ISAS - INTERNATIONAL SCHOOL
FOR ADVANCED STUDIES

MECHANISMS OF ACTIVATION OF THE INTEGRATED PROMOTER OF THE HUMAN IMMUNODEFICIENCY VIRUS TYPE 1

Giuseppe Marzio

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Supervisor:
Dr. Mauro Giacca

External examiner:
Dr. Ben Berkhout

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ΜΕΛΕΤΑΝ ΟΥΝ ΧΡΗ ΤΑ ΠΟΙΟΥΝΤΑ ΤΗΝ ΕΥΔΑΙΜΟΝΙΑΝ, ΕΙΠΕΡ ΠΑΡΟΥΣΗΣ ΜΕΝ ΑΥΤΗΣ ΠΑΝΤΑ ΕΧΟΜΕΝ, ΑΠΟΥΣΗΣ ΔΕ ΠΑΝΤΑ ΠΡΑΤΤΟΜΕΝ ΕΙΣ ΤΟ ΤΑΥΤΗΝ ΕΧΕΙΝ.

ΕΠΙΚΟΥΡΟΣ

Thus, we must take care of those things that create happiness, because when happiness is present we feel that we possess everything, in its absence we strive to obtain it.

Epicuros

CONTENTS

SYNOPSIS

A thorough understanding of the mechanisms regulating the activation and the rate of HIV-1 gene expression is of basic importance in the race towards effective treatment of AIDS. In this respect, the results reported in this thesis cover several critical aspects of the regulation of HIV-1 transcription.

Chapter 1 provides a general introduction on HIV-1 classification and structural and genomic organization. Here are described the structure of the viral promoter and the factors involved in transcription regulation of the virus after integration in the genome of the infected cells. This first chapter is also intended as a broad overview on the issues raised and dealt with in depth in the ensuing chapters.

In **Chapter 2** are reported the results of the study on the role of transcription factor USF in the modulation of LTR-driven transcription. They demonstrate that USF is a positive modulator of HIV-1 transcription and that this activity results from the specific binding of the protein to a *cis*-acting element present in the negative regulatory element of the LTR. Strikingly, binding of USF results in marked promoter bending. This observations is in agreement with previous reports that other LTR-binding factors are able to induce DNA-bending upon binding.

A novel function of the viral transactivator Tat is described in **Chapter 3.** It is demonstrated that Tat binds p300 and CBP, two proteins with histone-specific acetyltransferase activity able to remodel the chromatin scaffold on the LTR. These proteins are specifically directed by Tat to the HIV-1 promoter for viral transactivation. These data provide a molecular explanation for the long-standing observation that Tat increases the rate of transcription initiation. Moreover, they represent the first evidence that an RNA-targeted transactivating factor such as Tat is able to recruit histone acetyltransferases.

Chapter 4 describes a quantitative system for monitoring HIV-1 nucleic acids in infected samples. The system combines the sensitivity of PCR with the precision deriving from the use of a single competitor for viral DNA and RNA quantitation and normalization to cellular standards. The system was used to monitor HIV-1 activation in two different *in vitro* models of infection.

In the **Appendix** to this chapter the application of this competitive PCR procedure is described for the study the correlation between the proliferative state of infected lymphocytes and HIV-1 activation. Following repeated cycles of mitogen stimulation, the level of HIV-1 gene expression is shown to follow closely that of cellular activation. Furthermore, viral transcription is rapidly shut off after the cells return to a non-proliferative state but can be rapidly re-activated upon a second cell stimulation.

In **Chapter 5** conclusions are drawn based on the results described in the previous chapters.

Most of the results presented in this thesis have already been published in the following international journals:

- d'Adda di Fagagna, F., **Marzio, G.**, Gutierrez, M. I., Kang, L. K., Falaschi, A. and Giacca, M. (**1995**) Molecular and functional interactions of transcription factor USF with the Long Terminal Repeat of Human Immunodeficiency Virus type 1. *J. Virol.*, 69, 2765-2775.

- Comar, M., **Marzio, G.**, D'Agaro, P. and Giacca, M. (**1996**) Quantitative dynamics of HIV-1 expression. *AIDS Res. Hum. Retrov.*, 12, 117-126.

- **Marzio, G.**, Tyagi, M., Gutierrez, M.I. and Giacca, M. (**1998**) HIV-1 Tat transactivator recruits p300 and CREB-binding protein histone acetyltransferases to the viral promoter. *Proc. Natl. Acad. Sci. USA*, 95, 13519-13524.

Chapter 1

GENERAL INTRODUCTION

1. Human Immunodeficiency Virus type 1 and AIDS

Since the isolation and identification of the human immunodeficiency virus type 1 (HIV-1) as the etiologic agent of the acquired immunodeficiency syndrome (AIDS) in the early '80s (11, 68), a remarkable body of knowledge has accumulated about the mechanisms of HIV-1 infection and the evolution of the disease. 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.

HIV-1 belongs to the Retroviridae family, lentivirus genus (for a recent review on HIV-1 classification, see ref. (116)). The viral genome consists of two RNA molecules of approximately 10 kb each encapsidated in the virion particle. 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. (190, 194). The 5' LTR acts as a strongly inducible promoter, originating a single genomic transcript which terminates at the 3' LTR. By multiple alternative splicing, more than 30 different RNA molecules arise from the full length genomic transcript (177). In addition to this complex splicing mechanism, 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 (55, 143, 152) and increasing viral load in plasma has been demonstrated to be a reliable marker for disease progression (28, 131). 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 (93, 149, 205). Inhibition of new rounds of infection by combined antiretroviral therapy results in a dramatic decrease in viral load in plasma and lymphoid districts (93, 205). However, this rapid drop is followed by a lower rate decrease, which has been ascribed to the chronic persistence of latently infected cells (34, 149). HIV-1 is in fact able to establish a latent infection in resting memory CD4+ lymphocytes, where it can be reactivated by external stimuli acting on the infected cell (61). These lymphocytes may remain for many months in a non-dividing state and thus represent a reservoir for HIV-1 that decays very slowly (128). 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. (80)).

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 upregulate viral gene expression from latency is essential for understanding the pathogenesis of the disease.

2. Regulation of HIV-1 gene expression

The regulation of HIV-1 gene expression is an area of intensive investigation. Expression of the provirus integrated in the genome of the infected cells is governed by complex regulatory mechanisms that involve both cell- and virus-encoded factors as well as the incorporation of the viral promoter into the chromatin scaffold. Cellular transcription factors are able to interact with distinct regulatory regions present in the LTR promoter and modulate viral transcriptional activation. In addition, virally encoded proteins are essential to control HIV-1 gene

expression. In spite of data accumulated so far, a number or critical questions concerning the mechanisms which regulate HIV-1 expression still remain unanswered. What factors are responsible for the increase in viral gene expression in response to T-lymphocyte activation? Which mechanisms are involved in transcriptional repression of the integrated provirus in quiescent cells? How can the virus escape this strong silencing effect? How does HIV-1 transactivator protein stimulate viral transcription? During the course of my Ph.D. research I have addressed these and other related questions and approached the issue of HIV-1 transcriptional regulation by several complementary strategies.

The HIV-1 promoter

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, though, 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. (71)).

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 SP1 factor (88, 103), located upstream of an RNA polymerase II TATA box sequence (69), and an initiator element at the transcription start site, whose functional significance is still controversial (104, 124, 125). These three elements are essential for minimal promoter activity. *The enhancer region* contains two binding sites for cellular factor NFkB and is responsible for the strong inducibility of the LTR promoter by a variety of stimuli triggering cell activation and proliferation (51, 138, 193). The region extending upstream of the enhancer exerts an overall negative effect on viral transcription, as demonstrated by deletion studies, and was therefore termed *the negative regulatory element* (NRE) (162). Several binding sites for human nuclear proteins were mapped within the NRE, including among the others LEF (110), NFAT (182), c-Myb (43), and AP-1 (67). 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 (42). Members of this family of transcription factors include c-Myc (137), Max (19), Mad (8), TFEB (29), and USF (171).

Transcription factor USF, for upstream stimulatory factor, was initially characterized for its ability to bind the upstream element of the Adenovirus major late promoter, thus 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 able to bind the consensus CACPuTG E box. USF has all the structural characteristics of its family members (Fig. 1.3). 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 (59). *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 (46). 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 (45).

In Chapter 2, I report the results of my studies on the interaction between transcription factor USF and the LTR promoter. Besides defining the binding specificities of the protein on the LTR, I demonstrate the functional role of USF in the context of HIV-1 transcriptional regulation. Binding of recombinant USF to its binding site results in a several fold 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 and promoter activation

Efficient activation of the LTR requires the virally-encoded transactivator of transcription (Tat), a 14 kDa protein arising from multi-spliced transcripts (for a comprehensive review, see ref. (105)). 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 some laboratory strains (HXB2 clone derived) produce a 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 (162) and located on the 5' untranslated region of all viral RNAs (position +1 to +60 downstream of the transcription start site) (16). TAR RNA folds in a complex three-dimensional structure

able to recruit Tat to the promoter (Fig. 1.4). The key recognition determinant of the TAR hairpin motif is a three nucleotide bulge present in the stem (47, 87, 164). 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 (113).

Several distinct structural and functional domains are present in the protein (Fig. 1.5). The arginine-rich basic domain of Tat is responsible for binding to TAR (25) and nuclear localization of Tat (115), but can be functionally replaced by an heterologous nucleic acid binding domain (72). Full transactivation by Tat relies on the integrity of the amino-terminal, cystein-rich and core domains of the protein, which altogether form an independent *trans*-activation domain (160). Accordingly, the first 72 aa of the protein, encoded by exon 1, are sufficient for *trans*-activation (6, 136). Tat is 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 polymerase (39). A number of converging observations have recently indicated that the role of Tat in transcriptional elongation can be ascribed to its specific interaction with protein complexes possessing protein kinase activity (38, 79, 145, 204, 213). These complexes are able to phosphorylate the carboxyl-terminal domain of the large subunit of RNA polymerase II, an essential step for the recruitment of processive transcriptional complexes on the LTR promoter (Fig. 1.6).

While these data contribute to the elucidation of the processivity-enhancing functions of Tat, they leave some important questions unanswered. For one, it is not clear how these interactions are allowed to occur at the integrated proviral LTR, which has been demonstrated to be tightly bound by nucleosomes and strongly silenced by chromatin structure (see Chapter 3 for an extensive discussion). In particular, the HIV-1 LTR sequence, acting as a very strong promoter when analyzed as naked DNA *in vitro* (145), 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 activity of the promoter is down-regulated (123).

My results reported in Chapter 3 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. This effect is indirect and requires the interaction of Tat with chromatin modifying activities, as discussed in the following section.

Chromatin and HIV-1 transcription

A central question in eukaryotic transcription is how the transcriptional machinery is able to gain access to specific loci tightly packed in chromatin. Nucleosomes negatively affect transcription initiation and elongation by restricting DNA accessibility to transcription factors and impeding elongation by the cellular RNA polymerase II enzyme (146, 195, 196, 206). 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 (187, 201). Few cellular proteins have been so far demonstrated to possess histone acetyltransferase (HAT) activity (23, 208). Interestingly, they do not share a common domain responsible for their HAT activity, and show different histone substrate specificities (Fig. 1.7). In particular, p300 and CBP are the only human nuclear HATs so far identified able to acetylate all the four core histones (Fig. 1.7).

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 aminoacid level, especially in the domains important for their enzymatic and protein-recognition properties (Fig. 1.8). P300/CBP were originally identified as transcriptional coactivators (33, 52). 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 (78, 100). 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 (108, 210). 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. (78, 108)).

As any cellular gene, the HIV-1 provirus is also incorporated into a highly compacted chromatin structure (199, 200) (Fig. 1.9).
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 (54, 197). As described in Chapter 3, I have investigated the effect of chromatin on the activation of HIV-1 gene expression and the molecular mechanisms that allow the virus to escape its transcriptional silencing effect. The results of my research work demonstrate that the Tat protein of HIV-1 associates in the cell nucleus with a HAT activity that I identified as p300 and CBP. Tat binds p300 and CBP and specifically recruits these enzymes to the nucleosome-bound integrated promoter for transactivation. Consistently, overexpression of p300/CBP results in a potentiation of the Tat-mediated transcriptional activation of the integrated LTR.

3. Dynamics of HIV-1 expression

It is now recognized that HIV-1 replication in infected individuals is continuously active at all stages of the disease (55, 143) and that the natural history and the pathogenesis of the HIV-1 infection are closely linked to the replication of the virus *in vivo* (9, 10, 147, 166). Clinical stage is significantly associated with all measures of viral load, including infectious virus titres in blood, viral antigen levels in serum, and viral nucleic acid content of lymphoreticular tissues, peripheral blood mononuclear cells and plasma (reviewed in ref. (27)).

Important insights into the complex aspects of pathogen-host interaction in HIV-1 infection have recently come from a surprisingly straightforward approach, consisting in the direct measurement of circulating levels of free virus. The subsequent development of rapid, sensitive and accurate methods for quantitating virus particles in the blood has greatly facilitated the study of the AIDS pathogenesis and the management of patients infected with HIV-1. Detection tests based on the polymerase chain reaction (PCR) are able to identify HIV-1 DNA in circulating infected cells even during the initial phase of the infection, when the organism has not yet developed antibodies against any viral antigens. RT-PCR allows the quantification of low abundance viral RNA in blood and other biological fluids to detect genomic viral RNA which is present at two copies per virion (152). Since the rate of clearance of free virus from the blood is largely independent of disease stage and other factors, the level of viral RNA in plasma reflects the rate of virus production. Furthermore, the sensitivity of this type of assay allows the precise quantitation of HIV-1 viral load at the beginning and during the course of the anti-retroviral therapy. This facilitates the choice by the physician of the most appropriate antiretroviral protocol and the assessment on the patient of the efficacy of the therapeutic regimen. Thanks to this continuous surveillance, it is possible to limit the risks of appearance of drug-resistant variants.

I contributed to the development of a quantitative system based on competitive PCR and RT-PCR for the precise quantitation of HIV-1 nucleic acids in infected samples. The procedure, described in Chapter 4, is based on a single competitor and allows the detection and quantitation of proviral DNA and of viral full length genomic, single and multi-spliced transcripts. Moreover, the values obtained can be directly normalized to a reference amount of a single-copy cellular gene or a constitutive cellular transcript. I applied this technique to quantitatively monitor the kinetics of HIV-1 expression after activation of experimentally infected peripheral blood lymphocytes (PBLs) and latently infected promonocytic U1 cells. Despite the biological differences between the two experimental systems, in both infection models production of infectious virus is accompanied by a remarkable increase in the levels of unspliced viral mRNAs and by a consequent switch in the abundance of the differently-spliced transcript classes. As a further step, I focused on the events that follow lymphocyte activation and that lead to the activation of the integrated provirus, as described in the Appendix. Using the same quantitative procedure, I demonstrated that the level of viral transcriptional activation is a function of the proliferative state of the infected cells and that when the cells return to the Go phase of the cell cycle also viral gene expression is virtually shut off. However, the virus could be rapidly re-activated by a subsequent activation of the cells.

