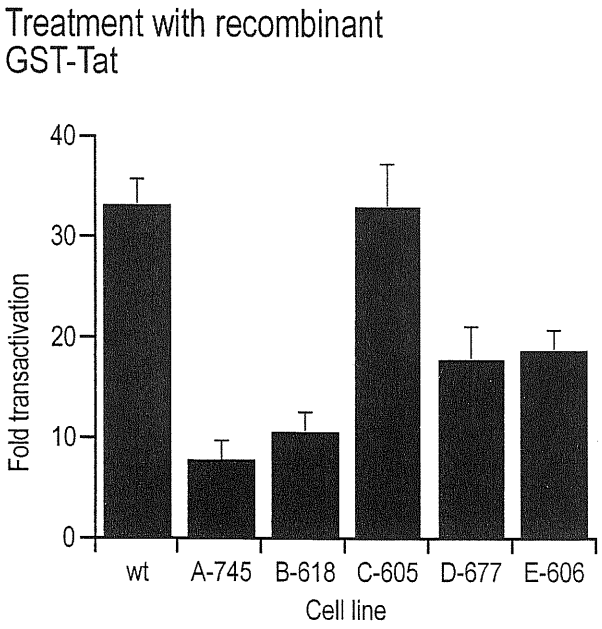
Wild type CHO K1 cells and the indicated cell clones mutated at different steps in GAG biosynthesis were treated with 1 μg/ml GST-Tat-GFP and analyzed by flow cytometry after 2, 5, 9 and 24 hrs of treatment. In each panel, the uppermost FACS profile shows fluorescence of a CHO K1 cells clone stably expressing Tat-GPF (see Figure 7). The main recognized defect of each cell line is indicated.

uptake. Despite a dramatic reduction in $^{35}SO_4$ incorporation, the mutant synthesizes sulfated heparan and chondroitin chains by using the inorganic sulfate produced from oxidative metabolism of cystein and methionine (94).

To demonstrate the involvement of HS-proteoglycans in Tat entry, the wild type and mutant CHO cell lines were treated by the addition of 1 μg/ml GST-Tat-GFP protein to the cell culture medium. Cell-associated fluorescence was analyzed at different time intervals by flow cytometric analysis. As indicated in Figure 3.5, internalization of the GST-Tat-GFP protein was clearly detectable in the wild type CHO cells and it was proportional to the time of treatment. In pgs A-745 (no proteoglycan production) Tat entry was undetectable, and it was severely impaired also in pgs B-618 cells, in which proteoglycan production is strongly reduced. Cell line pgs C-605, which is deficient in sulfate transport, behaved like wild type cells, consistent with the normal production of proteoglycans in this cell line. Interestingly, almost no GST-Tat-GFP protein internalization was observed in pgs D-677 cells, which do not produce any HS but possess three times more chondroitin sulfate proteoglycans than the wild type CHO cells. Additionally, Tat entry was impaired also in the mutant pgs E-606 cell line, which produces an undersulfated form of HS, further supporting the notion that N-sulfation of HS is an important determinant for Tat/HS recognition (263).

The ability of these cells to support Tat-mediated LTR transactivation was also tested by transfecting an LTR-reporter gene construct and measuring transactivation either after treatment with recombinant Tat (Figure 3.6 A) or co-transfection of a Tat-expressing plasmid. All cells efficiently supported Tat functions when this was expressed as an endogenous protein, indicating that the cellular mechanisms mediating Tat transactivation were intact in all these cell lines (Figure 3.6 B). However, mutants pgs A-745 and pgs B-618, not producing or producing only 15% proteoglycan respectively as compared to wild type cells were clearly impaired in LTR transactivation by the exogenously added Tat protein (Figure 3.6 A). Consistent with the Tat-GFP internalization data, the specific role of HS proteoglycans in Tat entry was indicated by the results obtained with the mutant cell lines pgs D-677, not producing HS but with overproduction of chondroitin sulfate, and pgs E-606, producing an undersulfated form of HS. In both cell lines, LTR transactivation by recombinant Tat was clearly impaired. On the contrary, transactivation was normal in pgs C-605 cells, carrying a genetic defect in sulfate transport.

A



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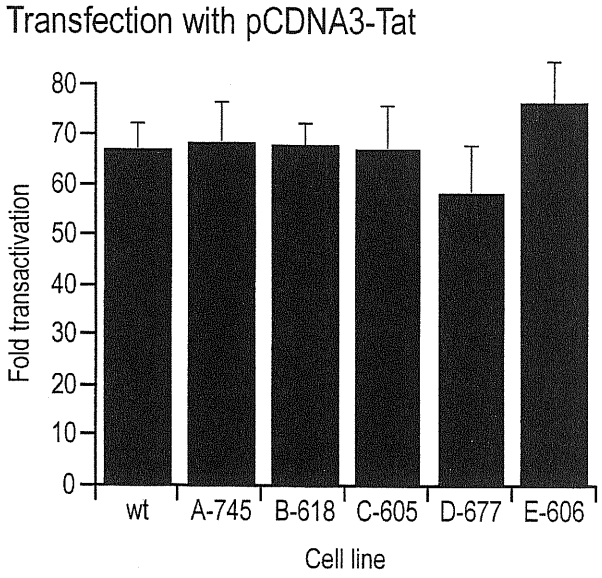


Figure 3.6
HIV-1 LTR transactivation in cells genetically defective in GAG biosynthesis

Wild type CHO K1 cells and the indicated cell clones mutated at different steps in GAG biosynthesis were transfected with an LTR-CAT reporter plasmid (10 μg) and either treated with recombinant GST-Tat (20 ng/ml; panel A) or co-transfected with a Tat expressing plasmid (1 μg; panel B). CAT assays were performed after 48 hrs. The results shown represent the average values and the standard deviations obtained in several (at least three) independent transfections.

Overall, these data indicate that HS (but not chondroitin sulfate) GAG moieties of cell surface proteoglycans are the major cellular receptors for Tat internalization. In addition, the reduced ability of Tat entry in pgs E-606 cells suggests that the degree of sulfation of HS is an important factor in determining the efficiency of this process.

Involvement of proteglycans in transcellular transactivation

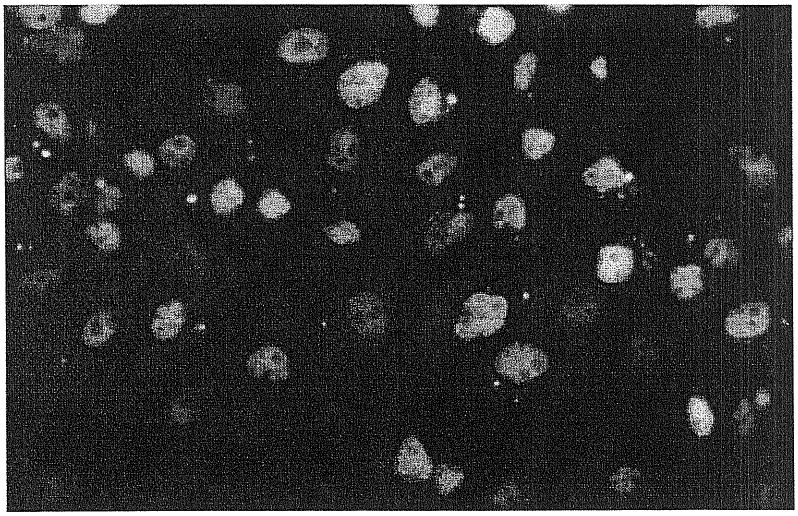
The results of the experiments reported above indicate a major role of HS proteoglycans in cell internalization of recombinant Tat added to the culture medium. To understand at what extent these findings also apply to the physiological extracellular trafficking of Tat, and to know whether cellular proteoglycans also play a role in Tat export from the cells, we obtained two cell lines constitutively expressing Tat-GFP. Wild type CHO K1 and proteoglycan-negative pgs A-745 cells were transfected with a plasmid vector expressing Tat-GFP from the CMV immediate early promoter and selected for antibiotic resistance. The two cell lines were undistinguishable by fluorescent microscopic examination, and showed nuclear localization of the expressed protein, with exclusion of the nucleolus (shown for CHO/Tat-GFP cells in the Figure 3.7 A).

When the wild type CHO/Tat-GFP cell line was co-cultured for 72 hours with normal CHO K1 or with HeLa cells previously transfected with an LTR-reporter gene construct (Fig. 3.7 B), transactivation of the LTR promoter contained in the latter cells could be readily detected, indicating release of the Tat protein by the constitutively expressing cells. In the same assay, when the reporter cells carrying the LTR construct were the proteoglycan negative pgs A-745 mutant, transactivation did not occur. This observation indicates that the conclusions drawn from the studies with the exogenously added recombinant Tat protein hold true also for the endogenously expressed protein released from producing cells.

Interestingly, pgs A-745/Tat-GFP cells stably producing Tat-GFP were still proficient in releasing the protein extracellularly, as concluded from the results obtained by co-culturing these cells with wild type CHO K1 or HeLa cells carrying the LTR-reporter gene construct, which showed normal transcellular transactivation.