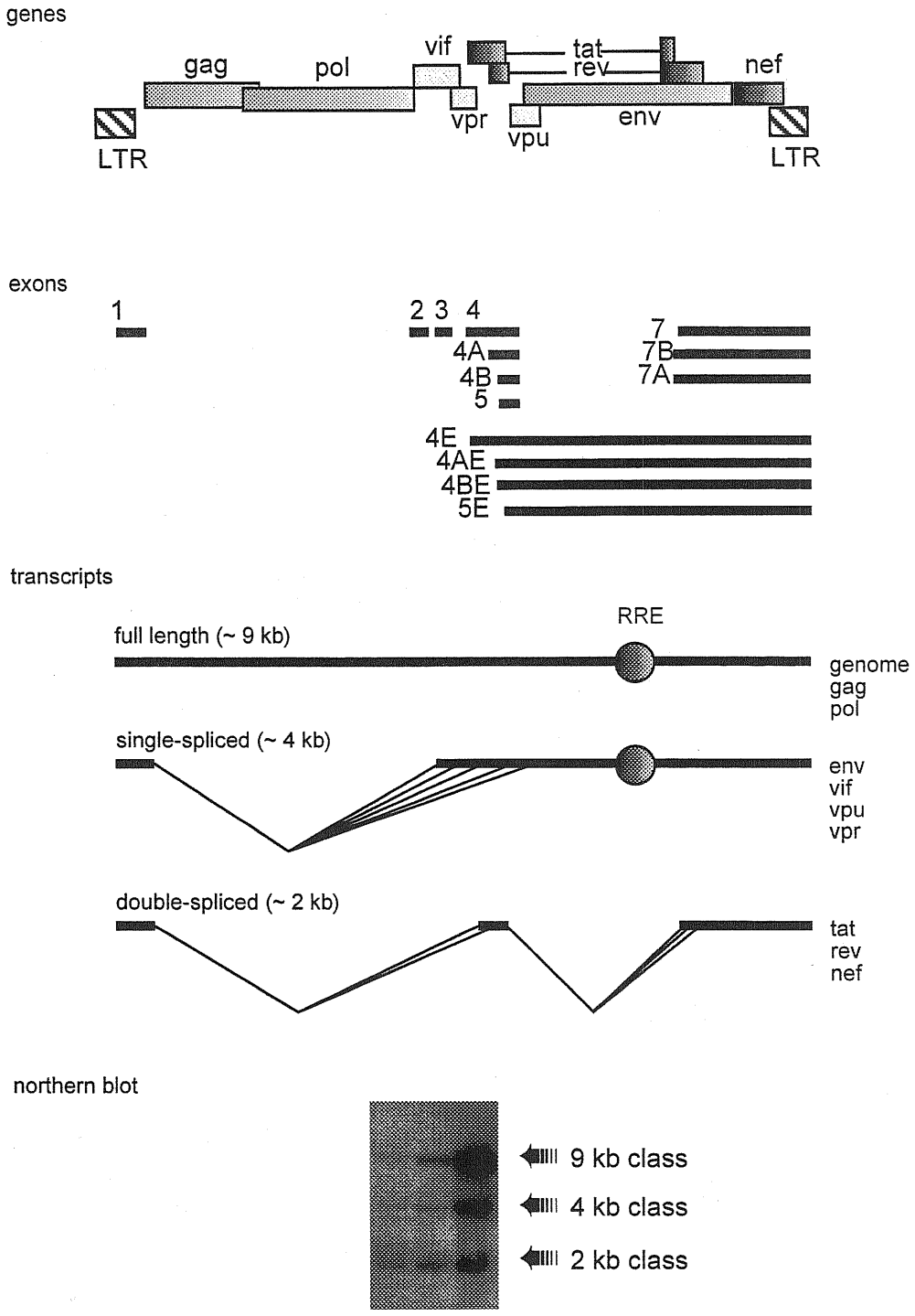

Figure 1.1
Genomic organization and transcripts of HIV-1

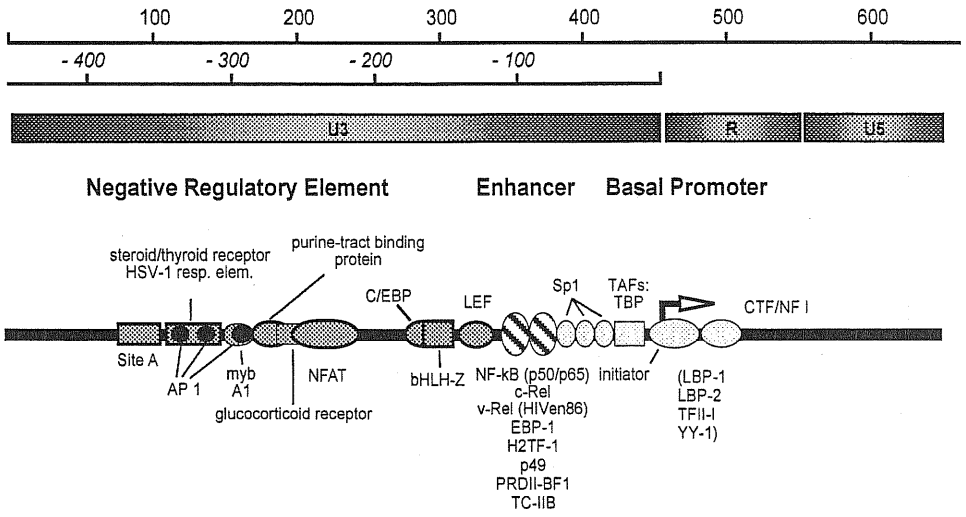

Figure 1.2
Transcriptional domains and protein binding sites at the HIV-1 Long Terminal Repeat

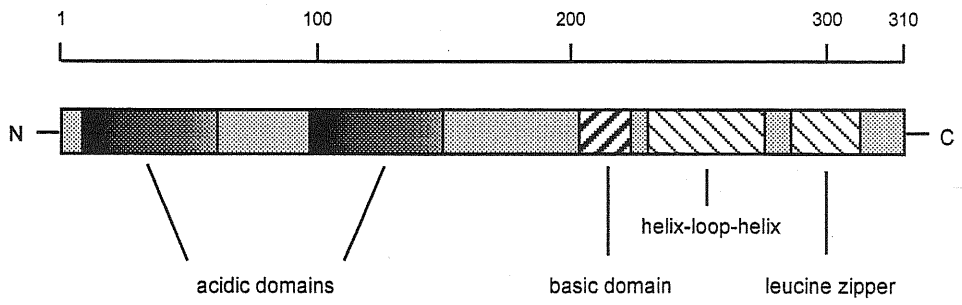

Figure 1.3
Structure of transcription factor USF

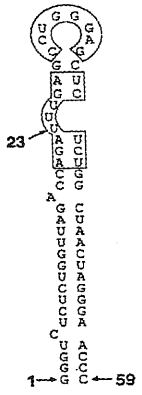

Figure 1.4
Sequence and structure of HIV-1 TAR RNA

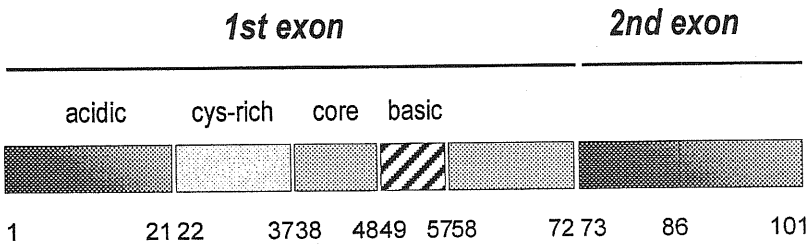

Figure 1.5
Structural domains of HIV-1 Tat protein

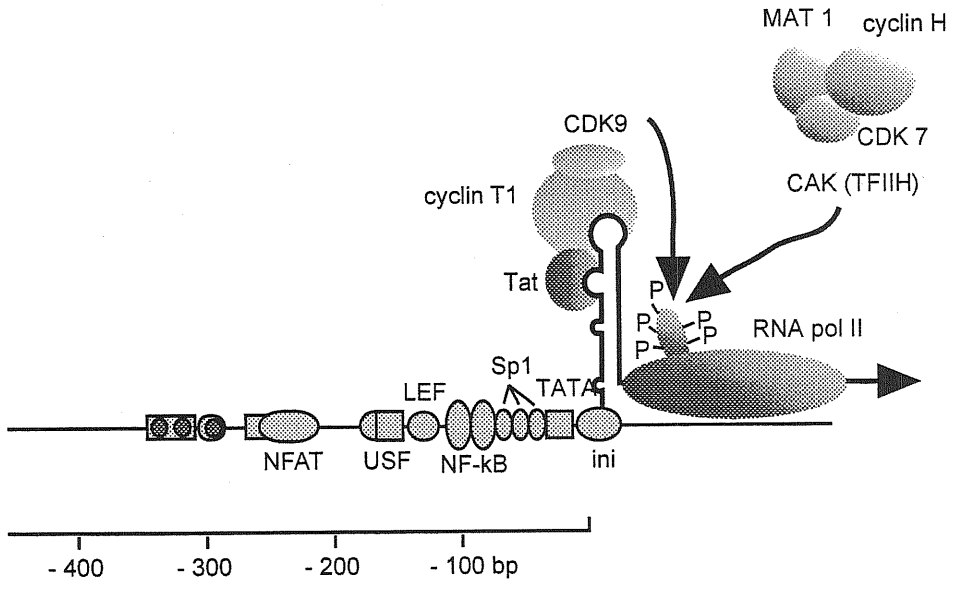

Figure 1.6
Increase of RNA pol II processivity by Tat-associated kinases

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 1.7
Known nuclear histone acetyltransferases

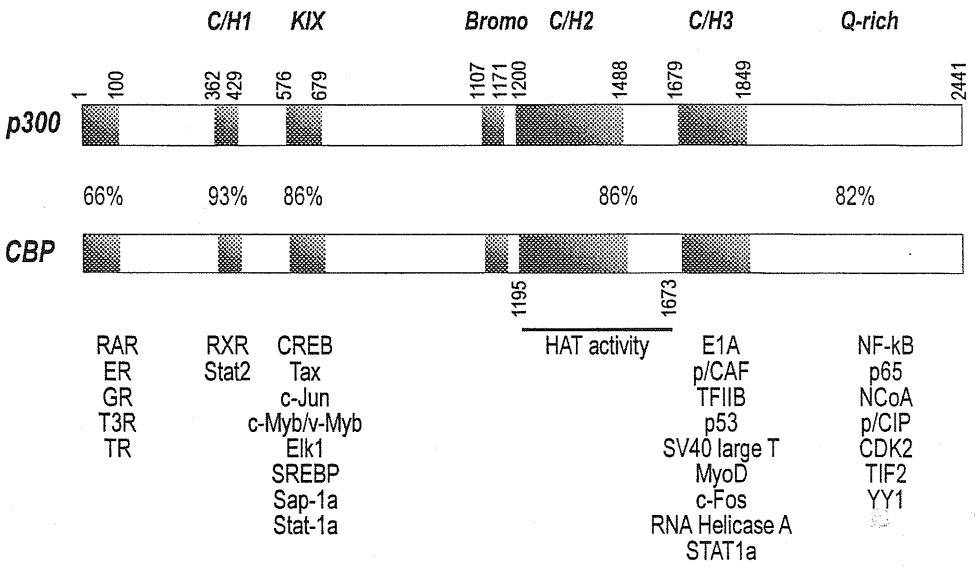

Figure 1.8
Structure of p300 and CBP, degree of homology, and interacting factors

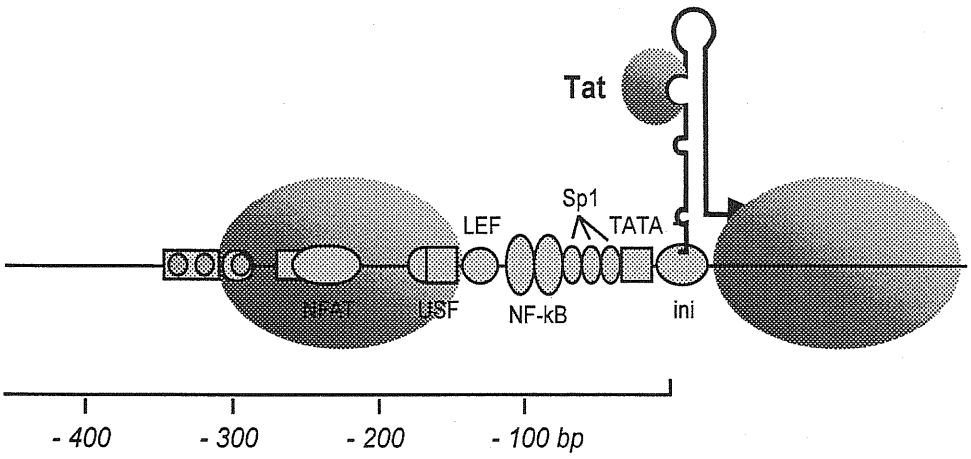

Figure 1.9
Nucleosomes positioned on the integrated HIV-1 LTR

Chapter 2

MOLECULAR AND FUNCTIONAL INTERACTIONS OF TRANSCRIPTION FACTOR USF WITH THE LONG TERMINAL REPEAT OF HUMAN IMMUNODEFICIENCY VIRUS TYPE 1

Fabrizio d'Adda di Fagagna, Giuseppe Marzio, Maria Ines Gutierrez, Li Ya Kang, Arturo Falaschi and Mauro Giacca

Journal of Virology (1995) 69, 2765-2775

ABSTRACT

The human transcription factor USF, purified from HeLa cells, and its recombinant 43-kDa subunit bind to the long terminal repeat of HIV-1. The proteins footprint over nucleotides from -173 to -157 upstream of the transcription start site, generating strong DNase I hypersensitivity sites at the 3' sides on both strands. As detected by methylation protection studies, the factor forms symmetric contacts with the guanines of the palindromic CACGTG core of the recognized sequence. Its binding ability is abolished by the mutation of this core sequence, and strongly reduced by the cytosine methylation of the central CpG dinucleotide. Upon binding, both recombinant and purified USF bend the LTR DNA template. The role of USF in the control of transcription initiation from the LTR was tested by *in vitro* transcription assays. Upon addition of the protein, transcription is increased from constructs containing an intact binding site, while the responsiveness is abolished in constructs with a mutated sequence. Furthermore, addition of a decoy plasmid which contains multiple repeats of the target sequence results in downregulation of transcription from the LTR. These results suggest that USF is a positive regulator of LTR-mediated transcriptional activation.

INTRODUCTION

Similar to all retroviruses, the rate of transcription of the integrated proviral form of the human immunodeficiency virus type-1 (HIV-1) is one of the major determinants of the levels of viral gene expression and replication. A single viral transcript originates from the transcription start site at the long terminal repeat (LTR) at the 5' end of the provirus and finishes at the polyadenylation site at the 3' LTR. From this genome-length transcript, which corresponds to the viral RNA eventually to be incapsidated, different gene products arise through extensive use of differential processing events, translation frame-shifts and proteolytic cleavage of precursor polypeptides. Several groups have shown that, although viral replication can be detected in all clinical stages of the infection (133, 143, 152), the levels of viral expression correlate with disease progression (9, 166). For this reason, it appears that the study of the molecular mechanisms controlling the rate of transcription initiation could offer useful insights into the mechanisms of disease development.

The regulation of HIV-1 transcription initiation is achieved through the recognition of the 5' LTR by human nuclear factors and through their interactions with the basal transcriptional machinery. In this respect, the regulation of transcription of the integrated provirus does not differ from that of any other cellular gene. Only one viral protein (the product of the *tat* gene) cooperates with this machinery to increase the rate of transcription initiation and elongation. A number of reports indicate that the U3 and R regions of the LTRs (~550 bp) contain the target sites for several nuclear factors and that many of these factors can bind to the LTR *in vitro* (for a review, see ref. (139)). With the use of an *in vivo* footprinting technique in infected cell lines, we have shown that most of the LTR regions are indeed engaged in protein-DNA interactions also *in vivo*, although the pattern of these interactions is different in different cell lines (45, 46). All the protein binding sites of the LTR are recognized by nuclear proteins physiologically controlling the expression of a variety of cellular genes, indicating that the LTR represents a very interesting biological example of evolutionary tinkering. The reasons for the need of such a complex regulation in the HIV-1 life cycle still remain to be fully understood.

We have previously described that a sequence of the LTR centered at position -164 upstream of the transcription start site contains the binding site for a constitutive binding activity of lymphoid and non-lymphoid cells (74). This region, which is located at the 3' end of the so-called negative regulatory element (NRE) of the LTR (162), contains the hexanucleotide CAC(A/G)TG (E box), which is the consensus target sequence of proteins of the B class of basic-Helix-Loop-Helix-Leucine Zipper (b-HLH-Zip) family (42). Members of this family are the c-Myc (137), Max (19), Mad

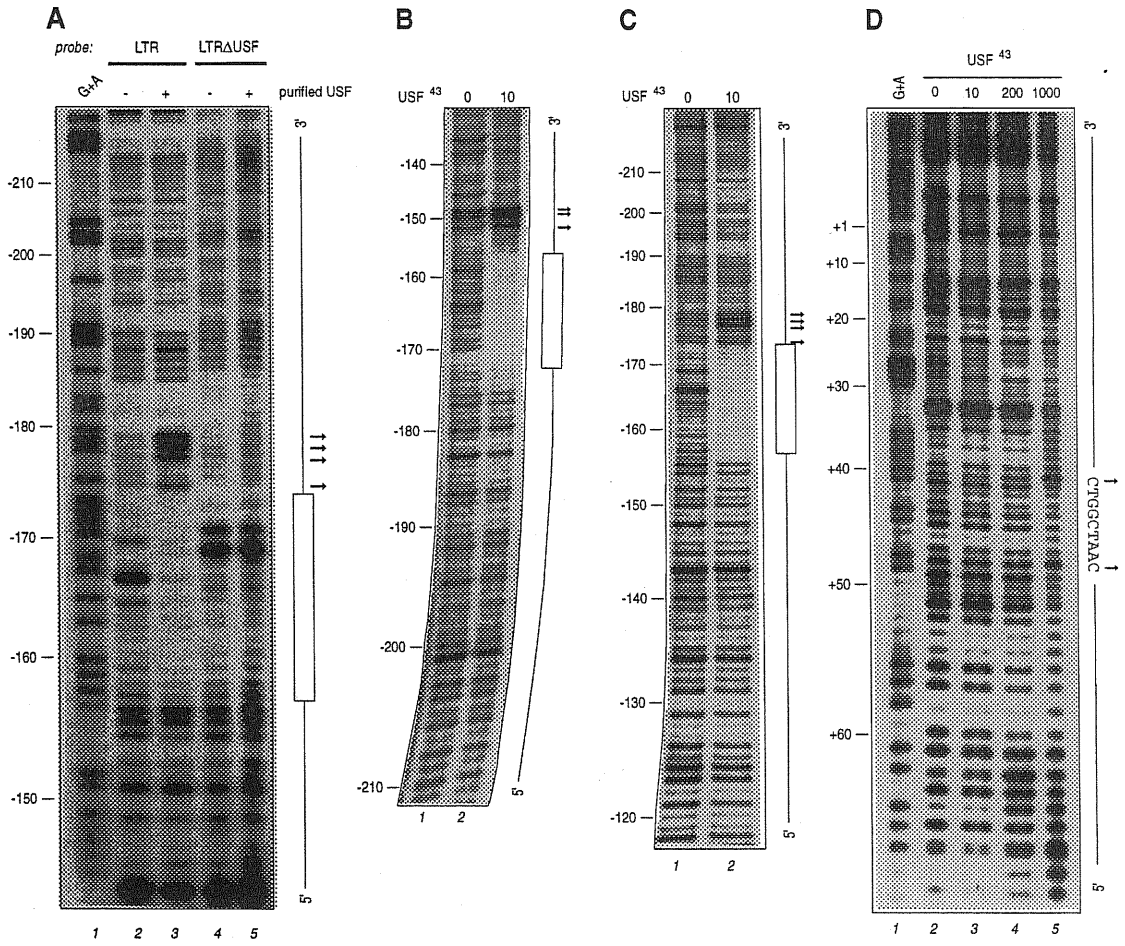

Figure 2.3
DNase I footprinting assays

A. DNase I footprinting using purified USF.
Lane 1: G+A chemical cleavage ladder; lanes 2 and 3: DNA fragment pattern generated by DNase I treatment after incubation of a 5'-end labelled probe in the absence (lane 2) or in the presence (lane 3) of USF; lanes 4 and 5: same as lanes 2 and 3, but using a DNA probe with a mutated E box.
The box and the arrows on the right side indicate the protected region and the hypersensitive nucleotides, respectively, of lane 3.

B. DNase I footprinting on the LTR coding strand using recombinant USF^{43}. Lane 1: 10 ng of GST added; lane 2: 10 ng of recombinant GST-USF^{43} fusion protein.
The box and the arrows on the right side indicate the protected region and the hypersensitive nucleotides, respectively, of lane 2.

C. DNase I footprinting on the LTR non-coding strand with recombinant USF^{43}. Lane 1: 10 ng of GST; lane 2: 10 ng of recombinant GST-USF^{43} fusion protein.

D. DNase I footprinting on the region encompassing the transcription start site with recombinant USF^{43}. Lane 1: G+A chemical cleavage ladder; lane 2: 10 ng of GST; lanes 3-5: 10, 200, 1000 ng of recombinant GST-USF^{43} fusion protein.
A sequence with altered DNase I sensitivity appearing using 1000 ng of protein, with the formation of two hypersensitive sites indicated by arrows, is shown on the right side.

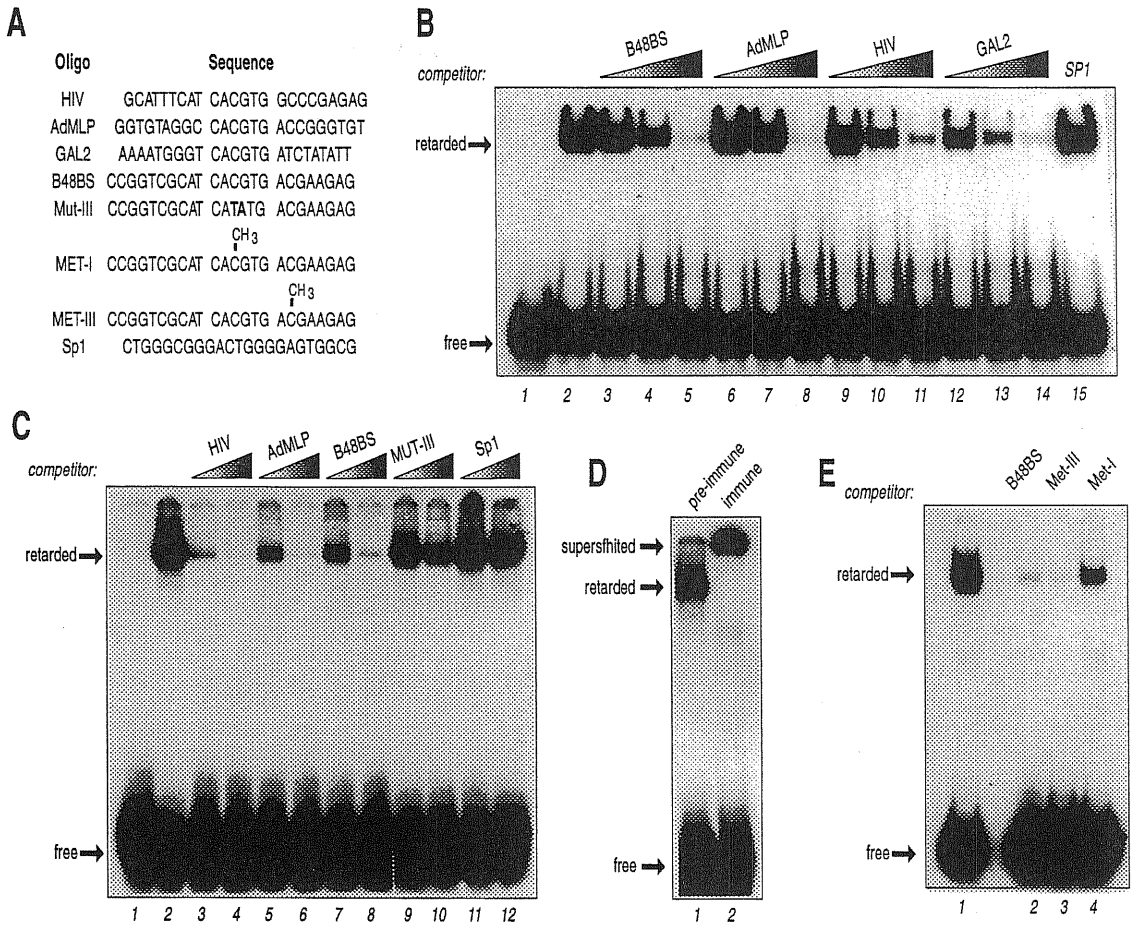

Figure 2.2
Gel retardation and competition assays.

A. Sequences of the upper strands of the oligonucleotides used for the gel retardation and competition assays (see Materials and Methods for their localizations). The E box sequence is boxed. Mutations of this sequence in the oligo MUT-III are indicated by bold typed letters. The localizations of the methyl groups in oligos Met-I and Met-III are indicated.

B. Gel retardation and competition assays with purified USF and oligo HIV as a probe. For the competition experiments, increasing amounts of competitor (6-, 20-, 60-fold molar excess with respect to the probe; 60-fold only for the Sp1 competitor) were added to the binding reactions as indicated on top of the Figure. The arrows indicate the positions of the free and retarded bands. Lane 1: probe without protein.

C. Gel retardation and competition assays with recombinant USF^{43} and oligo HIV as a probe. Lane 1: probe without protein. Competitor molar excesses were 6 and 30 fold for each competitor.

D. Gel retardation-supershift analysis with anti-rUSF^{43} antiserum. The binding reaction between oligo HIV and USF^{43} was incubated with anti-rUSF^{43} antiserum (lane 2) or preimmune serum (lane 1). The arrows indicate the position of the free, retarded, and supershifted bands.

E. Effect of cytosine methylation on USF^{43} binding to oligo HIV. Lanes 1-4: oligo HIV plus USF^{43}; lanes 2, 3, and 4: competition with a 30-fold molar excess of the indicated oligonucleotides.

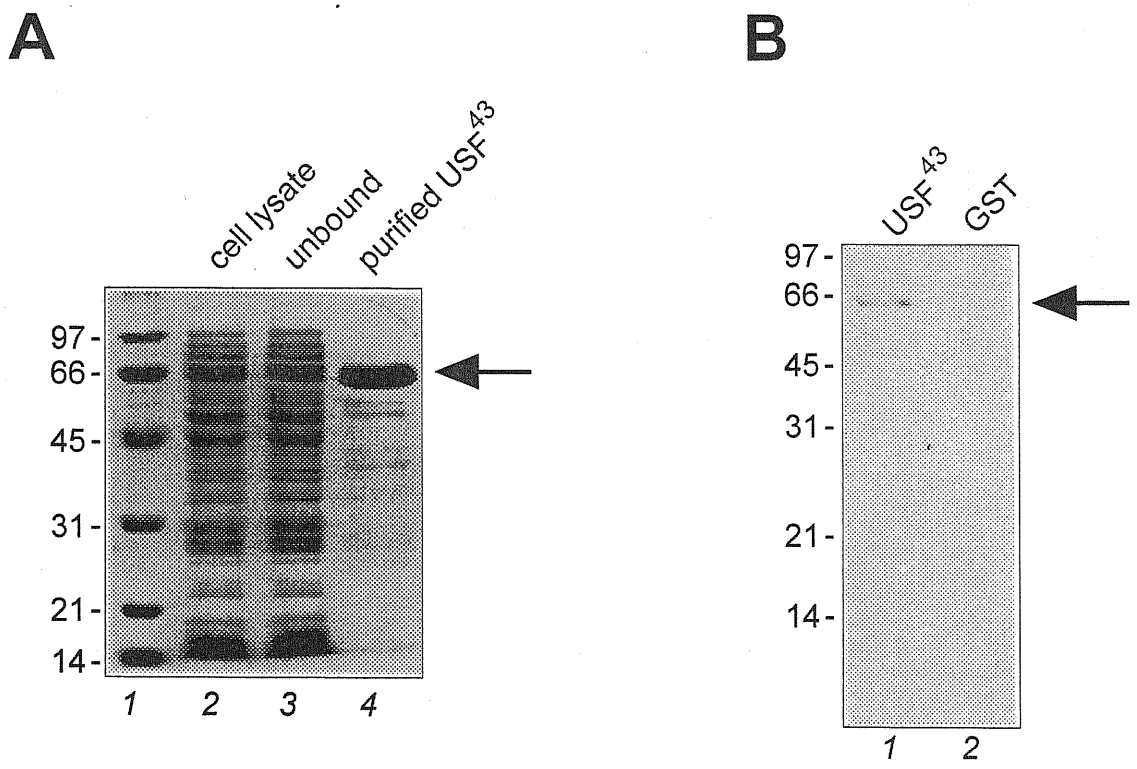

Figure 2.1
Expression and purification of USF^{43}

A. Single-step purification of recombinant USF^{43}
Protein samples were resolved on a 12% Laemli-gel and stained with Coomassie blue. Lane 1: protein molecular weight markers; lane 2: 10 µl of the supernatant of a bacterial lysate of the SF8 E. coli strain transformed with pGST-USF^{43} after induction with IPTG; lane 3: 10 µl of the flow-through fraction from the agarose-glutathione column; lane 4: 40 µl of a fraction obtained by elution of the column with free glutathione. The arrow indicates the position of the GST-USF^{43} fusion protein.

B. Western blot analysis.
Lane 1: purified USF^{43} (15 ng); lane 2: purified GST (15 ng). The arrow indicates the position of the GST-USF^{43} fusion protein. The positions of protein molecular weight markers are indicated.

(8), Mxi1 (212), USF (83, 186), TFE3 (12), and TFEB (29) proteins. All these factors are characterized by the presence of a specific DNA binding domain (basic region) and of two motifs involved in the formation of homo- and hetero-multimers (HLH and leucine zipper domains) (59, 137). Binding sites for b-HLH-Zip proteins and cognate binding activities are very conserved through evolution (73, 74) and they control the expression of several unrelated genes. For example, in the human genome, binding sites have been described in the globin locus control region (21), in the promoter regions of the L-type pyruvate kinase gene (198), of the heme oxygenase 1 gene (168), of the class I alcohol dehydrogenase gene (53), of the type 1 plasminogen activator inhibitor gene (159), of the human growth hormone gene (150), of the N-ras gene (192), of the topoisomerase I gene (91), in the enhancer of the insulin gene (158) and in a region which, as we have recently demonstrated, contains an origin of DNA replication (17, 37, 76). Additionally, it has been recently shown that the E box of the LTR of HIV-1 is also essential for the transcriptional control of the negative strand of the provirus exerted by the LTR at the 3' end (134).