From the results of these experiments, it can be concluded that, during the process of transcellular transactivation, Tat requires cell proteoglycans as receptors for cell

A



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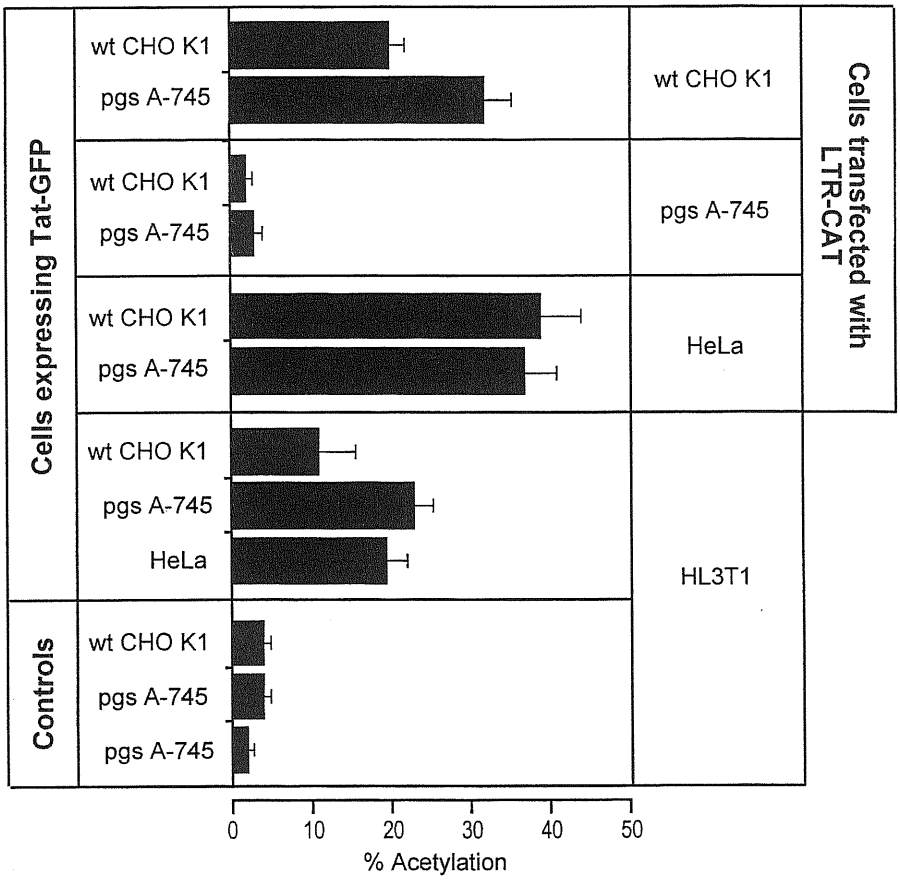

Figure 3.7
Transcellular transactivation

A. Fluorescence pattern of CHO clones producing Tat-GFP. Cellular clones stably expressing Tat-GFP were obtained for CHO K1 cells (shown in the figure) and pgs A745 cells, impaired in the production of proteoglycans. The protein showed predominant nuclear localization with exclusion of the nucleoli. The same pattern was observed for both cell lines.
B. Results of co-culture experiments. Wild type CHO/Tat-GFP and mutated pgs A-745/Tat-GPF cells were co-cultured (1:1 ratio) for 48 hours with wt CHO K1, pgs A-745, and HeLa cells which had been previously transfected with an LTR-CAT reporter plasmid. The histograms indicate the average levels and standard deviations of LTR-CAT transactivation obtained in three independent experiments. As a further control of the transcellular transactivation of the Tat-GFP protein produced by CHO and pgs A-745 cells, these were also co-cultured with HL3T1 cells, a HeLa derivative containing an integrated LTR-CAT construct.

internalization, while Tat export from the cells occurs by a still undefined mechanism not involving these proteins.

III - DISCUSSION

Intercellular trafficking of Tat

The HIV-1 Tat protein, an essential and potent activator of viral gene expression, has the unique property of being released from infected cells and entering neighboring cells in an active form. This mechanism may explain the burst in replication, during the early phase of HIV infection, where synchronised virion replication takes place (242). Tat also performs a number of biological effects, in addition to LTR transactivation, which might be responsible for some AIDS associated syndromes.

Expression of the HIV1-Tat gene in transgenic mice results in angiogenic skin lesions and increased lincidence of adenocarcinoma, lymphomas and hepatocarcinomas (59). Tat is a growth factor for Kaposi's sarcoma-derived cells and for endothelial cells (6, 19, 20, 91) and exerts angiogenic activity *in vivo* (6, 8, 60, 91). In addition, Tat could be responsible of some neurological complications associated with HIV disease progression (80, 142, 266, 308). The capacity of anti-Tat antibodies to delay HIV-1 replication in infected peripheral blood mononucleated cells (253, 332) and the direct correlation between the levels of natural anti-Tat antibodies and those of p24 antigen in HIV-seropositive subjects (253), suggest that extracellular Tat may be involved in the progression of the disease. On these bases, it has been recently suggested that Tat might be a specific target for AIDS vaccination (57).

It has long been known that heparin can antagonize some of the effects of extracellular Tat, suggesting that it could compete for binding of the protein to HS-containing GAGs, which are the major constituents of the proteoglycans present on the surface of cells and in the extracellular matrix (26). The results presented in this thesis actually provide genetic and biochemical evidence that cell surface HS proteoglycans are the major receptors for HIV-1 Tat protein internalization into the cell. The role of HS proteoglycans in Tat internalisation are supported by several findings. First, intracellular delivery of a recombinant Tat-GFP protein was completely inhibited by heparin but not by other soluble GAGs. Second, Tat entry was impaired after cell treatment with GAG lyases specific for HS, but not for chondroitin sulfate moieties on cell surface. Third, cell lines with genetic defects in the cellular pathway involved in the production of proteoglycans did not allow Tat-GFP entry inside the cells. In particular, the use of the

pgs D-677 cell line, which overproduces chondroitin sulfate GAGs but is deficient in HS synthesis, further indicated the specific role of HS proteoglycans for Tat uptake. The requirement of HS GAGs for Tat internalization is consistent with a large body of evidence that we and others have obtained in the last few years showing that free heparin inhibits the uptake of extracellular Tat and its HIV-1 LTR transactivation activity (201, 263-265, 306). Similarly, the angiogenic, mitogenic and chemiotactic activity of Tat on endothelial cells are also modulated by heparin (5, 6, 9, 19, 20, 44, 51, 59, 60, 91, 193, 202, 216). In addition, Tat is able to enter into a wide variety of human, rodents and simian cell line, indicating that it utilizes a ubiquitous cell surface molecule(s) for its cellular entry. This is consistent with the observation that proteoglycans are produced by most cell types and are ubiquitously expressed throughout the animal kingdom (48, 174).

In the competition assay with free GAGs, inhibition of Tat entry was completely abolished at concentrations of heparin higher than 2 μg/ml. At five time higher concentrations, chondroitin sulfate B (dermatan sulfate) also exhibited some activity. This effect can be explained, as this GAG is the only chondroitin that contains some iduronic acid monosaccharides, which are common to heparin/HS (93). Consistent with this consideration, it is also to be noticed that HIV-1 LTR transactivation by extracellular Tat was comparatively less impaired in case of pgs D-677 cell line, which overexpress chondroitin sulfate proteoglycans than pgs A-745 cell line, cells deficient in proteoglycan synthesis.

The cell line pgs A-745, which does not produce any proteoglycans, also demonstrated a weak CAT activity. This finding suggest that either Tat utilizes other yet unidentified cell surface molecule for its internalization or that the very low level of xylotransferase activity, which is still present in pgs A-745 cells (95, 96) is sufficient for the production of limited amounts of proteoglycans. In this respect, it is commonly observed that few molecules of Tat protein are sufficient to produce a significant level of transactivation. An alternative explanation for the LTR transactivation observed in the pgs A-745 cells is that transcriptional activation in these cells is not actually due to physical translocation of Tat to the cell nucleus, but is mediated by intracellular second messengers. In this respect, extracellular Tat has been shown to interact on other cell types with integrin receptors $\alpha_5\beta_1$ and $\alpha_V\beta_3$ (20, 303), with the Flk-1/KDR receptor (9), with CCR2 and CCR3 chemokine receptors (7) and with a still unidentified membrane

protein of 90 kDa (307). Whether some of these receptor molecules are expressed in CHO cells and at what extend they could mediate intracellular signaling leading to LTR activation is a subject for further investigation.

A common peptidic motif for HS proteoglycan binding consists of a region rich in basic amino acids flanked by hydrophobic residues (259). The basic domain of Tat also contains this kind of configuration, and mutagenesis studies indicated that it is actually required for heparin binding (265). The extent of GAG sulfation is important for this interaction. Previous studies have indicated that selective 2-O-, 6-O-, total-O-desulfation, or N-desulfation/N-acetylation dramatically reduced the capacity of heparin to bind GST-Tat (263). Consistent with this finding are two observations made in the course of this work. First, the pgs E-606 cell lines, producing under sulfated heparan sulfate, is impaired in Tat internalization as compared to wild type cells. Second, treatment of wild type cells with high concentrations of dextran sulfate, a highly sulfated GAG analogue containing three sulfate groups per residue, competitively inhibited Tat entry. These findings indicate that, in addition to size and structure of the GAG backbone, also the degree of sulfation of HS is an important determinant of specificity for Tat interaction. It is conceivable that positively charged basic residues in the basic domain of Tat, which is known to interact with heparin, may support electrostatic interactions with negatively charged sulfate groups of HS proteoglycans. These interactions are not randomly scattered, but are likely to be specific not only to the degree of sulfation, but also to the arrangement of the sulfate groups along the polysaccharide chain. For example, polysulfated β-cyclodextrin is unable to bind Tat and to inhibit its LTR-transactivation activity, although it carries 14 sulfate groups per molecule (265). The above notion is also supported by the observation that GAGs with a similar SO_3/COO^- ratio (e.g. chondroitin sulfate A, C and dermatan sulfate), but which differ in their backbone conformation and charge distribution display different inhibitory activity on Tat transactivation (263).

The specificity of HS proteoglycans for Tat internalization into the cell was also confirmed by the GAG lyases assays. Treatment of cells with heparitinase (heparinase-III) impaired the cellular internalization capacity in a dose dependent manner. In contrast, treatment with chondroitinase-ABC, which cleaves out all types of chondroitin sulfates from the cell surface, was not able to inhibit Tat uptake, and same was true also for Chondroitinase-AC, which digests specifically chondroitin sulfates A and C.

The lower activity of heparinase-I in this assay can be explained by its lower enzymatic activity for heparan sulfate GAGs as compared to heparitinase (78, 79).

Cell surface proteoglycans, especially those contain HS GAGs carbohydrate chains, are rapidly internalized and degraded (323). Thus membrane proteoglycans may act directly to internalize ligands that bind to their GAG chains by a receptor mediated endocytosis pathway (93). Such a mechanism of entry has already been described for other HS proteoglycan ligands, including basic fibroblast growth factor and lipoprotein lipase (257, 271). Consistent with this finding, chloroquine, which has been used in most of the experiments presented in this thesis, favors Tat uptake by modifying the pH of endolysosomal vesicles thus preventing protein degradation (201). Chloroquine is also known to inhibit the intracellular degradation of the heparan sulfate proteoglycans by the same mechanism (153).