Among the members of the b-HLH-Zip family, transcription factor USF (also known as MLTF (30) or UEF (135)) was originally identified as a human cellular factor which binds to the upstream sequence of the adenovirus major late promoter (MLP) at position -63 to -52 upstream of the transcription start site and stimulates transcription *in vitro* (170). USF is the major binding activity detected by *in vitro* assays with crude nuclear extracts from several cell types and species, including T cells and monocytes (45). After extensive purification, it appears to be composed of two polypeptides with an apparent molecular weight of 43 and 44 kDa (37, 171), both capable of binding independently to the DNA recognition site, even if they preferentially bind as heterodimers (59). The 43-kDa form of USF (USF^{43}) was cloned and shown to belong to the b-HLH-Zip family (83). Very recently, the full length RNA of the 44-kDa form was also isolated, showing a striking similarity to USF^{43} (186).

In this paper, we report the results of our studies on the molecular features of the *in vitro* interaction of both purified human USF and of recombinant USF^{43} with the E box of the HIV-1 LTR, as well as the effects of this interaction on the regulation of transcription.

MATERIALS AND METHODS

Purification of USF from HeLa cells. The procedure for the purification of USF from HeLa cells has already been reported (37). Briefly, a nuclear extract was precipitated by 35% ammonium sulfate; the precipitated protein fraction of was subsequently purified through BioRex 70, HiLoad S-Sepharose and Mono Q columns. The active fractions from the Mono Q column were loaded on a specific DNA-Sepharose affinity column containing ligated concatenamers of the E box site. The purified binding activity consists of a protein doublet with a relative molecular weight of 43 and 44 kDa. The binding activity was monitored throughout the purification by gel retardation and south-western assays.

Cloning, expression and purification of USF^{43}. The plasmid pGST-USF^{43} was constructed by cloning the coding region of the 43-kDa form of USF (USF^{43}) in the commercial vector pGEX2T (Pharmacia, Uppsala, Sweden). This plasmid expresses the glutathione-S-transferase (GST) gene in *E. coli*, under the control of a promoter inducible by isopropyl-galacto-pyranoside (IPTG). The coding sequence of USF was rescued by polymerase chain reaction (PCR) amplification from the plasmid pBSA1MLTF (a kind gift of Dr. Giuseppe Biamonti), which contains the coding sequence of USF^{43} under the control of the human A1 gene promoter (unpublished). The primers for amplification (RUSF1, 5'-CGAGGATCCAAGGGGCAGCAGAAAACA-3' and RUSF2, 5'-GCTGAATTCCTTAGTTGCTGTCATTCTTGATGAG-3') were designed in order to generate a DNA fragment containing the restriction sites for *Eco*R I and *Bam*H I at the two extremities allowing oriented cloning in the vector. Amplification was carried out in 50 µl of a solution containing Tris 10 mM (pH 8.0), KCl 50 mM, $MgCl_2$ 1.5 mM, each dNTP 200 µM, the two primers 1 µM, 1 ng of template plasmid DNA and 2.5 units of *Taq* DNA polymerase (Perkin Elmer Cetus, Norwalk, CT), with 35 repetitions of the following cycle: 45 sec at 94°C, 45 sec at 52°C, and 45 sec at 72°C, in a Perkin-Elmer 480 Thermal Cycler. The sequence of the plasmid insert was verified by DNA sequencing.

A colony of the SF8 strain of *E. coli* transformed by pGST-USF^{43} was grown overnight in 100 ml of Terrific Broth containing 50 µg/ml ampicillin at 30°C. The culture was diluted by the addition of 900 ml of fresh medium and was allowed to grow until the optical density of 0.6-0.8 at 600 nm was

reached; protein expression was then induced by the addition of IPTG (Sigma, St. Louis, MO, 1 mM final concentration). After an additional 3 to 5 hours of incubation, the culture was centrifuged at 5000 x g at 4°C, and the bacterial pellet was resuspended in 10 ml of cold phosphate buffered saline (PBS) containing 4 mM dithiothreitol (DTT). Cells were sonicated in ice by three pulses of 20 sec each. After centrifugation of the lysate, the supernatant was mixed with 1 ml of a 50% (v/v) slurry of glutathione cross-linked agarose beads (Sigma). The fusion protein was allowed to bind to the beads at 4°C on a rotating wheel for 1 hour. The suspension was then loaded on an empty plastic column (BioRad) letting the unbound proteins pass through, and the beads were subsequently washed with 50 ml of PBS containing 4 mM DTT. Finally, USF^{43} was eluted in 1 ml 100 mM Tris containing 4 mM DTT and 20 mM free glutathione (Sigma). With this procedure, 2-3 mg of protein per liter of medium were usually obtained. The purity and integrity of the protein was checked by SDS-PAGE and Coomassie staining.

Western blotting. Fifteen ng of recombinant protein were resolved on a 12% SDS-polyacrylamide gel and transferred to a nitrocellulose filter by electroblotting. The filter was then incubated in 10% milk-TBS buffer (10% w/v non-fat dried milk in 125 mM NaCl, 10 mM Tris, pH 7.4) for 1 hr at 37°C. Incubation with antibodies against full-length $rUSF^{43}$ (kindly donated by Dr. R.G. Roeder) was performed in 5% milk-TBS for 1 hr at room temperature. The filter was then washed in TBS-0.1% Tween 20 and incubated in 5% milk-TBS with alkaline phosphatase-conjugated swine anti-rabbit immunoglobulins for 1 hr at room temperature. After several washes, bound antibodies were revealed using BCIP/NBT colour development solution (BioRad, Richmond CA, USA).

Gel retardation and competition assay. All 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 double-stranded oligonucleotides B48BS, AdMLP, HIV (74), and GAL2, Met I, and Met III (73) have already been described. Oligo Sp1 contains the sequence of the HIV-1 LTR from nucleotides -69 to -46 upstream of the transcription start site, containing two binding sites for transcription factor Sp1. Oligo MUT is a derivative of oligo HIV, where the core E box sequence (CACGTG) was mutated to CATATG.
The synthesized oligonucleotides were resolved by polyacrylamide gel electrophoresis, eluted from the gels, purified and annealed with the complementary strand and end-labelled with [γ^{32}P]-ATP (Amersham, UK; 3000 Ci/mmol; 10 mCi/ml) and T4 polynucleotide kinase.
Gel retardation assays were carried out by the incubation of end-labelled DNA probes (10^4 cpm) with 1-5 ng of purified USF^{43} or 2 µl of purified HeLa USF (Mono Q fraction (37)) and 0.25 µg of poly[d(A-T)]:poly[d(A-T)] or 1.5 µg of poly[d(I-C)]:poly[d(I-C)], respectively, in binding buffer (20 mM Hepes, pH 7.3, 50 mM NaCl, 4 mM DTT, 0.2 mM EDTA, 5% glycerol), in 10 µl final volume. The addition of 1 µl of heat-treated (95°C for 5 min) normal human serum to the binding reaction was used to stabilize the protein-DNA complex. After 20 min incubation at 30°C, samples were resolved by 5% polyacrylamide gel electrophoresis in 0.5 x TBE; the gel was then dried and exposed. Under these conditions, 1 ng of USF^{43} is able to generate a retarded complex which can be detected after an overnight exposure, with an estimated molar DNA/protein ratio of 1/5.6.
Competition experiments were carried out by mixing a 6 to 150 fold excess of cold oligonucleotides to the probe before the addition of the protein. Supershifting of the retarded complex was obtained by the addition to the reaction mixture of 1 µl of a 1:10 dilution of immune serum before the addition of the protein.

Circular permutation assay. A set of six probes (B1 to B6, Fig. 2.6 A) for the circular permutation assay was generated by PCR amplification using the pLTRCAT plasmid (74) as a template. The localization on the LTR of the oligonucleotides utilized for the amplifications are indicated in Fig. 2.6 A. Amplifications were carried out in 50 µl of a solution containing Tris 10 mM (pH 8.0), KCl 50 mM, $MgCl_2$ 1.5 mM, gelatin 0.01%, each dNTP 200 µM, both primers 0.1 µM, one of which previously end-labelled with [g^{32}P]-ATP and T4 polynucleotide kinase, 1 ng of template plasmid DNA and 2.5 units of *Taq* DNA polymerase, with 35 repetitions of the following cycle: 45 sec at 94°C, 45 sec at 60°C, and 45 sec at 72°C. PCR products were resolved on a 5% polyacrylamide native gel and eluted for a few hours at 65°C in Tris 10 mM (pH 8.0) and EDTA 0.1 mM. Curve fitting was performed using the Curve Fit program on a Macintosh computer by the application of a cosine function.

DNase I footprinting. The probes for DNase I footprinting were generated by PCR amplification of the HIV-1 LTR from the plasmid pLTRCAT. For the experiments with purified USF, the primers for amplifications were FOOTP (5'-GCAAGCTTGAAGAGGCCAAT-3') and USF1 (5'-AGCAAGCTCGATGTCAGCAGTTCTT-3'); for the experiments with recombinant USF^{43}, the primers used were from position -256 to -220 and from -46 to -70 respectively, relative to the LTR sequence numbering of transcription start site. One of the two primers was end-labelled with [γ^{32}P]-ATP and T4 polynucleotide kinase before PCR amplification in order to generate an asymmetrically-labelled DNA fragment.
Plasmid pLTRΔUSF was obtained by PCR amplification from plasmid pLTRCAT with primers pLTR-GlessI (5'-GCGAATTCTAGGGCGAATTGGGTACC-3') and ΔUSF (5'-GCTCTCGGGGCGAATTCATGAAATGCTAG GCGGC-3'), the latter bearing the CACGTG sequence mutated into an *Eco*R I (GAATTC) restriction site. The amplification product was purified, cut with *Ava* I and *Kpn* I and ligated between the *Ava* I and *Kpn* I sites of pLTRCAT, therefore substituting for the wild type sequence.
In the experiments with USF purified from HeLa cells, about 10^5 cpm of the probe were incubated with 20 µl of purified protein (mono Q fraction), with 2 µg of poly[d(I-C)]:poly[d(I-C)], in the same buffer as that used for the gel retardation assays. After 20 min incubation at room temperature, the sample was mixed with an equal volume of a solution containing 5 mM $CaCl_2$ and 10 mM $MgCl_2$. DNase I (Boehringer Mannheim, Germany) was added to a final concentration of 6 ng/ml. After 1 min, DNase I activity was stopped by the addition of SDS and EDTA (1% and 25 mM final concentrations respectively). Proteins were removed by phenol extraction and the DNA fragments were precipitated, dissolved, denatured and loaded on a 8% polyacrylamide sequencing gel. As a control, the probe was incubated in the same conditions as above without the addition of the protein. In this case, DNase I was used at a concentration of 2 ng/µl.
When testing the recombinant protein, 10 ng of USF^{43} or GST (in the control reaction) were mixed with $5x10^4$ cpm of the probe in the same conditions as above. DNase I was allowed to digest for 30 sec at a final concentration of 0.5 ng/ml.
A G+A ladder of the probe, obtained according to the Maxam and Gilbert chemical cleavage method (126), was loaded alongside to align the DNase I digestion products.

Methylation protection assay. Methylation protection assays with dimetyl sulfate (DMS) were carried out essentially as described (144). Briefly, 5-fold scaled-up gel retardation assays were assembled; after electrophoresis, the gel was immersed in a 0.2% (v/v) DMS solution for 4 min; methylation reaction was stopped by soaking the gel in a 0.5 M β-mercaptoethanol solution. After autoradiography, the free and retarded bands were excised separately, DNA was eluted and cleaved by piperidine treatment (126). The products were resolved on a sequencing gel alongside a G+A ladder.

***In vitro* transcription assays.** The LTR-containing G-less plasmids for *in vitro* transcription assays are derivatives of the pUGL400 plasmid, which contains a *Eco*R I-*Xho* I fragment from plasmid Syn-O-TG (175), carrying a G-free cassette of ~380 bp (169), cloned between the *Eco*R I-*Sal* I sites of pUC19. The HIV-1 promoter element to be cloned upstream of the G-less cassette was obtained by PCR amplification from plasmid pLTRCAT (74), with primers pLTR-GlessI, containing a *Eco*R I restriction site at the 5' end continuing with the HIV-1 sequence upstream of the LTR) and primer pLTR-GlessII (5'-CGGAGCTCAGGCAAAAAGCAGCTGCTTA-3', containing a *Sac* I site at the 5' end in the correspondence of the LTR transcription start site); the amplification product obtained was cloned between the *Eco*R I and *Sac* I sites of pUGL400 to obtain plasmid pGLA. Plasmid pGLE was obtained following the same strategy, with the exception that the plasmid pLTRDUSF was the template DNA for PCR amplification. As a consequence, the nucleotides at position -162 to -167 are GAATTC (containing a *Eco*R I site) instead of CACGTG.
Plasmid pFN2, containing the fibronectin promoter upstream of a ~200 nt G-less cassette, was used as an internal control for the *in vitro* transcription experiments. It is a derivative of plasmid pUGL200, which was obtained by cloning a PCR amplification product between the *Eco*R I and *Hind* III sites of pUC19. The amplification product derives from plasmid pUGL400 and was obtained by using the M13-universal primer and primer GL202 (5'-GGAAGCTTGGATCCCGGGATAAGATTG-3'). The latter contains at the 3' end a region complementary to the sequence from 187 to 108 of the ~380 nt G-less cassette (169); PCR amplification was followed by digestion with

*Eco*R I and *Hind* III. Plasmid pFN2 was obtained by cloning of a ~710 bp fragment obtained by partial digestion with *Aat* II and *Sac* I of plasmid p-220 (a kind gift of Dr. Alberto Kornblihtt), into the corresponding sites of pUGL200. The cloned fragment contains the human fibronectin promoter .

HeLa nuclear extracts for *in vitro* transcription assays were prepared according to the protocol of Hattori et al. (90) and modified as follows: cells were washed twice in PBS, once in RSB (10 mM Hepes, 10 mM KCl, 3mM $MgCl_2$, 0.1 mM EDTA, 0.74 mM spermidine, 1 mM DTT, 0.5 mM PMSF, 2 μg/ml aprotinin (Sigma) and leupeptin (Sigma), 5 mM bestatin (Sigma)) and resuspended in RSB; following a 20 min incubation on ice, cells were lysed using a Dounce homogenizer.

Transcription reactions were carried out in 25-30 μl final volume containing 250 ng (in the experiments monitoring the effects of recombinant USF) or 750 ng (in the competition experiments with the E box decoys) of DNA template, 75 μg of nuclear extract, 500 μM ATP and CTP, 25 μM UTP, 10 μCi of [a-^{32}P]-UTP (Amersham, UK; 3000 Ci/mmol; 10 mCi/ml), 0.1 mM *o*-methylguanosin, 10 mM *N* - 2-hydroxethylpiperazine-*N'*-2-ethanesulfonic acid (Hepes; pH 7.4), 7.5 mM $MgCl_2$, 5 U RNase T_1 (Boehringer), 30 U RNasin (Promega, Madison, WI), 4 mM DTT. Nuclear extract was equilibrated for 15 min at 30°C with the addition of the appropriate amounts of protein or decoy template. DNA was then added, preincubated for 10 min at 30°C and the reaction was initialized by the addition of the above described reaction mix. Transcription was allowed to run at 30°C for 45 min and stopped by adding 175 ml of a solution containing 300 mM Tris (pH 7.4), 300 mM sodium acetate, 0.5% SDS, 2 mM EDTA, 3 mg/ml tRNA. Proteins were then extracted with 200 μl of PCI (Phenol/Chlorophorm/Isoamilic alcohol 25/24/1), RNA was precipitated with ethanol, dried and run at 12 W on a denaturing 8% polyacrylamide gel in 1xTBE and 0.1% SDS. Finally, the gel was dried and exposed for autoradiography.