Internalization of several ligands of HS proteoglycans, including AAV-2 (283), is favored by the interaction with cell surface integrins. The second exon of Tat actually contains an RGD motif (20, 33), the minimal sequence required for interaction with different integrins. However, extracellular Tat protein with or without the second exon display identical transactivation activity (201, 263), suggesting that interaction of Tat with integrins through the RGD motif is not a major determinant for Tat internalization. Tat can also bind to integrin $\alpha_V\beta_5$ in an RGD-independent manner through its basic domain (303). For adenovirus, $\alpha_V\beta_5$ integrin, although not required for viral attachment, was shown to be involved in both viral internalisation and endosome escape by inducing membrane permeabilization. (316, 317). This two-step mechanism (cell attachment followed by cell internalization) could hold true also for Tat. Tat could bind to the cell surface via interaction with heparan sulfate and then internalization could favored by $\alpha_V\beta_5$ integrin interaction. A two-step mechanism is supported by the observation that, in L8 cells, antibodies against Tat are able to inhibit Tat attachment to the cells but not Tat uptake, and by the finding that cells lacking $\alpha_V\beta_5$ integrin are still able to bind Tat (33, 303). Additionally, a peptide encompassing the basic domain of Tat significantly enhanced uptake and transactivation of the Tat protein; this effect was obtained by promoting endocytosis of the protein, since transactivation was not enhanced when the peptides were scrape loaded with Tat (201). Finally, while the results presented in this work indicate that Tat internalization requires the function of surface HS, it is still unclear what is the mechanism that determines escape of Tat from

the internalized endosomal vesicles. $\alpha_V\beta_5$ or other integrins are possible candidates for this activity. Further investigations are clearly required to address these issues.

Previous observations had shown that polysulfated compounds, including sulfated GAGs, may act as inhibitors of HIV infection. This activity is partially accounted for by the prevention of HIV adsorption and internalization in CD4+ positive cells (16, 319) as a consequence of the disruption of the interaction of gp120 with CD4 (192, 217) and/or with cell associated HS (43, 211). The finding that HS proteoglycans are essential for Tat internalization adds another possible mechanism by which these polysulfated compounds may work. Pharmacological interference preventing Tat-HS interactions may be a target for blocking AIDS-associated pathologies, including Kaposi's sarcoma and, possibly, HIV replication itself. Specifically tailored HS analogs with high affinity for Tat protein might allow the development of potent extracellular Tat antagonist of possible therapeutic value. In particular, the capacity of HS analogs to interact with the highly conserved basic region of Tat may overcome the problems related to the heterogeneity and high rate of mutation that hampers the currently available drug treatments of HIV infection.

Chapter IV

TAT AND DNA-PK

IV - INTRODUCTION

Phosphorylation is the most common and important mechanism of acute and reversible regulation of protein function. Studies of mammalian cells metabolically labeled with (^{32}P) orthophosphate suggest that as many as one third of all cellular proteins are covalently modified by protein phosphorylation (140, 151). In particular, phosphorylation is a well defined mechanism also for the modulation of gene expression. This notion also specifically applies to the transcriptional regulation of the HIV-1 LTR.

More specifically related to the functions of the HIV-1 Tat protein is the finding that the elongation properties of Tat are connected to its association with kinases able to phosphorylate the CTD domain of human RNA polymerase II (for discussion, see above). One of the kinases that are known to phosphorylate the RNA polymerase II CTD is the DNA-dependent protein kinase (DNA-PK), a nuclear kinase that specifically requires association with DNA for its activity (12, 47, 186). DNA-PK plays a pivotal role in the regulation of different DNA transactions, including transcription, replication and DNA repair. The DNA-PK holoenzyme consists of a 350-kDa catalytic subunit (DNA-PKcs) (136), which is a serine/threonine kinase, and a regulatory component known as Ku autoantigen (127). Ku is a heterodimer composed of a 70 kDa (254) and a 80 kDa (324) subunits. The 70 kDa subunit possesses ATPase as well as DNA helicase activities (293). Ku and DNA-PK have been implicated in transcriptional repression (122, 178), DNA repair (187, 285), and immunoglobulin gene rearrangements (VDJ) recombination (30, 100). Mutations in DNA-PKcs and Ku subunits causes the mouse SCID phenotype (30, 227, 285).

DNA-PK-activity is widely distributed, at least among higher eukaryotes. So far, however, DNA-PKcs has been purified extensively only from human cells. Recent studies have shown that DNA-PK is much more abundant (more than 50 times) in cultured somatic cells from primates than in such cells from rodent or insects (13), suggesting that DNA-activated phosphorylation has become much more important in primates. DNA-activated phosphorylation has not been reported in extracts of plants, yeast or bacteria. DNA-PKcs is one of the best characterized member of PIKL (PI3-kinase-like) family, but like several other members of the family, DNA-PKcs is not able

to phosphorylate inositol phospholipids. Thus, unlike its other familymates, DNA-PK is exclusively a protein kinase.

The DNA-binding component of DNA-PK is the heterodimeric Ku factor, one of the most abundant DNA-end binding protein in human cells. Ku was discovered as a protein in normal human cells that react with the sera from some patients suffering from the autoimmune diseases lupus erythematosus or scleroderma-polymyositis overlap syndrome (215). *In vitro*, Ku binds to the ends of naked DNA and translocates along DNA fragments in an ATP-independent manner (72). Importantly, and consistent with genetic data indicating that Ku70 and Ku80 are functionally interdependent, neither subunit alone can bind DNA efficiently (229). Ku generally binds non-specifically to free dsDNA ends of a variety of DNA structures; however, it does not bind to closed circular DNA (85, 328).

The notion that DNA-PK can phosphorylate the RNA pol II CTD and that both DNA-PKcs and Ku are components of the RNA polymerase-II holoenzyme (199), the observation that Ku specifically recognizes stem-loop structures and, in particular, that it binds to HIV-1 TAR RNA (164), and the finding that Tat interacts *in vitro* with DNA-PK (54) prompted us to investigate whether the Tat protein itself could be a substrate for DNA-PK phosphorylation.

IV - RESULTS

The Tat protein is a substrate for DNA-PK phosphorylation

The analysis of the primary amino acid sequence of Tat revealed that Tat carries three potential target sites for phosphorylation by DNA-PK (Fig. 4.1). In fact, each serine residue at positions 16, 62 and 75 was found to be followed by glutamine, which makes the configuration of one of the most common target sites of DNA-PK (18). To test whether HIV-1 Tat protein is a substrate of DNA-PK *in vitro*, we employed DNase/RNase treated recombinant GST-Tat in our phosphorylation assays using purified DNA-PK (Fig. 4.2). As shown in figure 4.3, the Tat protein is an efficient substrate for DNA-PK in the presence of DNA both when fused to the GST moiety and after its separation by proteolytic cleavage with thrombin. These results clearly demonstrate that phosphorylation relies strictly on the presence of DNA (lanes 2 and 4), being Tat phosphorylation was undetectable in the absence of it (lanes 1 & 3).

Serine residues at positions 16 and 62 are the target sites for DNA-PK phosphorylation

To precisely map the position of the phosphorylation site(s) in the HIV-1 Tat protein, we exploited a set of 8 peptides, each one 20 amino acids long, spanning the entire Tat sequence and overlapping by 10 amino acids. The results of the phosphorylation assays using these peptides are shown in Figure 4.4. Phosphorylation of each peptide was assayed in the presence and in the absence of DNA. The peptides encompassing the serines at positions 16 or 62 were phosphorylated by DNA-PK in presence of DNA (lanes 2, 4, 12 and 13) while, the peptide including the DNA-PK target site at position 75 was not (lane 16).

Phosphorylation of Tat *in vivo*

To test whether Tat is a substrate for phosphorylation *in vivo*, we performed *in vivo* phosphorylation assays. We transfected cells either with a Tat expressing plasmid or we provided exogenous recombinant GST-Tat to the cell culture medium. Cellular proteins were then labeled with ^{32}P as described in Experimental Procedures. After five

A

1 Met Glu Pro Val Asp Pro Arg Leu
9 Glu Pro Trp Lys His Pro Gly Ser
17 Gln Pro Lys Thr Ala Cys Thr Asn
25 Cys Tyr Cys Lys Lys Cys Cys Phe
33 His Cys Gln Val Cys Phe Ile Thr
41 Lys Ala Leu Gly Ile Ser Tyr Gly
49 Arg Lys Lys Arg Arg Gln Arg Arg
57 Arg Pro Pro Gln Gly Ser Gln Thr
65 His Gln Val Ser Leu Ser Lys Gln
73 Pro Thr Ser Gln Ser Arg Gly Asp
81 Pro Thr Gly Pro Lys Glu

B

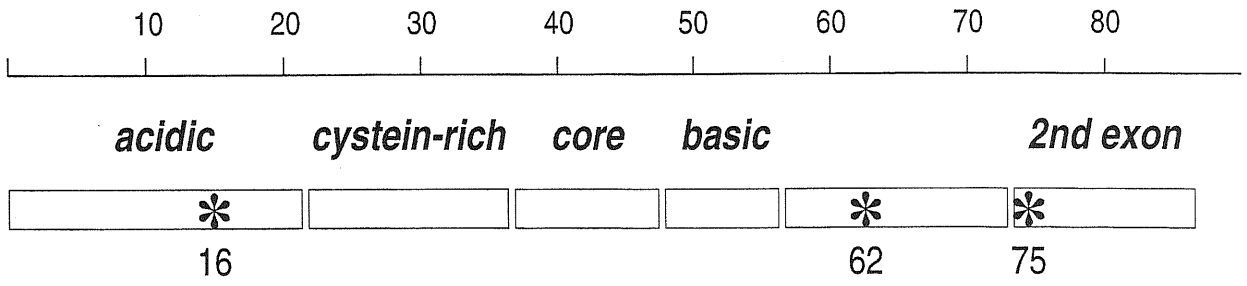

Figure 4.1
HIV-1 Tat (isolate HXB2): potential phosphorylation sites by DNA-PK

A. Sequence analysis of Tat protein with indication of three most prevalent DNA-PK targeting sites (Ser/Thr followed by Gln) at positions 16, 62 and 75.