Plasmid pUF128 (a gift of Dr. Fabio Cobianchi) is a pUC19 derivative containing 128 copies arranged in tandem of a 65 bp *Alu* I-*Fnu* D2 sequence from the insert of plasmid pL15 (74), containing the E box present in the human lamin B2 origin of DNA replication (18, 76).

RESULTS

Purified USF and recombinant USF^{43} bind to the long terminal repeats of HIV-1

The presence of a human nuclear factor binding to the HIV-1 LTR region centered around nucleotide -164 upstream of the transcription start site has been originally identified by DNase I footprinting using HeLa cell nuclear extracts (70). Subsequently, we have shown that the protein interacting with this sequence also binds to the upstream element of the adenovirus MLP and to several other sequences containing the hexanucleotide consensus sequence CAC(A/G)TG (74), which is the E box target of members of the B class of the bHLH family of proteins (42). By means of a combination of ion-exchange and sequence-specific affinity chromatography techniques (37), we have purified to homogeneity a protein complex binding to the E box. This complex is composed of two polypeptides of 42- and 44-kDa; its size, heat stability, and target DNA sequence suggest that it corresponds to the transcription factor USF, a member of the b-HLH-Zip family (83, 186); furthermore, the 42-kDa polypeptide is recognized by antibodies raised against 43 kDa-USF (37).

In order to study in further detail the interactions of this transcription factor with the HIV-1 LTR, we expressed and purified USF^{43} from bacteria as a recombinant protein fused to glutathione-S-transferase (GST). On the basis of the available cDNA sequence (83), the coding region of USF^{43} was cloned in plasmid pGEX2T, at the 3'-end of the GST gene maintaining the same open reading frame. The resulting plasmid, pGST-USF^{43}, expresses the USF^{43} protein as an extension of the C-terminus of GST under the control of a promoter inducible by IPTG. In Fig. 2.1 A the results of a single-step purification of USF^{43} from bacteria are shown. The identity of the purified protein was further confirmed by western blot analysis using antibodies raised against rUSF^{43} (153) (Fig. 2.1 B).

The interactions of USF purified from HeLa cells and of recombinant USF^{43} with the E box of the HIV-1 LTR and other E boxes are shown in Fig. 2.2. A double-stranded oligonucleotide corresponding to the sequence from -174 to -151 of the LTR (oligo HIV) specifically binds to purified USF in a gel retardation assay resulting in the formation of a retarded complex (Fig. 2.2 B, lane 2). This complex can be competed by the addition of an excess of cold oligonucleotides with the

same sequence (lanes 9-11) or corresponding to other E box sequences (B48BS, contained in a human origin of DNA replication (76); AdMLP, the MLP upstream element; GAL2, a sequence upstream of the yeast GAL2 gene (73)). An oligonucleotide encompassing the two downstream-positioned Sp1 sites of the LTR (oligo SP1, lane 15) is ineffective in the competition assay.
Similar to the purified factor, the recombinant USF^{43} protein also binds to the HIV oligo in gel retardation assays (Fig. 2.2 C, lane 2). The addition of anti-rUSF^{43} antibodies to the binding reaction causes a supershift of the protein-DNA complex, in analogy with the effect described for the purified protein (37) (Fig. 2.2 D). Again, the specificity of binding was challenged by the addition of a 6 to 30 fold excess of other cold oligonucleotides to the binding reactions. Competition was obtained with the same HIV oligonucleotide (Fig. 2.2 C, lanes 3 and 4) and with the related oligonucleotides AdMLP and B48BS (lanes 5-8), but not with the oligonucleotide containing the Sp1 sites (lanes 11-12).

An oligonucleotide carrying a TpA dinucleotide in the core position of the E box instead of CpG (CATATG, oligo MUT-III) is not able to compete for binding (lanes 9 and 10). This result indicates that these nucleotides are essential for the specificity of sequence recognition by the protein as already suggested (14, 42). Since the CpG dinucleotide is the target for physiological cellular methylation, we investigated the role of cytosine methylation within the E box by competition experiments using methylated oligonucleotides with the same sequence of B48BS (Fig. 2.2 E). The results indicated that, whilst methylation on both strands of a CpG outside of the E box consensus sequence (oligo Met-III) has no effect on the competing ability of the oligonucleotide with respect to the unmethylated sequence (compare lanes 2 and 3), methylation of the core CpG dinucleotide of the E box (oligo Met-I) greatly affects competition (lane 4). These results obtained with the recombinant USF^{43} protein reflect those described for crude nuclear extracts from HeLa cells (73).

Interactions of USF with the E box of the HIV-1 long terminal repeat

Further details of the interactions between purified USF or recombinant USF^{43} and the HIV-1 LTR were obtained by a DNase I footprinting assay. As shown in Fig. 2.3 A, the incubation of a LTR DNA probe labelled at the 5'-end of the non-coding strand with the purified factor (lane 3) prevents DNase I cleavage of a box centered around the CACGTG sequence (nucleotides from -173 to -157 upstream of the transcription start site). Lanes 4 and 5 show the results of DNase I digestion of a probe derived from plasmid pLTRΔUSF, in which the CACGTG motif was mutated into an *Eco*RI site (GAATTC). In this case, no difference can be detected in the DNase I digestion pattern obtained in the presence or absence of the protein, again indicating that the E box is absolutely required for protein binding. Protein binding causes the appearance of four strong hypersensitive sites at the 3' end of the recognized box, corresponding to nucleotides G_{-179}, A_{-178}, T_{-177}, and T_{-174} (arrows on the right side of Fig.2.3 A). A similar pattern of footprinting is produced by the binding of recombinant USF^{43} (Fig. 2.3 C). On the upper, coding strand, the recombinant protein protects the E box form DNase I digestion, footprinting over nucleotides -157 to -172, again generating three strong hypersensitive sites at the 3' end (nucleotides C_{-152}, G_{-150}, and C_{-149}, Fig. 2.3 B). The generation of these DNase I hypersensitive sites is most likely due to a structural alteration of the double helix upon protein binding resulting in a widened groove where nucleotides are more accessible to DNase I digestion.

It has been reported that USF^{43}, in addition to E box elements, can also bind to the initiator elements of the adenovirus MLP and of HIV-1 (49, 163). Therefore, we challenged a DNA probe encompassing the HIV-1 transcription start site in DNase I footprinting experiments with increasing amounts of highly purified (>95% homogeneity) USF^{43} (from 10 ng to 1000 ng, Fig. 2.3 D). No changes were observed using up to 200 ng of protein, in contrast with the clear protection of the E box which is complete with even 10 ng, panels B and C). Only upon addition of 1 μg of protein, some changes in the footprinting pattern were detected in the region from nucleotides +42 to +47 relative to transcription start site, with the formation of two hypersensitive sites at nucleotides C_{40} and C_{48}. However, it should be considered that this sequence does not fully match the previously reported Inr elements of the HIV-1 promoter (49), and that these changes could be non specific, due to the very high amount of protein used.

A further insight into the pattern of interaction of USF with the HIV-1 LTR was obtained by methylation protection experiments with dimethyl sulfate (DMS), a sensitive chemical method to probe DNA-protein contacts which allows the determination of the purines

protected from methylation by DNA-bound proteins. The results of methylation protection experiments with USF^{43} and the HIV-1 LTR as a probe are shown in Fig. 2.4 A for the upper, coding strand and B for the lower, non-coding strand. Guanines at positions -162 and -164 on the coding strand, and -165, -167, and -170 on the non-coding strand are clearly protected from methylation, while guanine at position -161 on the coding strand is hypersensitive.

The results obtained by the DNase I and methylation protection experiments are summarized in Fig. 2.5. It is evident that the E box consensus sequence CACGTG is centered at the 2-fold rotational axis of symmetry of protein-DNA interaction, with major contacts occurring at the 3' half of the DNA sequence on both strands. This pattern closely resembles the one produced by USF binding to the MLP upstream element (135, 170).

Template LTR bending upon USF binding

The generation of sites of increased sensitivity to DNase I symmetrically located on both strands and the presence of a site hypersensitive to methylation on the coding strand (see Fig. 2.5), suggest that DNA undergoes a structural distortion upon protein binding.

The ability of USF to bend the LTR template upon binding was tested by circular permutation assay, a method based on the position-dependent effect of DNA bends on the electrophoretic mobility of DNA fragments (209). To exploit this technique, a set of six probes (B1 to B6) was obtained by PCR amplification using the LTR as a template. All these probes have approximately the same length (from 244 to 246 bp) but differ because of the position of the USF binding site (Fig. 2.6 A). They were tested in gel retardation assays both with USF^{43} and with purified USF (Fig. 2.6 panels B and C respectively). As expected, all the probes gave rise to a retarded complex upon incubation with both protein species. However, the relative mobility of the retarded bands of the probes containing the binding site in the middle were lower than those of the probes containing the binding site at one extremity, suggesting that the template DNA becomes bent upon protein binding. The centre of the flexure was mapped by plotting the mobility as a function of the distance of the binding site from the end of the probe (Fig. 2.6 panels B and C, lower graph) nd turned out to correspond to the position of the CACGTG box.

Estimation of the bending angle according to Thompson and Landy (191) indicates values of 90°C of bending for purified USF and 110° degrees for recombinant USF^{43}, as evaluated on the average values obtained from three independent experiments. As a negative control, the Ku protein, which binds to DNA without an apparent sequence specificity giving rise to several retarded complexes due to multiple protein dimers bound to the probe (37), was also tested in gel retardation assays with the same probes. As shown in Fig. 2.6 D, the retarded bands generated by all the probes migrate with the same mobility.

***In vitro* transcription assays**

The role of USF in the control of transcription from the HIV-1 LTR was determined by *in vitro* transcription assays. For this purpose, the LTR region upstream of nucleotide -1 was cloned upstream of a ~380 nt G-less cassette. Each *in vitro* transcription experiment was simultaneously performed by the addition to the same tube of a plasmid template containing a shorter G-less cassette (~200 nt) under the control of the fibronectinpromoter, to be used as an internal control.

The addition of increasing amounts of highly purified recombinant USF^{43} to HeLa cell nuclear extracts progressively upregulates transcription from the LTR in a concentration-dependent manner, while it does not affect the transcription driven by the fibronectin promoter (Fig. 2.7 A, lanes 2-6). Quantification by scintillation counting and optical scanning of the autoradiograms indicated a reproducible three-to fourfold increase in LTR-driven signal intensity after normalization to fibronectin; the ratio between the intensity of the RNA bands for the two constructs for each experimental point is reported in the graph below the gel . Mutation of the CACGTG hexanucleotide to an unrelated *Eco*R I restriction site abolishes responsiveness of the LTR construct to protein addition (Fig. 2.7 B, lanes 2-6). Furthermore, the transcription efficiency of this mutated promoter is invariably lower than that of the wild type LTR (compare lanes 1 in panels A and C with lanes 1 in panels B and D).

The HeLa cell extract used in the *in vitro* transcription experiments contains, among other E box binding factors, an endogenous amount of USF (estimated in the range of 0.15-1.5 ng/μg of extract (153)), which presumably contributes to the basal level of transcriptional activation from the LTR template. In order to saturate the endogenous

E box binding proteins, increasing amounts of a plasmid (pUF128) carrying 128 copies of a human E box containing sequence were added to the transcription reactions (Fig. 2.7 C, lanes 2-5). The addition of this decoy progressively decreases the level of transcription from the LTR as compared to the fibronectin promoter. As a control, the same molar amounts of vector pUC19 are ineffective (lanes 6-9). Again, mutation of the E box of the LTR abolishes responsiveness of the LTR construct to the specific decoy addition (Fig. 2.7 D, lanes 2-5).

Altogether, these results show that the E box in the context of the U3 LTR region is a positive *cis*-acting element in the control of transcription and that USF is most probably a *trans*-acting factor contributing to this function.

DISCUSSION

The regulation of the rate of transcription of the HIV-1 provirus is achieved by the interaction of several human transcription factors with the LTR DNA and of the viral Tat transactivator with the 5' end of the nascent RNA. Although the LTR appears as a highly inducible promoter responsive to a number of stimuli which trigger cellular activation of proliferation, interestingiy most of the human factors binding to the LTR are constitutively present in most cell types and tissues. The reasons for this evolutionary outcome have still to be understood. It is conceivable that this situation reflects the need either for responsiveness to a variety of cellular environments, or for fine tuning of viral transcription in response to a variety of cellular events.

One of the LTR regions which are recognized by a constitutive binding activity in human nuclear extracts is the E box centered at position -164 upstream of the transcription start site. This site is actively engaged in protein-DNA interactions in an actively producing T-cell line *in vivo* (46) and appears to be strictly conserved, despite the overall HIV-1 sequence variability, in the DNA amplified directly from the peripheral blood lymphocytes of AIDS patients over several years (130). Furthermore, this same site appears to play an active role in the control of transcription by the recently described negative strand LTR promoter (134).

These observations, as well as the evolutionary conservation of the E box motif and the cognate binding proteins in the upstream sequences of several cellular genes (73, 74), suggest that this sequence has an important biological role in the context of the control of HIV-1 expression.

Interactions of USF with the LTR E box target site

Several nuclear proteins are capable of specifically binding to the CACPuTG sequence. In south-western experiments, at least three species (of ~43/44, 70, and 110 kDa apparent molecular weight) are detectable (37, 74) and, in the last years, the cDNA sequences of several proteins of the b-HLH-Zip family interacting with this E box were cloned. The major DNA-binding activity detectable by gel retardation assays in nuclear extracts corresponds to transcription factor USF, which is purified as a heterodimer composed of two polypeptides of 43 and 44 kDa encoded by two different genes (83, 186). We show, with the experiments reported in this work, that purified human USF specifically binds to the E box at position -162/-167 of the HIV-1 LTR. Indeed, the 39 kDa-HIV-TF1 factor binding to this sequence which was purified by Maekawa *et al.* is likely to correspond to USF (122).

The 43- and 44-kDa polypeptides preferentially bind as a heterodimer to the target site (59), even if each one is individually able to bind to DNA (153, 186), as demonstrated also by south-western analysis (37). We exploited the available cDNA sequence for the 43-kDa form of USF to produce a recombinant fusion USF^{43} protein in bacteria. Both the purified ($USF^{43/44}$) and the recombinant (USF^{43}) proteins show the same binding specificity for the HIV-1 LTR and other targets containing an E box sequence. As expected, USF^{43} binding is affected in the same way by the mutations that abolish DNA binding of the eukaryotic protein.

Similar to the interactions occurring at the MLP upstream element (135, 170), the G residues which exhibit decreased methylation in the presence of USF are symmetrically situated in the two halves of the region of dyad symmetry. This symmetry is consistent with the proposed binding of the factor as a dimer to the target sequence, with each monomer contacting the DNA sequence on the 3' site of the dyad axis of the E box element (59). It is likely that the protein dimer binds at the centre of the palindromic site with the basic domains extending symmetrically into the major grooves of each half site, as suggested (63).

With analogy to the sensitivity of the native factor (73, 202), binding of recombinant USF^{43} to the LTR is abolished by methylation of the core CpG dinucleotide of the E box. This observation suggests that a possible mechanism of control for the function of USF (which is constitutively and ubiquitously expressed (185)) could be through epigenetic modification of the target site, namely by altering the methylation state of DNA. Since it has been shown that methylation of the LTR represses HIV-1 transcription (13), it is likely that the E box could be one of the target sites mediating this effect. Finally it is interesting to observe that the mechanism for preventing binding by target site methylation is not restricted to USF, since binding by other members of the b-HLH-Zip family is also sensitive to methylation of the core CpG sequence of the E box (157).