B. Schematic representation of the positions of DNA-PK targetting sites in different Tat domains.

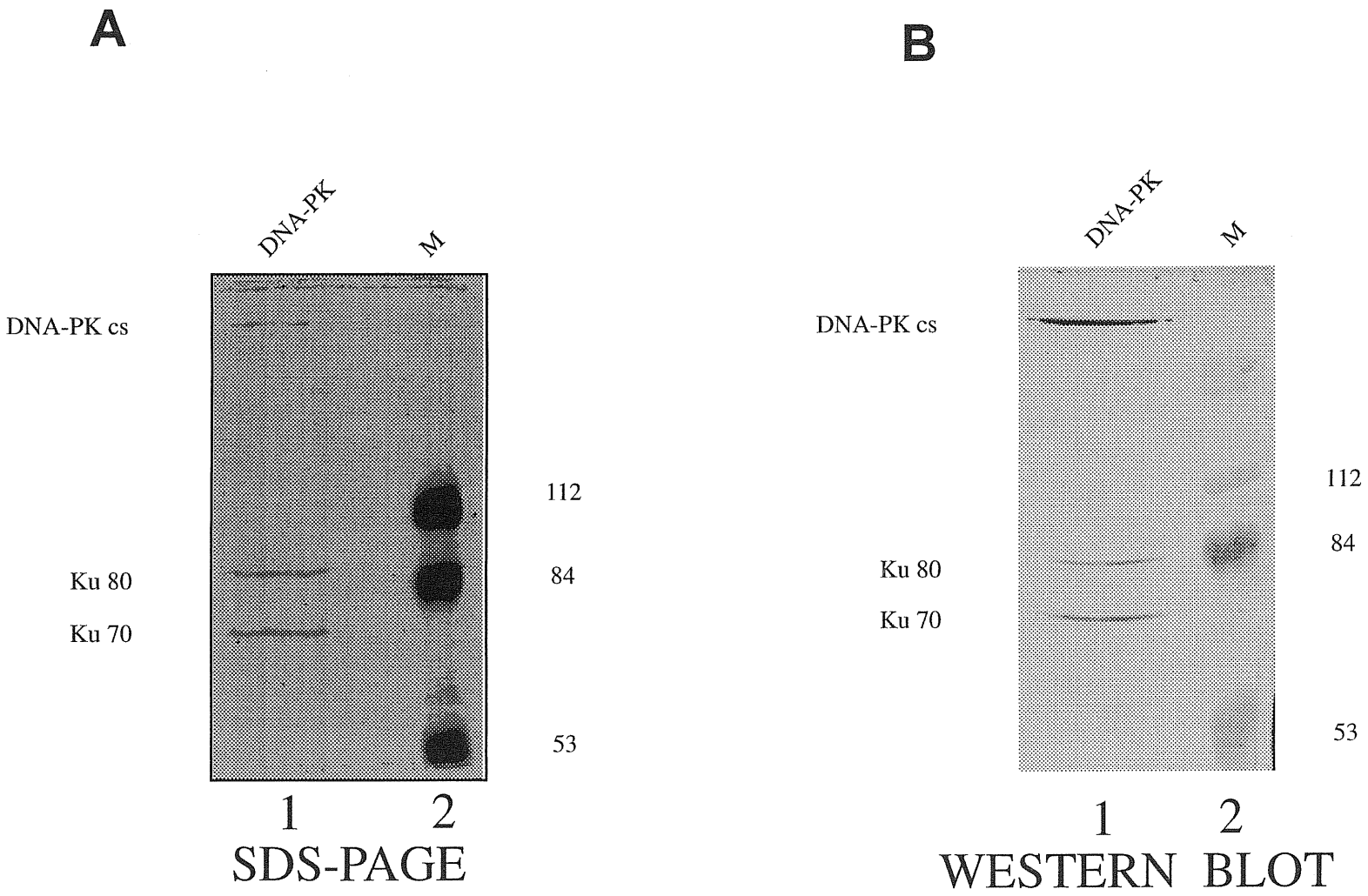

Figure 4.2
Purification of DNA-PK

A. Purified DNA-PK proteins were resolved on a 10% SDS-PAGE and stained with Coomassie blue.
Lane 1, 25 μl of purified DNA-PK; Lane 2, Protein molecular mass standards.

B. Western blot analysis of the purified DNA-PK by using specific antibodies and developed, as described in Experimental Procedures.
Lane 1, 10 μl of purified DNA-PK; Lane 2, Prestained protein molecular mass standards.

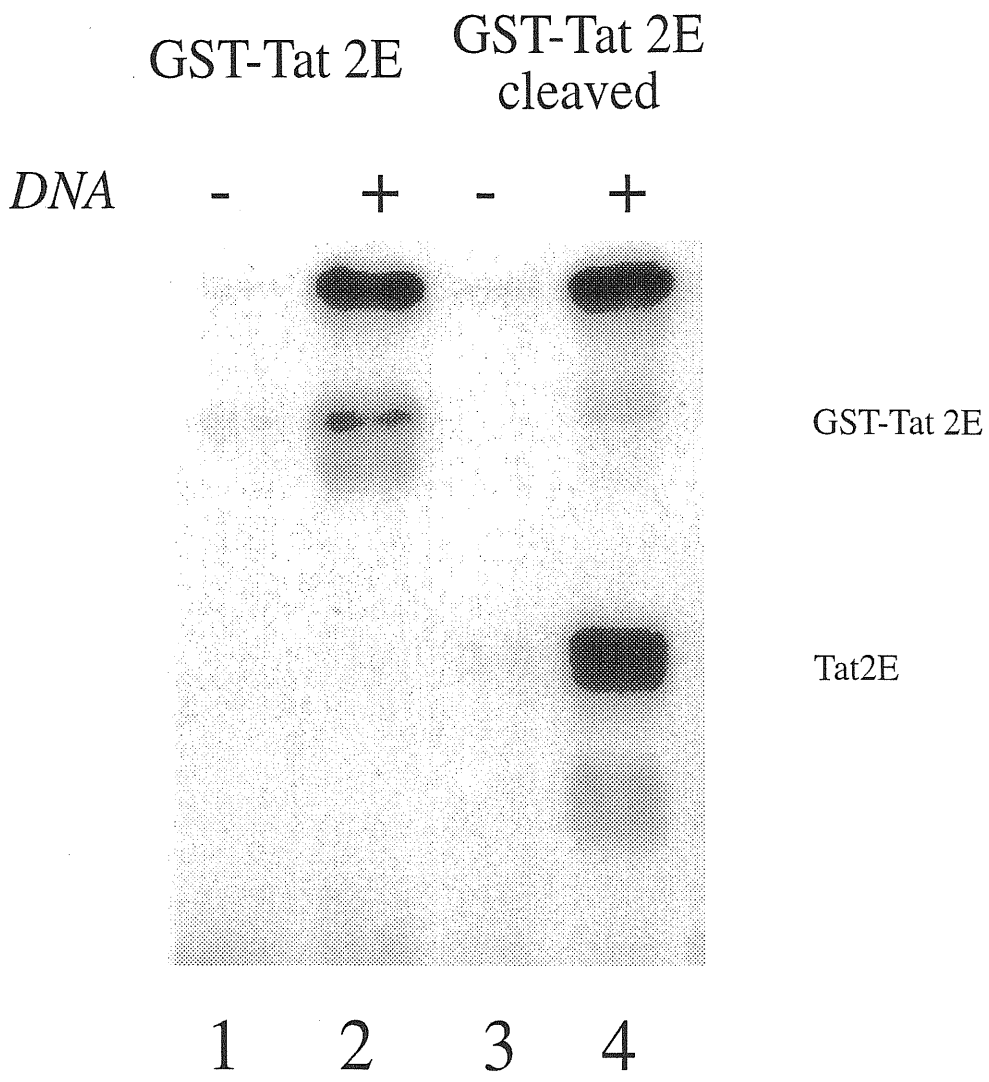

Figure 4.3
Purified DNA-PK phosphorylates recombinant Tat protein

Lanes 1 and 2, Phosphorylation of GST-Tat protein by DNA-PK in the absence (1) or in the presence of DNA (2).

Lanes 3 and 4, Phosphorylation of thrombin-cleaved GST-Tat protein by DNA-PK in the absence (3) or in the presence of DNA (4).

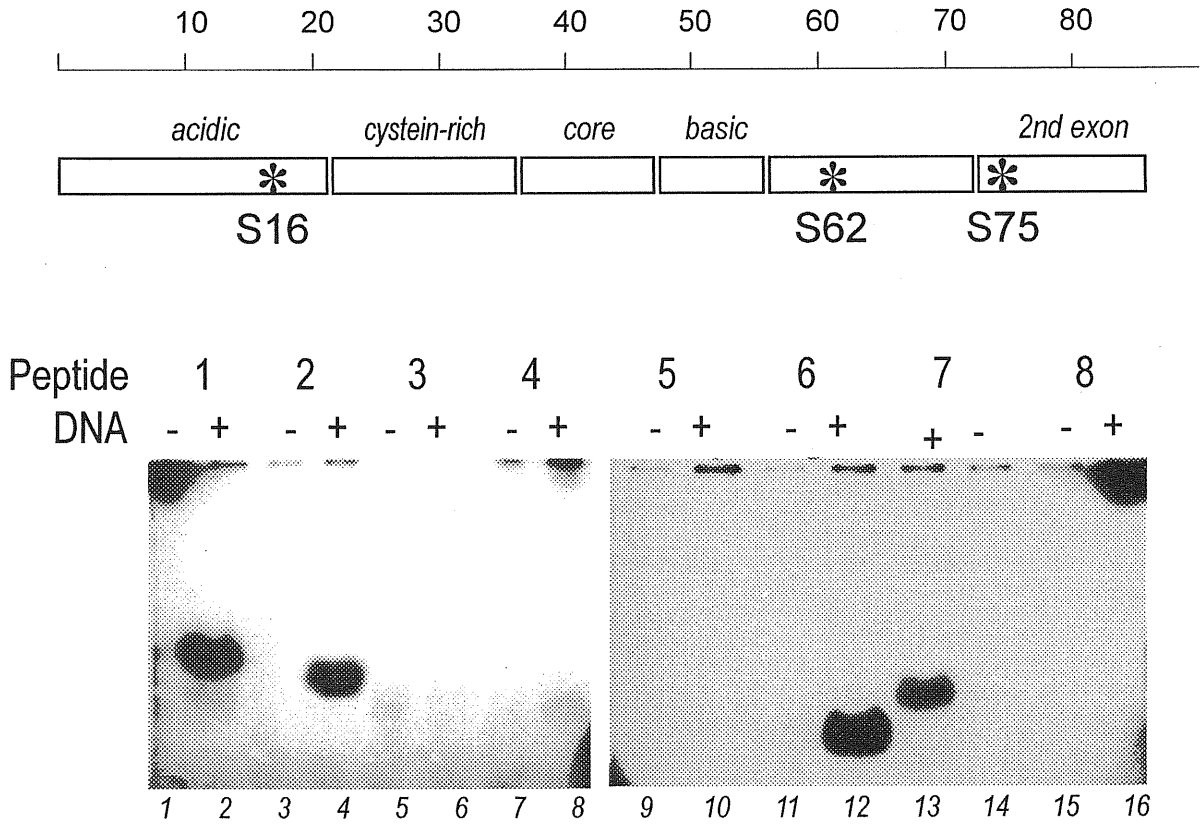

Figure 4.4
Purified DNA-PK phosphorylates Tat-derived peptides

Eight different peptides, of 20 amino acids each and spanning the whole Tat sequence with 10 amino acids overlapping the preceding one, were phosphorylated by purified DNA-PK only in the presence of DNA (+).