Template LTR bending upon USF binding

The circular permutation analyses performed, indicate that both USF purified from HeLa cells and recombinant USF^{43} bend DNA upon binding to the target site. It is likely that the sites of hypersensitivity to DNA methylation and DNase I digestion detected in the methylation protection and footprinting experiments are generated by an increase in the groove width, typical of DNA bending upon protein binding. Although circular permutation assay alone does not allow the distinction between DNA bending and increased DNA flexibility, the detected extent of bending (90°-110°) cannot be due only to a mere increase in flexibility. DNA bending by a number of b-HLH-Zip proteins (including USF and Max) has been reported (63, 203). However, crystal analysis of the complex of DNA with Max (60) and with the USF b-HLH-Zip regions (59) could not show any net bend in the double helical axis. The reasons for this discrepancy are likely to be related to the crystal packing mode of the two structures.

In our experiments, the calculated bending angles induced by HeLa-purified USF and USF^{43} were 90° and 110°, respectively. A trivial explanation for this difference could be that recombinant USF^{43}, bearing the GST extension, has a different shape and a greater size than HeLa-purified USF. However, it was demonstrated that there is no significant correlation between the molecular weight of the protein and the extent of the induced DNA bending (109). Therefore, our preferred interpretation is that the difference in the bending ability is due to the different molecular compositions of the dimers interacting with DNA: an obligate USF^{43} homodimer in the experiments with the recombinant protein and a preferred USF$^{43/44}$ heterodimer in the experiments with the purified factor. In the latter case, the bending angle results from the vectorial sum of the bending angles induced by the two different monomers. In this respect, there are also other examples of differential bending by monomeric or heteromeric forms such as Myc/Max and Fos/Jun heterodimers (109, 203).

In the context of HIV-1 transcriptional regulation, the studies on the functional role of LTR DNA bending by transcriptional factors probably deserve further scrutiny. In fact, it should be considered that, in addition to USF, other transcription factors that also bind to the LTR (Jun/Fos (109), NF-kB (176), TBP (96), YY1 (125, 140), Sp1 (98)) are able to bend the target DNA sequence. As a consequence, the traditional picture of the LTR as a linear structure should be replaced by a more realistic 3D-view of a highly structured promoter where also non adjacent proteins, by DNA bending and looping, can interact among themselves and with the basal transcriptional machinery. According to this view, it has been recently reported that Sp1 interacts with NF-kB (151), that both Sp1 and Tat bind to TBP (56, 107), that YY1 physically interacts with Sp1 (119), and that Fos is able to bind to the 44 kDa form of USF (20). In this respect, it is also interesting to observe that the b-HLH-Zip domain of USF was reported to exist as a bivalent tetramer, potentially able to bind simultaneously to two independent sites, with a possible role in DNA looping (59). However, despite reported evidence showing interactions between USF and the initiator element of the HIV-1 LTR (49, 163), we were not able to obtain a clear footprint on this region, even with the addition of an amount of protein of 100 fold as that needed to footprint over the E box. Accordingly, the analysis of the USF binding sites by the random oligonucleotide selection procedure showed that the E box motif is almost absolutely required for binding (14).

Functional significance of USF binding to the LTR

In vitro transcription experiments, performed either by the addition of recombinant USF^{43}, or by the subtraction of endogenous E box binding proteins by E box decoys, indicate that USF acts as a positive regulator of transcription driven by the LTR promoter. This effect is strictly and solely dependent upon the presence of an intact E box, since mutation of

the E box impairs the responsiveness of the promoter in both types of experiments and weakens its basal strength.

We have previously reported (74) that a human binding site for bHLH proteins, contained in a human origin of DNA replication (17, 76), acts as a downregulator of transcription when cloned upstream of the enhancer region of the LTR, therefore replacing the whole negative regulatory element, in agreement with the postulated negative function exerted by the site in the context of the infectious virus (120). Several explanations could be proposed to explain this apparent contradiction between the *in vitro* and the *in vivo* experiments. Since the E box is the potential target of different factors, the possibility cannot be ruled out that USF is not the major protein binding to the LTR site within the cell. Alternatively, it could be argued that the major E box binding factor interacting with the human origin sequence is different from the one binding to the HIV-1 LTR, since also the sequences flanking the E box are important to determine the affinity of different b-HLH-Zip proteins (14). Finally, we have recently shown that also other proteins can modulate binding of USF to its target site (214): similarly, the *in vivo* function of the USF site could necessitate the presence of a complex combination of other DNA-binding factors, a situation which is hardly reproducible in the *in vitro* transcription. Further experiments are obviously needed to clarify these points.

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Chapter 3

HIV-1 TAT TRANSACTIVATOR RECRUITS P300 AND CREB-BINDING PROTEIN HISTONE ACETYLTRANSFERASES TO THE VIRAL PROMOTER

Giuseppe Marzio, Mudit Tyagi, Maria Ines Gutierrez and Mauro Giacca

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ABSTRACT

In cells infected with the HIV type 1 (HIV-1), the integrated viral promoter is present in a chromatin-bound conformation and is transcriptionally silent in the absence of stimulation. The HIV-1 Tat protein binds to a stem-loop structure at the 5' end of viral mRNA and relieves this inhibition by inducing a remodeling of the nucleosome arrangement downstream of the transcription-initiation site. Here we show that Tat performs this activity by recruiting to the viral long terminal repeat (LTR) the transcriptional coactivator p300 and the closely related CREB-binding protein (CBP), having histone acetyltransferase (HAT) activity. Tat associates with HAT activity in human nuclear extracts and binds to p300 and CBP both *in vitro* and *in vivo*. Integrity of the basic domain of Tat is essential for this interaction. By a novel quantitative chromatin immunoprecipitation assay we show that the delivery of recombinant Tat induces the association of p300 and CBP with the chromosomally integrated LTR promoter. Expression of human p300 both in human and rodent cells increases the levels of Tat transactivation of the integrated LTR. These results reinforce the evidence that p300 and CBP have a pivotal function at both cellular and viral promoters and demonstrate that they can be recruited also by an RNA-targeted activator. Additionally, these findings have important implications for the understanding of the mechanisms of HIV-1 latency and reactivation.

INTRODUCTION

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 (146, 195, 196, 206). In cells infected with HIV type 1 (HIV-1), the integrated proviral genome is also tightly packaged into chromatin. 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. (145)), is almost silent when integrated into the cellular genome in the absence of stimulation (101, 156). Nuclease accessibility studies of the proviral chromatin structure indicate 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 (188, 199, 200). Through genomic footprinting studies, we have shown that in the silent LTR several critical protein-DNA interactions are still preserved in this region (44, 45).

The Tat protein of HIV-1 is a powerful activator of viral gene expression from the integrated LTR. The protein binds to TAR, a highly structured RNA element located at the 5' end of all viral transcripts (16), and is 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 polymerase (for reviews, see refs. (39, 105)). In an exciting *crescendo* of findings, a converging number of observations have recently indicated that the role of Tat in transcriptional elongation can be ascribed to the specific interaction of the factor with protein complexes possessing protein kinase activity and being able to phosphorylate the carboxyl-terminal domain of the larger subunit of RNA polymerase II (38, 79, 145, 204, 213). This is an essential step for the recruitment of processive transcriptional complexes on the LTR promoter.

While these data contribute to the elucidation of the functions of Tat in transcriptional processivity, some important questions are still unanswered. 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 (200). In particular, remodeling of the chromatin structure can be induced by Tat, but not by other stimuli acting through the

upstream enhancer sequence (54). 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, induced by proteins having histone acetyltransferase (HAT) activity, weakens histone-DNA interactions, thereby relieving the repressive effects of the chromatin scaffold (for reviews, see refs. (187, 201)). Consistently, the silent, integrated LTR also can be strongly activated by drugs inducing sustainedly high levels of histone acetylation in latently infected cell lines (117, 118, 197).

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. Our results demonstrate that Tat associates with p300 and with the closely related CREB-binding protein (CBP) HATs both *in vitro* and *in vivo* and that it targets these proteins to the integrated LTR promoter. Overexpression of p300 both in human and in rodent cells increases Tat-mediated transactivation of the integrated LTR promoter.

MATERIALS AND METHODS

Plasmids. Plasmid pCMV-Tat101 was constructed by cloning the cDNA of wild-type 101-aa Tat in pcDNA3 (Invitrogen, Carlsbad, CA). Plasmids 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 pCMVβp300, 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 (74).

Recombinant proteins. Glutathione S-transferase (GST), GST-Tat and GST-Tat mutants were prepared as already described (44). Plasmids pBS-KS+hTAF32 and pcDNA3-p300 were used as templates to produce the *in vitro* ^{35}S-labeled hTAF32 and p300 proteins, respectively, using the TNT Reticulocyte Lysate System (Promega, Madison, WI) according to the manufacturer's protocol.

***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 MgCl2, 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 phenilmethylsulphonyl fluoride) 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 phenilmethylsulphonyl fluoride) 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 (22). 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 extensive 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 (Gibco BRL Life Technologies LTD, Paisley, Scotland). HL3T1 cells, kindly donated by B. Felber (58), 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 (167). All transfections were adjusted to the same content of transfected plasmid (and of CMV promoter sequences) by addition of the appropriate amounts of pcDNA3. CAT assays were performed 48 hours after transfections; the results shown in Fig. 2.5 represent the average values obtained in several (at least three) independent transfections.

Treatment with recombinant proteins. HL3T1 were grown overnight to reach about 80% confluence. Cells were then treated with 1 μg/ml GST or GST-Tat proteins and 100 μM chloroquine or Lipofectin (Gibco BRL) according to a published procedure (44). After a 5 hour incubation, 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 phenilmethylsulphonyl fluoride, 1 μM leupeptin, 1 μM pepstatin). The cell lysate was passed through a 24-gauge needle and centrifuged for 10 minutes at 14,000 rpm at 4°C in Eppendorf bench centrifuge. The cleared supernatant was used for immunoprecipitation.

Immunoprecipitation and immunoblotting. Cleared cell lysates were incubated with the appropriate antibodies overnight at 4°C. After incubation, 40 μl of a 50% suspension of protein-A sepharose beads (Pharmacia) 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. (142). Chromatin pellets were resuspended in 500 μl RIPA lysis buffer 50 with protease inhibitors (500 μM phenilmethylsulphonyl fluoride, 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) in 0.5% SDS, 100 mM NaCl and for 6 hours at 65°C to revert cross-links. DNA was extracted with phenol/chloroform/isoamylic 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 (44), B48 and B13 (76) and β-globin (35). The multicompetitor DNA fragment was constructed by a recombinant PCR procedure as already described (75) and outlined in Fig. 3.4. 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 (75, 148).

RESULTS

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

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. 3.1A, left), by exploiting the property of the protein to enter intact cultured cells (44, 66). Five hours after protein delivery, cell lysates were obtained, 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 Tat cDNA (Fig. 3.1 A, right). Consistently, immunoprecipitation with an anti-Tat antibody specifically recovered HAT activity, whereas background activity only was 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 severalfold 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. 3.1 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 (present in several primary HIV isolates), wt Tat 86 (HXB2 clone), and some mutated derivatives of the latter [Tat 86 Δ(1-21), lacking the amino-terminal acidic domain; Tat 86 R(49-57)A, with 6 arginines in the basic domain mutated to alanines; and Tat 86 C(22-27)A, with 3 cysteins mutated to alanines in the cystein-rich domain]. As shown in Fig. 3.1 C, both wild type 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 86C(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 and CBP *in vivo*

The results reported above demonstrate that Tat associates with a HAT activity 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 review, see references (23, 207)). Among the known nuclear HATs, only p300 and CBP are capable of acetylating all four core histones (52, 121, 141). 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. 3.2). 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 occurs also within the cell.

Tat binds p300 *in vitro*

Does Tat directly interact with p300/CBP 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 p300 but not another protein such as hTAF32 (Fig. 3.3 A) or luciferase (not shown) specifically binds to Tat. Thus, there is probably a direct interaction between the two proteins, although we cannot exclude the possibility that an unknown component of the translation lysate mediates indirect binding.

We mapped the sites of interaction of Tat with p300 using a series of mutated Tat derivatives (Fig. 3.3 B and 3.3 C). The transcriptionally active proteins (Tat 101, Tat 86 and Tat 72), as well as the protein mutated in the cystein-rich domain efficiently bound to p300 (Fig. 3.3 B). On the contrary and in good agreement with the results obtained studying the association of Tat with HAT activity (Fig. 3.1 C), the interaction of Tat with p300 strongly depended on the integrity of the arginine-rich domain (Fig. 3.3 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. 3.3 C). The same was observed in a Tat protein bearing a single point mutation in this domain (histidine at position 13 to glutamine; Fig. 3.3 B). Consistently, this mutant is a very poor transactivator of the LTR (less than 5% of wild type; not shown). Mutation at this residue was recently detected in the defective integrated provirus of the latently infected U1 monocytic cell line (57). 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 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. 3.4). This technique is based on the crosslinking of protein-DNA and protein-protein complexes within the cell by formaldehyde treatment (142), followed by chromatin sonication, immunoprecipitation with specific antibodies and precise quantification of the immunoprecipitated DNA segments by competitive PCR (Fig. 3.4 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, and B13, ~7 kb away from the origin), and one region in the β-globin gene (Fig. 3.4 A)]. We used as a competitor 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. 3.4 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 both cases, 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. 3.4 E; the actual competitive PCR results for the quantification of anti-USF immunoprecipitates in GST-treated cells are shown in Fig. 3.4 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, 41). 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. 3.4 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.

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. 3.5, 50 ng of Tat per plate). This is in agreement with the well established notion that rodent cells do not support efficient Tat transactivation (3, 89). 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.

DISCUSSION

The results described in this work suggest that an additional function of HIV-1 Tat is to relieve chromatin inhibition on transcription by recruiting p300 and CBP histone acetyltransferase to the LTR promoter. This conclusion is in agreement with several observations showing that inhibitors of histone de-acetylation, such as sodium butyrate (117, 118), trapoxin and trychostatin (197), cause a remarkable activation of viral gene expression. 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 (52, 121). 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-kB p65 (for a recent review, see ref. (78)). Given the pivotal role of p300/CBP in the control of gene expression, it is not surprising that several viruses encode proteins targeting the two factors. The adenovirus E1A, the HTLV-I Tax, and the SV40 large T proteins (7, 52, 77) are among these viral products.

Tat specifically associates with p300 and CBP *in vitro* and within the cells. Moreover, by *in vivo* quantitative chromatin crosslinking experiments, we also show 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. In this respect, this demonstrates that p300/CBP is also recruited to a promoter by an RNA-targeted activator. 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 (101)), associates with components of the basal transcriptional machinery (e.g. TBP (107)), induces NF-kB which in turn binds to the LTR (44), and recruits protein kinases phosphorylating the carboxyl-terminus of RNA polymerase II (102). 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.

Our findings provide a molecular explanation for the long-standing observation that, in addition to its function in promoting the recruitment of processive RNA polymerase II complexes, Tat has a role in increasing the rate of transcriptional initiation at the LTR (39). It appears plausible that chromatin remodeling and the increase in polymerase processivity are two highly coordinated processes 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 (99). The former factor also can stimulate the TFIIH-dependent phosphorylation of the carboxyl-terminal domain of RNA polymerase II (95, 127). This provides another possible indirect link between Tat-mediated promoter activation and the increase in efficiency of transcriptional elongation.