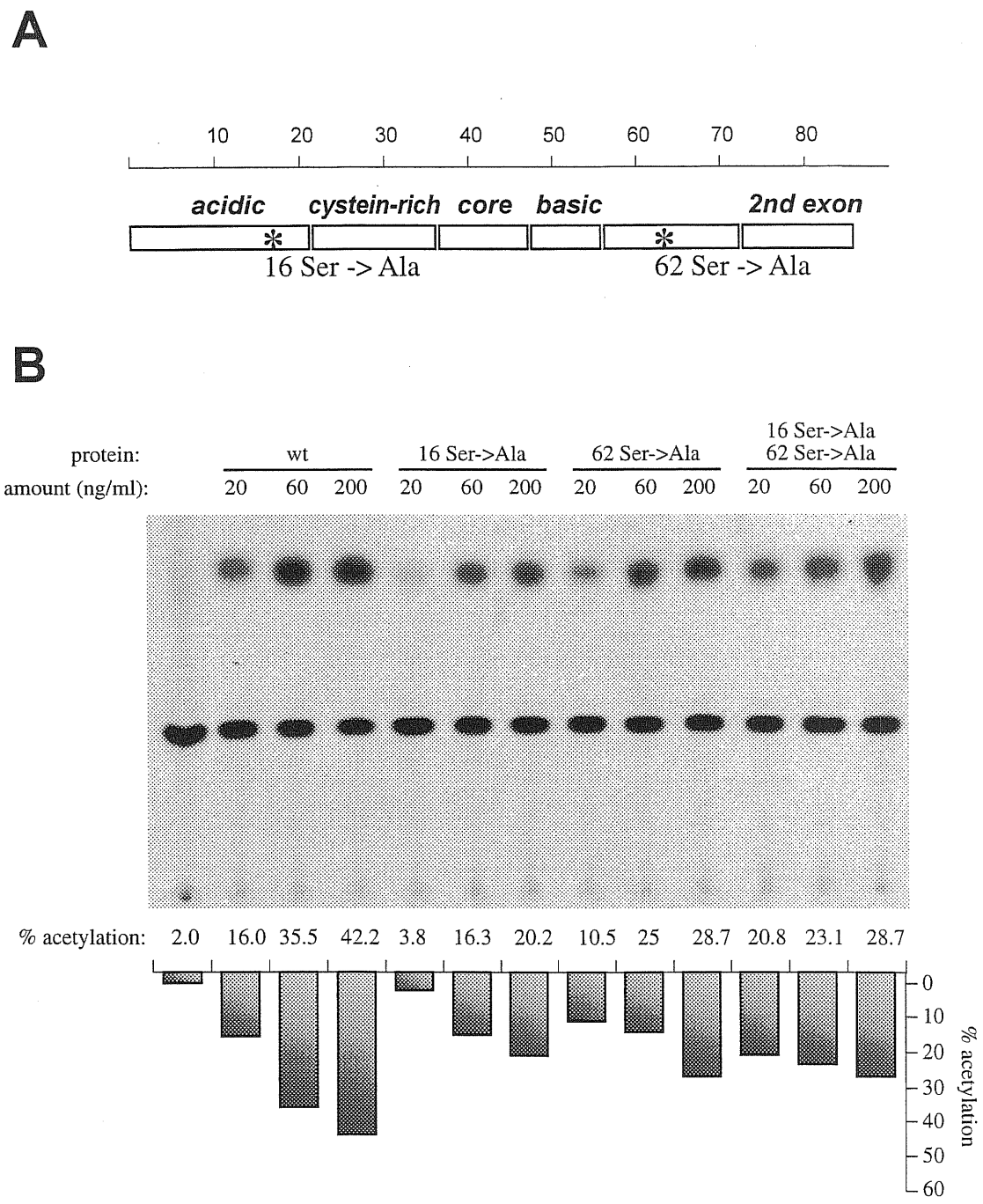

Figure 4.5
Mutations in the DNA-PK phosphorylation sites of Tat decrease transactivation of HIV-1 LTR

Panel **A.** Schematic representation of the domain structure of the HIV-1 Tat protein, with the indication of the positions of S16 and S62, which were both mutated to alanines in the experiment of panel B.
Panel **B.** Results of CAT transactivation assays performed by treatment of HL3T1 cells with the increasing amounts of recombinant wild type as well as the point mutants of Tat protein. All proteins were obtained and purified as GST-fusion products, as detailed in the Experimental Procedures.

hours, cells were lysed and Tat was immunoprecipitated with a specific antibody. The immunoprecipitated proteins were resolved by SDS-PAGE, blotted, and reacted with anti-Tat antibody. While the immunoprecipitated protein was clearly detected by immunoblotting (data not shown), still it did not result labeled even after prolonged exposure at the phosphoimager.

Physiological significance of Tat phosphorylation by DNA-PK

Since, serine at position 62 lies immediately adjacent to the basic region of Tat, this suggested that Tat phosphorylation might influence its ability to transactivate the HIV-1 LTR by affecting the interaction of the protein with TAR, by inducing a modification of its cellular localization, or both. The serine residue at position 16 lies in the N-terminal domain, which is required for the Tat transactivation. To establish whether phosphorylation of these residues could have a role in Tat activity, we performed a mutational study. Site directed mutagenesis was used to change serines 16 and 62 to alanines, both individually or in combination. GST-fusion proteins for these constructs were obtained and purified on agarose-glutathione beads. Increasing amounts of the recombinant proteins were used in a standard LTR transactivation assay on HL3T1 cells, harboring an integrated LTR-CAT construct, in the presence of 100 μM chloroquine. As shown in Figure 4.5, all the three mutant proteins were impaired in LTR transactivation as compared to wt Tat 86. However, it should be observed that the extent of impairment in all cases was not dramatic, being less than two fold. This effect nevertheless was very reproducible in different experiments.

IV - DISCUSSION

Several direct and indirect evidences suggest that DNA-PK could be involved in the regulation of HIV-1 gene expression by exerting different interactions at the LTR promoter, as detailed as follows.

i) DNA-PK is one of the kinases shown to phosphorylate the carboxy-terminal domain (CTD) of RNA polymerase II and in turn to facilitate the transition from initiation to the elongation phase of the transcription reaction (68, 243). Consistently, DNA-PKcs and Ku (p70/p80) are components of an RNA polymerase-II holoenzyme purified from HeLa cell nuclear extract (199). Increase in elongation proficiency by RNA polymearse II is a property clearly required for the activation of LTR transcription (182). Tat has long been recognized to specifically associate with a kinase activity with specificity for the RNA polymerase II CTD (139).

ii) The Ku subunit of DNA-PK specifically recognizes stem-loop structures and, in particular, it binds to HIV-1 TAR RNA with a 5-fold higher affinity than for the ends of dsDNA (164). This raises the possibility that DNA-PK could be recruited at LTR promoter also *in vivo*.

iii) Among the many protein kinases that phosphorylate the transcriptional apparatus *in vitro*, DNA-PK is of particular interest because of its co-localization with the transcriptional template (11, 127), that facilitates the enzymatic reaction, and of its ability to phosphorylate many proteins that are relevant to transcription. These proteins include Sp1, RNA polymerase II, p53, SV40 large T antigen, Myc, Oct-1, Fos, Jun, SRF and histone H1 (reviewed in 278). In particular, the Sp1 factor plays a key role in the control of LTR-mediated gene expression and in Tat transactivation (165), and Tat specifically binds to Sp1 (159). Finally, Tat augments DNA-PK-mediated Sp1 phosphorylation in a contact-dependent manner (54).

iv) Tat direcly forms protein-protein contacts with DNA-PK (54).

The above reported considerations prompted us to ask the question whether Tat itself could be an *in vitro* substrate for DNA-PK phosphorylation. The preferred DNA-PK target consensus sequences, consist of a serine or threonine residue followed by (or, to a lesser extent, preceded by) a glutamine residue (S/TQ). Three such DNA-PK

target sites are present in the Tat protein at positions 16, 62, and 75. The results obtained by studying phosphorylation of the protein by a set of overlapping peptides clearly indicated that the serine residues at positions 16 and 62 are phosphorylated *in vitro* by purified DNA-PK in a DNA-dependent manner, while residue at position 75 is not. Other authors have observed that DNA-PK does not recognize all sequences conforming to the target consensus sequence (reviewed in 278). For the Tat protein, the arginine residue close to potential phosphorylation site at position 75 possibly inhibits phosphorylation by DNA-PK. Indeed, it has been reported that positively charged amino acids can negatively affect DNA-PK recognition on neighboring serines or threonines (18), probably due to conformational constraints.

The residues of Tat phosphorylated by DNA-PK (serines 16 and 62) lie in the amino-terminal domain and basic domain of the protein respectively, and the indispensability of these domains for the transactivation activity of Tat protein has already been well established. The amino-terminal domain is known to be important for transactivation, even if the only characterized factors binding to the region is NFAT1 (196). On the other hand, the basic domain has pleiotropic functions, ranging from Tat trafficking, binding to TAR, and interaction with several transcription factors and transcriptional co-activators, including p300/CBP (see above).

To understand the functional consequences of DNA-PK phosphorylation of the HIV-1 Tat protein on its transactivation activity, we mutagenizes the target serine residues to alanines. When the transactivation activity of these Tat mutants was compared with that of wild type Tat protein, we found that Tat mutants were still able to transactivate the CAT gene through the HIV-1 LTR promote, even if at a lesser extent. This reduction, albeit very reproducible, was quite modest (less that 2 fold). In this respect, however, it should be observed that the only phenotype so far studied for these mutants have been LTR transactivation. At what extent these mutations could interfere with the other several non transcriptional functions of the protein will need further investigation.