Our 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 (24, 55, 189). 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.

During the preparation of this manuscript, it was brought to our attention that similar results about the interaction of Tat with cellular HAT proteins had also been obtained by an independent study by K.T. Jeang and collaborators (15).

Note added in proof

While this manuscript was in proof, similar results on the interaction of Tat with p300 and CBP were reported also by Hottiger et al. (97).

ACKNOWLEDGMENTS

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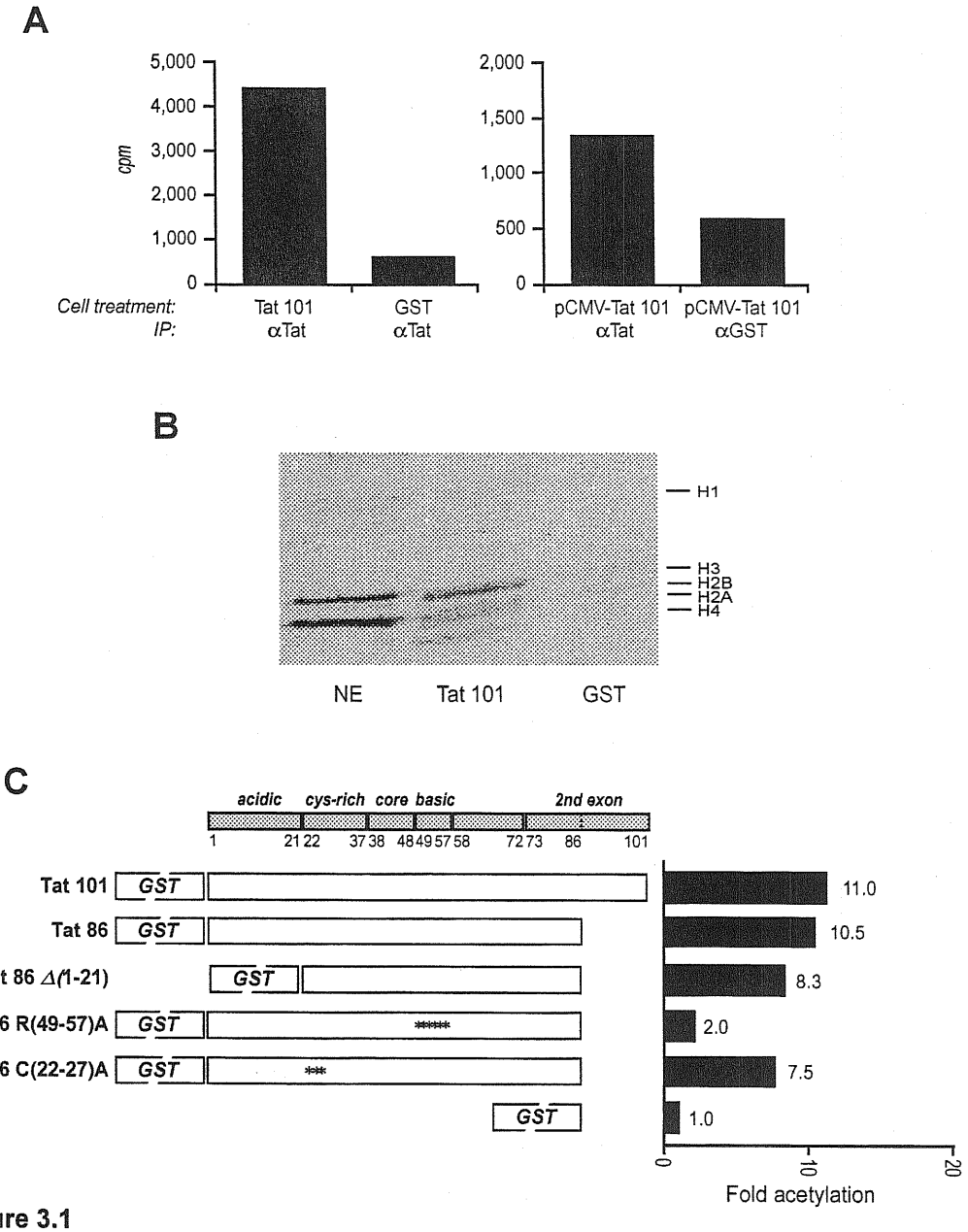


Figure 3.1
Association of Tat with HAT activity

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.

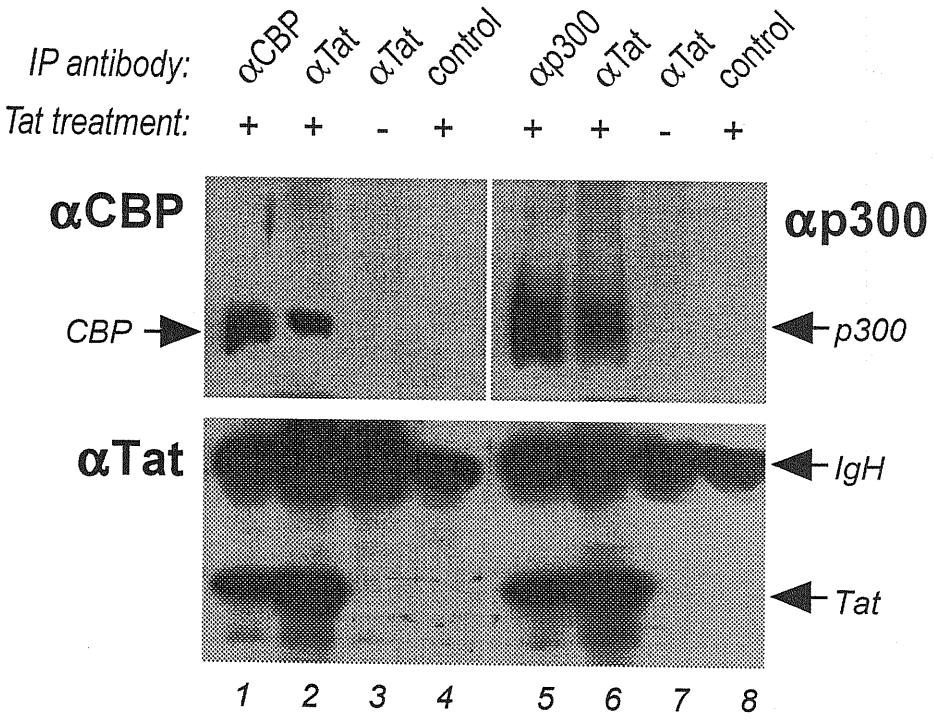


Figure 3.2
Association of Tat with p300 and CBP *in vivo*

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

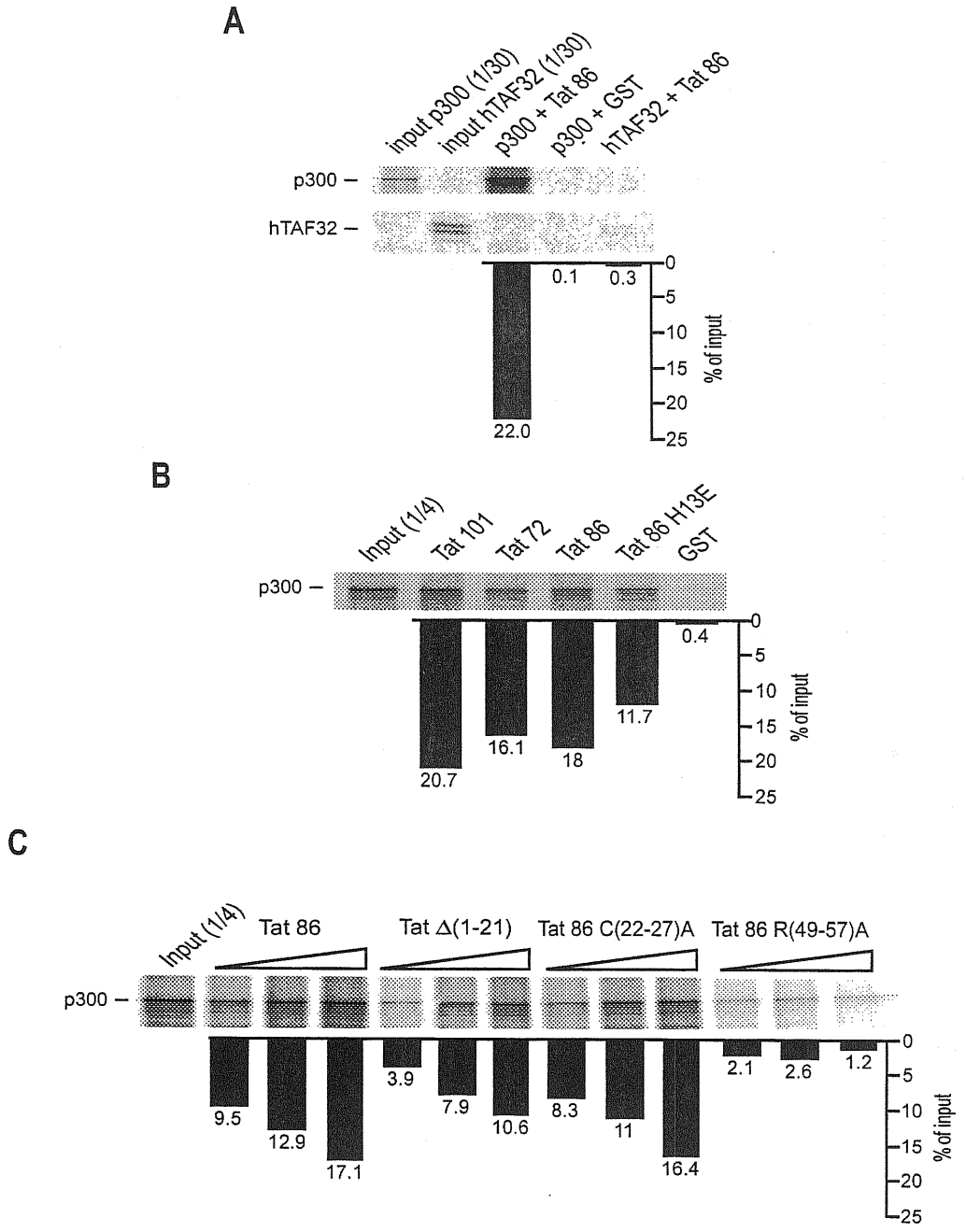


Figure 3.3
Interaction of Tat with p300 in vitro

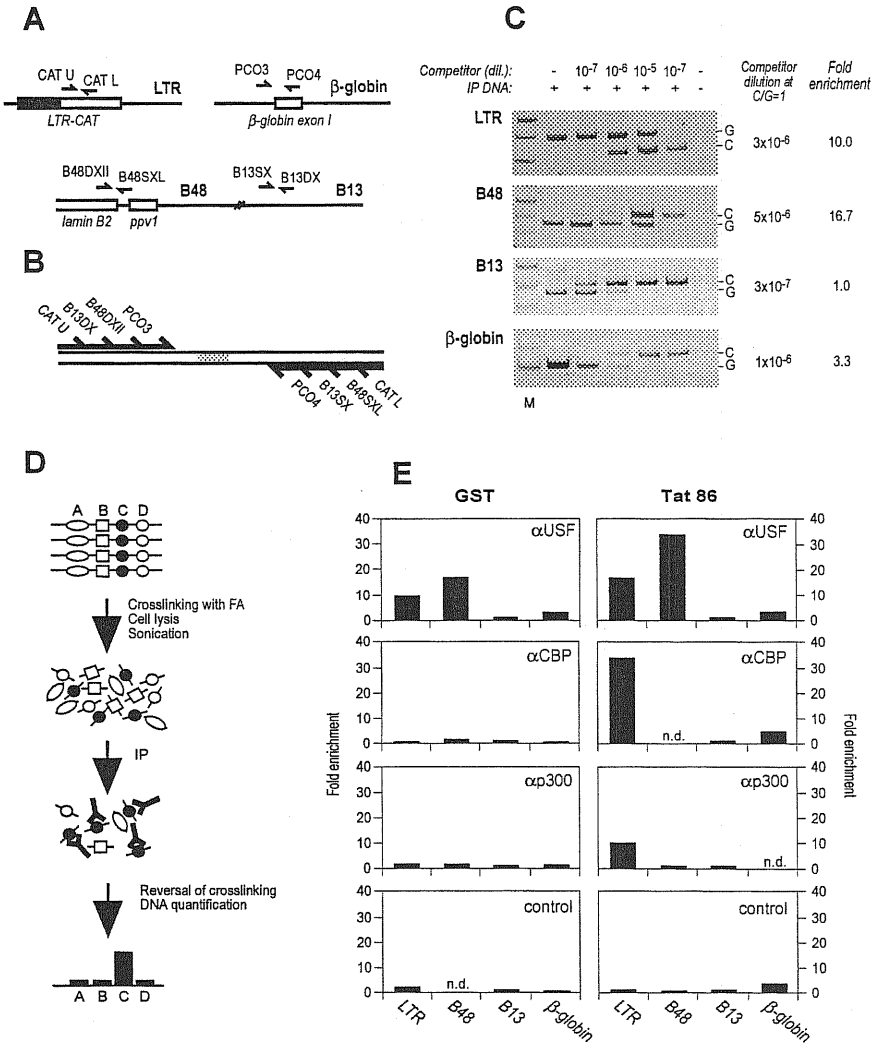
The indicated GST fusion proteins (5 μg) on agarose beads were incubated with [35S]-labeled p300 or hTAF32 (negative control). Bound proteins are expressed as percentages of the input radiolabeled protein.

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 [35S]-labeled p300. Samples were processed as in panel A.

Figure 3.4
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. 4e, 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

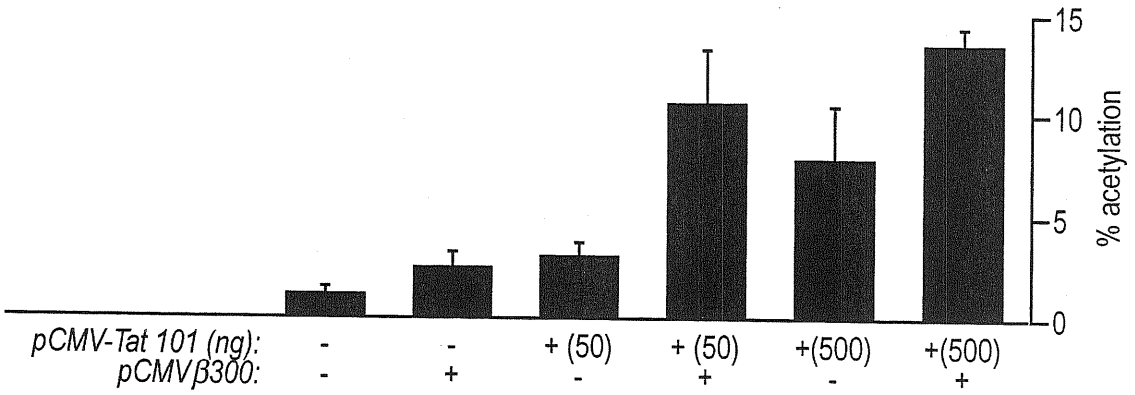


Figure 3.5
Effects of p300 expression on Tat activity

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 pCMVβp300 (10 μg where indicated) expression vectors. The results represent the average of at least three independent transfections.