The results presented in this study indicate that Tat is an *in vitro* substrate of DNA-PK. Thus, Tat can be also included in the list of the several other proteins which have been shown to be phosphorylated by DNA-PK *in vitro*. Among these proteins are several DNA-binding transcriptional activators, such as Oct1, Sp1, p53 and glucocorticoid receptor (reviewed in 12), the 34 kDa subunit of replication protein A (RPA) (37),

components of the DNA nonhomologous end-joining (NHEJ) machinery, such as XRCC4 (61, 185), the carboxy-terminal domain of the large sub-unit of RNA polymerase II, and several chromatin components including histone acetyltransferase hGCN5 (reviewed in 278). It should be noticed, however, that none of these proteins has been so far proved to be a significant physiological target of DNA-PK also *in vivo.*

DNA-PK is not the only protein kinase able to phosphorylate the Tat protein *in vitro.* At least two other kinases, protein kinases C (PKC) and the interferon-induced double-stranded RNA-activated kinase (PKR), have also been shown to phosphorylate Tat *in vitro* (34, 143). However, in agreement with our findings, the search of Tat as a phopshoprotein *in vivo* has always been negative (34, 132, 137). This negative finding, however, does not exclude that Tat phosphorylation could still play a regulative role in the cells, which could be transient or limited to a specific fraction of the protein and thus might have escaped experimental recognition so far. Taken all these considerations together, it can be concluded that it is questionable whether Tat is an *in vivo* substrate for DNA-PK. This doubtful conclusion still does not question the involvement of DNA-PK in the regulation of Tat-mediated LTR transactivation, both through the direct interaction with Tat and by the phosphorylation of cellular factors binding at the LTR DNA sequence, as recently described (54).

EXPERIMENTAL PROCEDURES

Reagents.

All general reagents, if not specifically mentioned, including the GAG lyases chondroitinase ABC, Chondroitinase AC, heparinase I and heparinase III (heparitinase) , the GAG analogue dextran sulfate (molecular weight, 5000) and all the soluble GAGs (heparin, from porcine intestinal mucosa; chondroitin sulfate A, from bovine trachea; chondroitin sulfate B, from porcine intestinal mucosa; and chondroitin sulfate C, from shark cartilage) were purchased from Sigma (Sigma, St. Louis, MO).

Plasmids.

Plasmid, pCMV-Tat101 was constructed by cloning cDNA of wild type 101-aa Tat in pcDNA3 (Invitrogen, Carlsbad, CA). Plasmid pBS-KS-hTAF32, containing the cDNA of human TBP Associated Factor 32 (TAF32), was kindly provided by R. Tjian (university of California, Berkeley). Plasmid pcDNA3-p300 was constructed by cloning the cDNA of p300 (obtained from plasmid pCMV-p300, a gift from D. M. Livingston, Dana-Farber Cancer Institute, Boston) in pcDNA3. Plasmid pULBLTR-CAT contains the chloramphenicol acetyltransferase (CAT) gene downstream of the LTR promoter (117) and the plasmid pBlue-LTR-CAT, which also contains the same configuration of genes, but in a different plasmid backbone was kindly provided by B. Berkhout (Academic Medical Center, Amsterdam).

Preparation of Tat Ser16Ala mutant

The N-terminal fragment of Tat containing mutated amino acid, 16 from Ser to Ala was amplified by PCR using the primers Tat-1 (Upper) and Ala 16 L (Lower).

The C-terminal fragment of Tat containing the mutated amino acid 16 from Ser to Ala was amplified by PCR using the primers Ala 16 U (Upper) and 2E-Tat (Lower).

The amplified products were gel purified, mixed together and annealed as they contained overlapping sequences. The annealed product (mutated Tat) was amplified by PCR using

Tat-1 and 2E-Tat as upper and lower primers respectively.

The molecular size and purity of the product was checked by agarose gel analysis. The

product was gel purified, digested with BamH-I and ECoR-I restriction enzymes and cloned in pGEX-2T vector (Pharmacia, Uppsala, Sweden,). The cloned product was used to transform competent SF-8 bacterial cells. The presence of mutation at appropriate site (Ser16Ala) was confirmed by nucleotide sequencing.

Preparation of Tat Ser62Ala mutant

Tat mutant Ser62Ala was prepared by the same procedure used for mutant Ser16Ala, but by employing the different set of primers.

The N-terminal of Tat containing mutated amino acid at position 62 was amplified by PCR using primers Tat-1 (Upper) and Ala 62 L (Lower).

The C- terminal fragment was amplified by using Ala 62 U and 2E-Tat as upper and lower primers respectively.

The two fragments were gel purified, annealed and amplified by using the primers Tat-1 (Upper) and 2E-Tat (Lower).

The PCR product was gel purified, digested with BamH-1 and ECoR-1 enzymes and cloned in pGEX-2T vector (Pharmacia, Uppsala, Sweden). The cloned product was used to transform competent SF-8 bacterial cells. The authenticity of the mutant was confirmed by nucleotide sequencing.

Preparation of Tat Ser16Ala, Ser62Ala double mutant

Single point mutated Tat at position 16 from Ser to Ala was used as template to prepare double point mutated Tat at both positions 16 and 62 from Ser to Ala. Second mutation at amino acid 62 was produced using the same procedure and set of primers employed to prepare Ser62Ala mutant using wild Tat as template.

Preparation of Tat His13 Ala, Arg, Glu and Tyr mutants

Point mutated Tat at position 13 from histidine to alanine, arginine, glutamic acid or to tyrosine were prepared using the methodology as explained above, but employing the different sets of primers.

The smaller fragments of Tat containing different mutations at position 13 from His to either Ala or Arg or Glu or Tyr were amplified separately by PCR using different sets of primers, namely Upper (Tat-1) along with Lower either (13 His-Ala,L) or (13 His-Arg,L) or (13 His-Glu,L) or (13 His-Tyr,L).

At the same time the bigger fragments of Tat containing different mutations at same site were amplified separately using different sets of primers Upper either (13 His-Ala,U) or (13 His-Arg,U) or (13 His-Glu,U) or (13 His-Tyr,U) along with Lower (2E-Tat).

The amplified products (eight) were gel purified, annealed accordingly and the annealed products (four) were amplified separately, but using same set of primers Upper (Tat-1) and Lower (2E-Tat).

The size and purity of the PCR products were estimated by agarose gel electrophoresis. The amplified products were gel purified, digested with BamH-I and EcoR-I restriction enzymes and cloned in different pGEX-2T vectors. The cloned products were separately transformed in the competent SF-8 bacterial cells. Presence of the right point mutations were confirmed by nucleotide sequencing.

GST-fusion protein purification

Luria Bertani (LB) broth (100 ml), supplemented with ampicillin (Sigma, St. Louis, MO) 50 μg/ml, were incubated overnight with shaking at 37°C. Overnight culture was diluted 1:10 with fresh LB broth (900 ml) and the incubation was continued till the bacteria achieved growth in the log phase. Fusion protein was induced by the addition of IPTG (isopropylthio-β-D-galactopyranoside) (Sigma, St. Louis, MO) to a final concentration of 0.1 mM and growth was continued for 5 more hours. Thereafter, bacteria were pelleted by centrifugation at 5,000g for 10 minutes at 4°C. The pellet was resuspended in 10 ml lysis buffer (50 mM Tris-Cl pH 8.0, 2mM EDTA and 5 mM DTT) and bacteria were lysed by sonication on ice, 30 seconds each for three times. The cell debris was removed by centrifugation at 12,000g for 15 minutes at 4°C. The supernatant was incubated with glutathione-S-sepharose beads (Pharmacia, Uppsala, Sweden), equilibrated with lysis buffer for one hour on end-over-end shaker at 4°C. Beads were washed with 100 ml lysis buffer containing 500 μM PMSF (phenylmethylsulfonylfluoride). The beads were incubated with 1 ml lysis buffer containing 20 mM free reduced glutathione for 30 minutes on end-over-end shaker.

The beads were pelleted out and the purified GST-fusion protein was collected as supernatant and stored in small aliquots at -80°C.

Cleavage of GST-fusion proteins by thrombin

Free glutathione from the GST-fusion protein was removed by dilysing about 100 μg of protein with 1 ml thrombin lysis buffer (Tris-Cl pH 7.5, 0.15 M NaCl and 2.5 mM $CaCl_2$) in a centricon column (Amicon, beverly, USA) having cut off 10 KDa. The volume of the glutathione free protein was made up to 400 μl and 2 μg thrombin was added to the the protein solution and allowed to digest for 2 hours at 30°C. The digestion was confirmed by running a small part of the protein on a SDS-PAGE and the protein was stored at -80°C.

Gel retardation assays

The oligonucleotides used in this work were synthesized by the ICGEB oligonucleotide synthesis service on an Applied Biosystem 380B Synthesizer using phosphoramidite chemistry. The sequence of the TAR probe has already been described (117). The synthesized oligonucleotides were resolved in polyacrylamide gel electrophoresis, eluted from the gel, purified and annealed with the complimentry strand and end-labeled with γ^{32}P-ATP (Amersham, UK; 3000Ci/mmol; 10 mCi/ml) and T4 polynucleotide kinase.

Gel-retardation assays with Tat were carried out by incubation of 1 ng of TAR probe (about 6 X 10^6 cpm/μg) with different amounts of GST-Tat protein in a buffer containing 25 mM Tris-Cl pH 8.0, 50 mM NaCl, 1 mM $MgCl_2$, 0.5 mM DTT, 0.1 % triton X-100, 0.5 μg of salmon sperm DNA, 10 μg of BSA and 10 U Rnasin (Promega, Madison, Wisconsin). Binding was allowed for 20 minutes at 25°C and then protein-RNA complexes were resolved on 5% polyacrylamide gel/0.5X TBE buffer.

Labeling of cultured cells with $^{32}P_i$ and preparation of cell lysate for immunoprecipitation

HL3T1 cells were grown overnight in 5 cm diameter cell culture dishes to reach about 80% confluency. Next day cells were supplied with fresh medium and treated with 1

μg/ml GST or GST-Tat proteins along with 100 μM chloroquine. After 5 hours, the cells were washed three times with phosphate-free DMEM medium supplemented with 2% serum dialysed against phosphate-free saline (labelling medium) and incubated with 1.5 ml labelling medium containing $^{32}P_i$ to a final concentration of 1 mCi/ml. After eight hours, the cell labelling medium was discarded in the radioactive waste and cells were washed three times with cold Tris Buffered Saline (TBS, 136.8 mM NaCl /5.0 mM KCl /0.9 mM $CaCl_2$ /0.5 mM $MgCl_2$ /0.7 mM NaH_2PO_4 /25 mM Tris-cl, pH 7.4). One ml radioimmunoprecipitation assay buffer (RIPA buffer, 50 mM Tris-Cl /pH 7.5 /50 mM NaCl /1% NP-40 /1% deoxycholate /0.1% SDS /2 mM EDTA /1 mM DTT /0.02% NaN3 was added and left in the cell culture dish for 20 minutes at 4°C. The cell lysate was collected in a screw-cap microcentrifuge tube with the help of rubber policeman, and passed through a 24 gauze needle. The cell lysate was clarified by centrifuging for 30 minutes at the maximum speed of microfuge (14000 rpm) at 4°C. and supernatant was transferred carefully into a fresh microcentrifuge tube. Protease inhibitors (500 μM phenylmethylsulfonyl fluoride /1 μM leupeptin /1 μM pepstatin) along with phosphatase inhibitor (300 nM Okadic acid) were added to the cleared cell lysate and used for immunoprecipitation.

Immunoprecipitation and immunoblotting.

Cleared cell lysates were incubated overnight with appropriate antibodies at 4°C. Next day 40 μl of a 50% suspension of protein-A sepharose CL-4B beads (Pharmacia, Uppsala, Sweden) ware addedto the cell lysate and further incubated for another two hours at 4°C. Later, beads were washed five times each with 1 ml RIPA buffer 150 (RIPA Lysis Buffer at 150 mM NaCl). Depending on the experiment, the samples were then either assayed for HAT activity or antigen-antibody complexes were released by boiling the samples in SDS-denaturing loading buffer and the precipitated proteins were analysed on 15% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) along with the protein marker. The resolved protein bands were transferred to a nitrocellulose filter by electroblotting. The filter was then incubated in 5% milk-TBST buffer (5% non-fat dried milk in 125 mM NaCl, /0.1% tween-20, /10mM tris-Cl, pH7.4) for 1 Hour at 37°C. Later the specific antibodies diluted in 5% milk-TBST were added and incubated for one more hour at room temperature. The filter was washed five times for five minutes each with TBST. Thereafter, it was incubated with

either alkaline phosphatase or horse-reddish peroxidase conjugated secondary antibodies diluted in 5% milk-TBST and incubated for 1 Hour at room temperature with shaking. After several washes with TBST, the bound antibodies were revealed either by using BCIP/NBT colour development solution (BioRad, Richmond CA, USA) or by the ECL kit (Amersham International plc, Little Chalfont, UK).

The presence of corresponding *in vivo* radio-labeled phosphorylated GST-Tat protein band on the filter was checked by exposing the filter in phosphoimager. All antibodies were obtained from Santa Cruz Biotechnology, (Santa Cruz, CA, USA), with the exception of the anti-Tat antiserum (NIH AIDS Research and Reference Reagent Program of the NIH, contributed by B. Cullen) and of the anti-Tau antibody (kind gift of M. Novak).

CAT assay

Cat assays were performed according to the protocol of Gorman with some modifications (126) briefly, the protein extract was prepared by freezing and thawing for three times (three minutes each in dry ice and in 37°C). Inactivation of endogenous deacetylases was performed by heating the samples at 60°C for 5 minutes. After pelleting the debris, protein concentration of the supernatant was determined by the Bradford assay system (BioRad, Richmond CA, USA). The same amount of protein from each sample was used in one set of experiment. Samples were then incubated for one hour at 37°C with 2.5 µl ^{14}C-1-deoxychloramphenicol (50 mCi/mM, Amersham, Little Chalfont, Buckinghamshire), 0.5 M Tris-Cl, pH 7.8, and 0.57 mM Acetyl-CoA in a final volume of 140 µl. The reaction was stopped by extracting out chloramphenicol with 1 ml of ethyl acetate. The samples were lyophilized, resuspended in 10 µl of ethyl acetate and spotted on a TLC silica gel plate. The plate was developed by ascending chromatography in a chloroform : methanol (95:5) mixture. The percentage of acetylated chloramphenicol was determined by Phosphoimager.

DNA-PK phosphorylation assay

DNA-PK (DNA dependent protein kinase) assays were carried out by incubating the protein substrate with 1 µl of γ-^{32}P-ATP (Amersham, Little Chalfont, Buckinghamshire; 3000 Ci/mM; 10 mCi/ml) in a DNA-PK reaction buffer (127). In the reaction mixture 0.5-

1.5 µl of purified DNA-PK, either in presence or in absence of DNA, was added. The reaction was carried out for 10 minutes at 37°C and it stopped by boiling the samples in SDS-denaturing loading buffer. The samples were analyzed by SDS-PAGE electrophoresis.

Cell lines

The wild type CHO and CHO mutants, deficient in proteoglycan biosynthesis, were obtained from the American Type Culture Collection (Rockville, Md.). The hamster cells were transiently transfected with pBlueLTR-CAT plasmid, which contain CAT gene under the control of HIV-1 LTR, by using lipofectin (GibcoBRL Life Technologies LTD, Paisley, Scotland). Similarly, hamster cell lines containing Tat-GFP gene were obtained by Lypofection of the pCDNA3-Tat-GFP plasmid. The cell lines constitutively expressing Tat-GFP were obtained by the selection for neomycin-resistant clones with 500 µg/ml G418 (GibcoBRL Life Technologies LTD, Paisley, Scotland). A single clone was collected and propagated. HL3T1 cells, kindly donated by B. Felber (98), are a HeLa derivative cell line containing an integrated LTR-CAT construct. CHO and mutant CHO cell lines were grown in HAM'S F10 medium; HL3T1 cells were grown in Dulbecco,s modified Eagle's medium (DMEM). Both mediums were supplemented with 10% fetal calf serum, 2 mM glutamine and 50 µg/ml gentamicin.

Recombinant Proteins.

Glutathione S-transferase (GST), GST-Tat and GST-Tat mutants were prepared as described earlier (73). Plasmids pBS-KS-hTAF32 and pcDNA3-p300 were used as templates to produce *in vitro* ^{35}S-labeled hTAF32 and p300 proteins, respectively, using the TNT Reticulocyte Lysate System (Promega, Madison, WI) and manufacturer's protocol. To prepare pGEX2T-Tat-GFP construct, the Tat fragment without terminal stop codon was amplified by PCR (Polymerase Chain Reaction) using pGEX2T-Tat as template and by employing Tat-1 (upper) and 2E Tat Hind GFP (lower) primers.

Similarly, Enhanced Green Fluorescent Protein (EGFP) was amplified by PCR from

commercial vector pEGFP-N1 (Clontech, Palo Alto, CA) using GFP Hind Tat and GFP DE as upper and lower primers respectively.

The size and purity of the amplified products were checked on a gel and the fragments were gel purified. As the fragments contain overlapping ends, $1/100^{th}$ amount of the fragments were mixed in a tube and annealed in PCR thermo cycler using a specific programme for slow temperature decreasing. The annealed product was amplified by using Tat-1 and GFP DE as upper and lower primers respectively.

The amplified product was gel purified, digested with BamH I and ECoR I restriction enzymes and ligated to pGEX-2T vector (Pharmacia, Uppsala, Sweden). The size of the protein was checked by SDS-PAGE and the fluorescence of the protein was confirmed by observing the green emission of the cells treated with GST-Tat-GFP recombinant protein, under a Zeiss Axiophot fluorescence microscope (Carl Zeiss, Jena, Germany).

Recombinant protein treatment.

The cells were grown overnight to reach about 80% confluence. Cells were then treated with 1 μg/ml of GST or GST-Tat or GST-Tat-GFP, depending on the application along with 100μM chloroquine or Lipofectin (GibcoBRL Life Technologies LTD, Paisley, Scotland) according to a published procedure (73). Depending on the application, after defined time intervals, cells were washed four times with the PBS, trypsinized, again washed with PBS and protein uptake by cells was determined by flow cytometry with a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA). A total of 10,000 events per sample were analyzed. For CAT assay, the $1x10^6$ cells were seeded in 10 cm diameter dishes and incubated for 24 hours. Later, cells were transfected with either pBlue-LTR-CAT alone or along with either pCDNA3-Tat-GFP or pCDNA3-Tat by using lipofectin (GibcoBRL Life Technologies LTD, Paisley, Scotland) using of the procedure supplied by manufacturer and were further incubated for another 24 hours. After that, the cells were washed twice with PBS and fresh culture medium was added. The cells transfected only with pBlue-LTR-CAT were supplied with either GST-Tat or GST-Tat-GFP (from concentrations 20 ng/ml to 1 μg/ml depending on the application) along with 100 μM chloroquine and incubated for another 24 hours. Later the cells were washed twice with PBS and left again in fresh medium for 24 hours. At the end, the

cells were scraped off the dishes using a rubber policeman. Cell extracts were prepared and used for CAT assay, following a procedure discussed earlier. For immunoprecipitation, after 5 hours following protein treatment, cells were washed four times with ice-cold PBS, scraped off the plates and lysed in 1 ml RIPA Lysis Buffer 50 (50 mM Tris-Cl, pH 7.5, 50 mM NaCl, 1% NP-40, 1% deoxycholate, 0.1% SDS, 2 mM EDTA) with protease inhibitors (500 μM PMSF, 1 μM leupeptin, 1 μM pepstatin). The cell lysate was passed through a 24-gauge needle and centrifuged at 14,000 rpm for 10 minutes at 4°C in Eppendorf bench centrifuge. The cleared supernatant was used for immunoprecipitation.

Immunocytochemistry.

HL3T1 cells were grown to about 60% confluency on glass coverslips. The GST-Tat protein (1 μg/ml) was added to the cell culture medium in the presence of 100 μM chloroquine. After different time intervals, cells were washed 6 times with PBS and fixed with a cold acetone:methanol mixture (50:50) for 15 minutes. Cells were then washed 3 times with PBS containing 0.2 % TritonX-100 (PBS-TritonX-100) and then 5 times with PBS for 5 minutes each. Cells were then incubated with an anti-Tat monoclonal antibody (ADP352/NT3, obtained from the MCR repository for AIDS research) for 1 hour, washed 5 times with warm PBS (25 to 28°C) and incubated with rhodamine-conjugated secondary antibodies (Sigma) for 30 minutes. Cells were then washed three times each with warm PBS-TritonX-100 and with warm PBS for 5 min each time. For each immunostaining, one coverslip was incubated in secondary antibody alone, as a negative control for background immunofluorescence. Nuclei were counterstained with Hoechst No-33342 (10 μg/ml in PBS) for 5 minutes, coverslips were washed three times with PBS and mounted on glass slides. Slides were observed using Zeiss Axiophot fluorescence microscope (Carl Zeiss, Jena, Germany).

Enzyme treatment.

Enzymatic treatment of the cells was performed according to the procedure suggested by Summerford with some modifications (284). Briefly, the GAG lysaes were reconstituted in PBS. For enzymatic digestion of the cell surface GAGs, 5 x 10^5 CHO cells were washed and incubated with the indicated concentrations of the GAG lyases

in PBS containing 0.1% bovine serum albumin, 0.2% gelatin and 0.1% glucose (digestion buffer) for 40 minutes at 37°C in a CO_2 incubator. Cells were then washed gently six times with PBS. Thereafter, cells were cultured in Ham's F10 medium without serum along with 1 μg/ml GST-Tat-GFP protein and 100 μM chloroquine for five hours. Cells were washed four times with PBS, trypsinized, and washed with complete Ham's F10 medium. The cells were suspended in PBS and used for flow cytometeric analysis. Experiment was performed in triplicate.

GAGs treatment.

Lyophilised GAGs were dissolved in PBS. For flow cytometric analysis, cells (5×10^5) were incubated with defined amounts of GAGs along with 1 μg/ml GST-Tat-GFP protein and 100 μM chloroquine in fresh culture medium for different time intervals. Cells were washed four times with PBS, trypsinized, washed once again with PBS and suspended in PBS. For CAT assays, cells (3×10^5) were transfected with pBlue-LTR-CAT by using lipofectin (GibcoBRL Life Technologies LTD, Paisley, Scotland), as described above. After 48 hours, cells were supplied with fresh culture medium and incubated over night (12 hours) with different amounts of the GAGs along with 1 μg/ml GST-Tat-GFP and 100 μM chloroquine. On the next day, cells were washed four times with PBS and fresh culture medium was added, After 36 hours the cell lysates were checked for their CAT activity.

Co-culture experiments.

The different cell types were lypofected with either the pBlue-LTR-CAT or pCDNA3-Tat-GFP, by lipofectin, as described earlier. After 48 hours cells were trypsinized and seeded together in equal quantities in different combinations in a medium containing 100 μM chloroquine and left for 3 days with the addition of 100 μM chloroquine every day. Later, cells were scraped with rubber policeman off the dishes and the cell lysates were prepared and used for CAT assays.

In vitro binding assays.

To remove contaminant bacterial nucleic acids, recombinant proteins were pre-treated with nucleases (0.25 U/µl DNase I and 0.2 µg/µl RNase) for 1 hour at 25°C in 50 mM Tris/HCl pH 8, 5 mM $MgCl_2$, 2.5 mM $CaCl_2$, 100 mM NaCl, 5% glycerol, 1 mM DTT. Subsequently, GST fusion proteins immobilized on agarose beads were washed and resuspended in NETN buffer (20 mM Tris/HCl pH 7.5, 100 mM NaCl, 1 mM EDTA, 0.5% NP-40, 1 mM DTT, 1 mM PMSF) supplemented with 0.2 mg/ml ethidium bromide to impede the possible formation of aspecific interactions between residual DNA and proteins. Six hundred cpm of ^{35}S-labeled p300 or hTAF32 proteins were added and incubated at 4°C on a rotating wheel. After 1 hour, bound proteins were washed 5 times with 1 ml of NETN buffer and separated by 7% SDS PAGE. Dried gels were quantitated by Instant Imager (Packard, Meriden, CT).

GST pull-down assays for HAT activity.

Two micrograms of glutathione-agarose immobilized proteins in a final volume of 1 ml IPH buffer (50 mM Tris/HCl pH 8, 150 mM NaCl, 5 mM EDTA, 1 mM DTT, 0.5% NP-40, 1 mM PMSF) with the addition of 0.2 mg/ml ethidium bromide was incubated with 200 µg of Dignam nuclear extract at 4°C for 2 hours. Bound proteins were washed 5 times with 1 volume IPH buffer and subsequently assayed for HAT activity.

HAT assays.

Protein samples obtained by immunoprecipitation or GST pull down assay were analyzed as described earlier (35). Acetylated histones were detected by autoradiography after separation by SDS-PAGE. Alternatively, incorporated ^{14}C-acetyl groups were measured by scintillation counting after spotting the samples on p-81 filters (Whatman, Maidstone, UK) and extensively washing with 0.5 M $NaHCO_3$ pH 9.2.

Cells and transfections.

A hamster cell line containing the integrated LTR-CAT (CHO/LTR-CAT) was obtained by calcium-phosphate transfection of plasmid pULBLTR-CAT and pcDNA3 in CHO-K1 cells, followed by selection for neomicin-resistant clones with 500 µg/ml G418

(GibcoBRL Life Technologies LTD, Paisley, Scotland). HL3T1 cells, kindly donated by B. Felber (98), are a HeLa derivative cell line containing an integrated LTR-CAT construct. CHO and HL3T1 cells were grown in DMEM and Ham's F10 medium, respectively, supplemented with 10% fetal calf serum, 2 mM glutamine and 50 μg/ml gentamicin. Cells were transfected by the standard calcium-phosphate procedure (269). All transfections were adjusted to the same content of transfected plasmid (and of CMV promoter sequences) by the addition of appropriate amounts of pcDNA3. CAT assays were performed 48 hours after transfections. The corresponding results represent the average values obtained in several (at least three) independent transfections.

Immunoprecipitation and immunoblotting.

The cleared cell lysates were incubated overnight at 4°C with the appropriate antibodies. After incubation, 40 μl of a 50% suspension of protein-A sepharose CL-4B beads (Pharmacia, Uppsala, Sweden) in RIPA buffer were added. After a two hour incubation at 4°C, beads were washed three times with 1 ml RIPA buffer 150 (RIPA Lysis Buffer at 150 mM NaCl). Samples were then assayed for HAT activity or analyzed by western blotting using the indicated antibodies. The membrane was developed by the ECL kit (Amersham International plc, Little Chalfont, UK). All antibodies were obtained from Santa Cruz Biotechnology, Santa Cruz, CA, USA, with the exception of the anti-Tat antiserum (NIH AIDS Research and Reference Reagent Program of the NIH, contributed by B. Cullen) and of the anti-Tau antibody (kind gift of M. Novak, International School for Advanced Studies, Trieste, Italy).

Chromatin immunoprecipitation.

HL3T1 cells were treated with GST or GST-Tat as described above. After a 5 hour incubation, protein-DNA complexes were fixed by formaldehyde and treated as described in ref. 233. Chromatin pellets were resuspended in 500 μl RIPA lysis buffer 50 with protease inhibitors (500 μM PMSF, 1 μM leupeptin, 1 μM pepstatin; Sigma, St. Louis, MO) and subjected to 20 cycles of 10-sec sonication on ice. Sonicated samples were centrifuged to spin down cell debris and immunoprecipitated as described above. Protein-bound immunoprecipitated DNA was resuspended in 100 μl TE and digested

with 5U DNase free RNase (Boehringer Mannheim, Mannheim, Germany) for 30 minutes at 37°C. The samples were successively treated for 3 hours at 56°C with 300 μg/ml proteinase K (Sigma, St. Louis, MO) in 0.5% SDS, 100 mM NaCl and for 6 hours at 65°C to revert cross-links. DNA was extracted with phenol/chloroform/isoamyl alcohol, precipitated with ethanol and resuspended in 100 μl of water for quantification.

DNA quantification by competitive PCR.

Primer sequences and amplification conditions already have been described for LTR-CAT (75), B48 and B13 (121) and ß-globin (60). The multicompetitor DNA fragment was constructed by a recombinant PCR procedure as already described (120) and outlined in Fig. 2.4. Competitive PCR experiments were carried out by mixing a fixed amount of immunoprecipitated DNA with increasing amounts of competitor, followed by amplification with each of the four primer pairs. A detailed outline of the competitive PCR protocol was published elsewhere (120, 237).

Name and sequences of different primers used

NAME OF PRIMERS	SEQUENCE OF PRIMERS
Upper (Tat-1)	5'GTGGATCCATGGAGCCAGTAGATCCTA3'
Lower (2E-Tat)	5'GCGAATTCTCATTCCTTCGGGCCTGTC3'
Upper (Ala 16 U)	5'ATCCAGGAGCTCAGCCTAAAA3'
Lower (Ala 16 L)	5'TTGTAGGCTGAGCTCCTGGAT3'
Upper (Ala 62 U)	5'CCTCAAGGCGCTCAGACTCA3'
Lower (Ala 62 L)	5'TGA GTCTGAGCGCCTTGAGG3'
Upper (13 His-Ala,U)	5'CCCTGGAAGGCTCCAGGAAG3'
Lower (13 His-Ala,L)	5'CTTCCTGGAGCCTTCCAGGG3'
Upper (13 His-Arg,U)	5'CCCTGGAAGCGTCCAGGAAG3'
Lower (13 His-Arg,L)	5'CTTCCTGGACGCTTCCAGGG3'
Upper (13 His-Glu,U)	5'CCCTGGAAGGAACCAGGAAG3'
Lower (13 His-Glu,L)	5'CTTCCTGGTTCCTTCCAGGG3'
Upper (13 His-Tyr,U)	5'CCCTGGAAGTATCCAGGAAG3'
Lower (13 His-Tyr,L)	5'CTTCCTGGATACTTCCAGGG3'
upper (GFP Hind Tat)	5'GGCCCGAAGGAAAAGCTTATGGTGAGCAAGGG3'
lower (GFP DE)	5'GGCGAATTCTCTAGAGTCGCGGCCGCTTTA3'
lower (2E Tat Hind GFP)	5'CCCTTGCTCACCATAAGCTTTTCCTTCGGGCC3'

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