Chapter 4

QUANTITATIVE DYNAMICS OF HIV-1 EXPRESSION

Manola Comar, Giuseppe Marzio, Pierlanfranco D'Agaro and Mauro Giacca

AIDS Research and Human Retroviruses (1996) 12, 117-126

ABSTRACT

A competitive PCR and RT-PCR procedure was developed for the quantification of HIV-1 nucleic acids in infected biological samples, with particular reference to the study of the kinetics of production of differently processed viral transcripts. The procedure entails the utilization of a competitor plasmid DNA (on DNA samples) or of an *in vitro* transcription product obtained from this plasmid (on RNA samples) and allows the quantification of proviral DNA, viral genomic RNA and viral single- and multi-spliced mRNAs. Furthermore, it permits the direct standardization of these measurements to the amount of a reference cellular gene (for DNA quantification) or of a reference cellular transcript (for RNA quantification).

This quantification procedure was used to monitor the dynamics of HIV-1 transcriptional activation in the latently infected U1 monocytic cell line after stimulation with phorbol-12-myristate-13-acetate, and in experimentally infected peripheral blood lymphocytes. Despite the biological differences between the two experimental systems, in both cases production of infectious virus is accompanied by a remarkable increase in the levels of unspliced viral mRNAs (rising up to 20,000 fold in U1 cells) and by a consequent switch in the abundance of the differently-spliced transcript classes. These observations reinforce the notion that the control of infection is subjected also to post-transcriptional events and prompts for quantitative evaluation of HIV-1 transcript class abundance in infected individuals to define potential markers for disease progression.

INTRODUCTION

Infection with the Human Immunodeficiency Virus type 1 (HIV-1) is followed by viral dissemination in lymphoid tissues and by continuous viral replication in infected cells (133, 143). During the course of the disease, a clear correlation exists between progression and the presence of increasing amounts of infectious virus, viral antigens and virus-specific nucleic acids. This notion derives from studies that measured the number of infected cells in peripheral blood by quantitative virus culture (36, 85, 86), or the amount of proviral DNA and viral genomic RNA in serum by semi-quantitative polymerase chain reaction techniques (36, 86, 106, 174, 184), or the amount of viral nucleic acids in infected cells by *in situ* methods (55). The results obtained by these studies were more recently validated by the introduction of quantitative methods for measuring the concentration of viral nucleic acids (9, 93, 129, 152, 172, 205).

In particular, some of these studies drew a correlation between the levels of viral expression (as measured from the ratio between viral transcripts and proviral DNA in infected cells) and the clinical course of the disease (9, 86, 166). These observation suggest that disease progression is paralleled by an increase in the transcriptional rate of the provirus, and indicate that the measurement of HIV-1 transcripts in HIV-1 infected individuals could provide an interesting marker for clinical and prognostic assessment.

In HIV-1 infected cells, more than twenty different viral mRNAs are synthesized, deriving from multiple alternative splicing processes (161, 177, 178). Either during the infection of susceptible cell cultures (111, 114) or in cellular systems in which transcription is inducible (132, 156), a temporal regulation of the synthesis of these transcripts is detectable: initially, short (~ 2 kb), multi-spliced transcripts are prevalent, encoding for regulatory proteins (i.e. Tat, Rev and Nef); subsequently, intermediate (~4.5 kb), single-spliced mRNAs (coding for Env Vif, Vpr and Vpu) and full-length (~9 kb), unspliced mRNA (coding for Gag, Pol and representing the viral genome to be eventually assembled in the new virions) accumulate. This dual pattern for transcript processing is probably related to the presence of the viral Rev protein (40, 156), which is synthesized from the short transcripts.

Altogether, these observations suggest that this pattern of production of viral transcripts could be linked to the progression of the disease also in patients (155). However, controversial results were obtained by measuring transcript classes in infected individuals: the finding that multi-spliced mRNAs were prevalent at early times of the disease while unspliced mRNAs

predominated at late times, similarly to the *in vitro* infection (86, 173, 179) has been recently questioned (166). It should be observed, however, that the discrepant outcome of some of the above mentioned studies is possibly related to the intrinsic difficulty of quantitation of HIV-1 nucleic acids by conventional PCR techniques, especially when these techniques are applied to the quantification of viral intracellular mRNAs.

In the last years, we have extensively exploited the competitive PCR and RT-PCR methodology for the precise quantification of small amounts of nucleic acids (including HIV-1) in biological samples (48, 76, 81, 82, 129, 180, 181). This methodology involves the simultaneous reverse transcription and amplification of the sample nucleic acid with known quantities of a reference template, sharing the primer recognition sites with the target and thus acting as a competitor. By this approach, any unpredictable variable affecting either enzymatic reaction has the same effect on both molecular species (competitor and sample). As a consequence, the final ratio between the two amplification products precisely reflects the initial ratio between the input number of molecules.

In this work, we address the problem of the construction of competitor DNA and RNA molecules for the precise quantification of HIV-1 nucleic acids, with particular reference to the quantification of different transcript classes in infected cells. Furthermore, we report the results of quantification of viral mRNAs in two different experimental systems, both of which suggest that viral production is dependent on a remarkable switch of abundance of different transcript classes.

MATERIALS AND METHODS

Oligonucleotide primers. The sequences of the primers for amplification of HIV-1 DNA and RNAs, β-actin mRNA and β-globin gene DNA are reported in Table 4.I. The localization of the HIV-1 primers is shown in Fig. 4.1 B. Primer *rev* is complementary to the HIV-1 sequence from 6011 to 6030 (numbering refers to file *hivhxb2* of the LANL data bank of AIDS related nucleic acid sequences). The use of primers PCO3 and PCO4 for β-globin amplification (76, 165) and BA1 and BA4 for β-actin amplification (81, 82) has already been reported.

Construction of a competitor for HIV-1 DNA and RNA quantitation. Competitor plasmid pSPLI-II was constructed according to the following procedure, as schematically shown in Fig. 4.2. Viral genomic RNA from U1 cells was reverse transcribed and amplified with primers 1 and 2II (A, step a). Amplified cDNA was submitted to two separate amplification reactions with primer sets 1-2L and 2U-2II (A, steps b and c). Primer 2L contains 20 nt at the 3' end complementary to HIV sequence from 816 to 797 (referred to file *hivmn* of the LANL data bank of AIDS related nucleic acid sequences), and 22 nt at the 5' end identical to the sequence of primer 5. Primer 2U contains 20 nt at the 3' end identical to HIV sequence from 817 to 836 (contiguous, on the opposite strand, to the 3' end of oligo 2L) and 22 nt at the 5' end complementary to the sequence of oligo 5 (A, step b). The two PCR products were resolved by polyacrylamide gel electrophoresis, eluted from the gel, mixed, denatured, annealed by virtue of the region of complementarity corresponding to primer 5, and amplified with primers 1 and 2II (A, step d). The resulting amplification fragment (240 bp) contains the sequence of primer 5 in the middle portion of the lower strand. This product was further amplified with primer 1 and primer 3L, which contains the sequences of primers 3 and 2 in a 5'-to-3' head-to-tail orientation (A, step e). Finally, this PCR product (A step f) was purified and cloned downstream of the T7 RNA polymerase promoter in the pCRtmII plasmid (TA Cloning KIT, Invitrogen Corporation, San Diego, CA; A, step f) to obtain plasmid pSPLI.

The DNA fragment to be used as competitor for β-actin quantification was obtained according to an already published procedure (48, 81, 82). This fragment corresponds to the amplification product obtained from the β-actin cDNA using primer set BA1-BA4, with the insertion of an extra 20 bp sequence in the middle (Fig. 4.2 B, step a). The fragment was re-amplified with modified BA1 and BA4 oligonucleotides carrying at the 5' extremities recognition sites for *Sac* I and *Kpn* I restriction endonucleases respectively. Additionally, the modified primer BA4 also contained a 18 nt poly-T sequence between the specific β-actin sequence and the *Kpn* I site. The amplification product (B, step b) was cloned between the two corresponding sites of plasmid pSPLI, 50 bp apart from the sequence of the previously cloned HIV-1 competitor, to obtain plasmid pSPLI-I. The competitor for β-globin DNA quantification was obtained similarly to the β-actin one, as already described (76). This competitor contains a β-globin gene fragment with an insertion of 20 extra nucleotides in the middle (Fig. 4.2 C, step a). For cloning purposes, it was re-amplified with modified PCO3 and PCO4 oligonucleotides containing *Hind* III and *Kpn* I restriction sites at the 5' ends respectively (C, step b). The obtained

product was cloned between the corresponding sites of plasmid pSPLI-I to finally obtain plasmid pSPLI-II. The nucleotide sequences of the final and intermediate constructs were verified according to established procedures.

Quantification of RNA and DNA competitor templates. For the competitive DNA-PCR experiments, a preparation of plasmid pSPLI-II was accurately quantified by spectrophotometry, and an aliquot was diluted and directly used as a competitive template.
For the competitive RNA-PCR experiments, the competitor RNA was obtained by *in vitro* run-off transcription from 50 ng of plasmid pSPLI-II after linearization with *Kpn* I. The *in vitro* transcription reaction was performed using a T7 RNA polymerase-based commercial kit (Promega, Madison, WI), with the addition of 2 µl of [^{32}P]-UTP (Amersham, UK; 3000 Ci/mmole; 10 mCi/ml), corresponding to 2.07×10^7 cpm, as experimentally evaluated by Cerenkov counting. After completion of the transcription reaction, template DNA was removed by DNase I digestion followed by purification of the newly synthesized RNA either by passage through an oligo-dT column (Promega) or after resolution by denaturing polyacrylamide gel electrophoresis and elution from the gel. An aliquot of the purified competitor RNA preparation was measured in a β counter, and, according to the U content of the transcript, concentration was evaluated from the final specific activity, as already described (81, 82).

Cells and virus. The U1 cell line is a U937-derivative promonocytic cell line carrying two HIV-1 proviral copies per cell (64, 65). Cells were maintained in RPMI medium supplemented with 10% fetal calf serum, 2 mM glutamine and 50 µg/ml gentamicin in a 5% CO_2 environment. Cells growing at a density of 10^6 per ml were induced in medium adjusted to 10^{-7} M phorbol-12-myristate-13-acetate (PMA, Sigma, St. Louis, MO).
Peripheral blood mononuclear cells from healthy donors were isolated from heparinized blood by Ficoll-Hypaque gradient centrifugation. Lymphocytes were activated by incubation in culture medium containing 1 mg/ml phytohemoagglutinin (PHA, Sigma) and 10% interleukin-2 (IL-2, Cellular Products Inc, San Diego, CA). Three days after stimulation, the cells were collected, resuspended at 2×10^6/ml in fresh medium, infected with the HIV-1_{IIIB} at a multiplicity of infection of 50 $TCID_{50}$/ml, washed twice with medium to remove residual free virus and cultured at 3×10^5 cells/ml in medium containing 10% IL-2. HIV-1 p24 core antigen concentration in the medium was determined by a commercial enzyme-linked immunosorbent assay (Cellular Products Inc).

DNA and RNA extraction. Stimulated U1 cells and infected peripheral blood lymphocytes were collected by centrifugation, washed once in phosphate-buffered saline (PBS) and resuspended at a concentration of 3×10^6 cells/ml in PBS. For DNA extraction, 100 µl of cell suspension were incubated overnight at 37°C in 500 ml of lysis buffer (100 mM NaCl, 10 mM Tris-HCl, 25 mM EDTA, 0.5% SDS pH 8.0, 0.1 mg/ml proteinase K) and DNA was extracted with a phenol-chloroform-isoamylic alcohol mixture (25:24:1) followed by ethanol precipitation using standard procedures (167).
Total RNA was extracted from other 100 µl of the cell suspension according to the guanidine thiocyanate procedure (32).

Competitive PCR and RT-PCR. Competitive PCR was carried out on 1 µl of sample DNA with the addition of increasing concentrations of the pSPLI-II competitive template, in 100 µl of PCR buffer (50 mM KCl, 10 mM Tris-HCl, 2 mM $MgCl_2$) containing the two primers (100 pmol each), the four dNTPs (200 µM each) and 2.5 U of *Taq* DNA polymerase (Perkin Elmer Emmeryville, CA). Samples were submitted to 50 cycles of amplification with the following cycle profile: denaturation at 95°C for 30 sec, annealing at 60°C (for both primer sets 1-2II and β-globin) for 30 sec, extension at 72°C for 30 sec. Before PCR analysis, DNA samples and *Kpn* I-digested linearized pSPLI-II competitor were heated to 95°C for 5 min to ensure complete denaturation.
For the RNA quantification experiments, the extracted RNA was reverse transcribed in the presence of the antisense oligonucleotides specific for each class of transcripts. Each reaction contained 1 µl of test RNA sample and 3 µl of RNA competitor (pSPLI-II *in vitro* transcription product, opportunely diluted) in 20 µl of reverse transcription mix, composed of 75 mM KCl, 50 mM Tris-HCl pH 8.3, 3 mM $MgCl_2$, 0.4 mM each dNTP (Pharmacia, Uppsala, Sweden), 2 mM antisense primer, 400 units MMLV-RT (Promega), 20 units RNasin (Promega). RNA was pre-heated at 65°C for 5 min and incubated with the reaction mix at 37°C. After 1 hour, the reaction was stopped by incubation at 95°C for 5 min and samples were cooled on ice. The 20 µl-reverse transcription reaction was then diluted in PCR buffer to a final volume of 100 µl,

containing both primers 100 pmol, the four dNTPs 200 μM each and 2.5 U of *Taq* DNA polymerase. Samples were submitted to 50 cycles of amplification with the following cycle profiles: denaturation at 95°C for 30 sec, annealing at 60°C (primer sets 1-2II, 1-5 and β-actin) or 59°C (primer set 1-3) for 30 sec, extension at 72°C for 30 sec.
After amplification, 15 μl of each PCR product were resolved on a 8% non denaturing polyacrylamide gel, visualized under UV light after ethidium bromide staining and photographed. Quantification of the amplification products was obtained by densitometric scanning of the ethidium-bromide stained gels.

RESULTS

Selection of primers for the detection of HIV-1 DNA and RNA

In order to develop a procedure for the quantification of HIV-1 nucleic acids by competitive PCR and RT-PCR, four oligonucleotide primers were selected on the HIV-1 genome (primers 1, 2II, 3, 5; Table I). The sequence of these primers (and, in particular, of their 3'-ends, which are critical for polymerase priming) is conserved among several clinical HIV-1 isolates of different geographical origin (not shown). One of these primers has the same polarity of the viral mRNA, and its sequence is present in all viral transcripts (primer 1). The other three primers are complementary to the viral mRNAs and are positioned within the *gag* gene (primer 2II), the *env* gene (primer 3), and the second coding exon of the *rev* gene (primer 5; Fig. 4.1 panels A and B). The position of primer 2 is downstream of the first splice donor site (localized at position 743), and thus RT-PCR amplification with primers 1 and 2 detects only the unspliced viral transcripts. Primer 3 is positioned downstream of the 5' splice site at position 6045, and thus RT-PCR with primers 1 and 3 allows amplification only of single-spliced transcripts (since the two primers are too far apart in the unspliced RNA to yield a product). Finally, since primer 5 is located downstream of the 3'-most splice acceptor site, amplification with primers 1 and 5 detects only double- or multi-spliced mRNAs. The localization of these primers with respect to the HIV-1 splice sites and the major amplifiable mRNAs for each transcript class are shown in Fig. 4.1 panels B, C and D.

An example of the amplification products obtained with these primer pairs on a cellular RNA sample from monocytic U1 cells, latently infected with HIV-1 (64, 65), is shown on panels D to F of Fig. 4.3. Amplification with primers 1-2II gives rise to a single PCR product of 218 bp (D lane 1), while amplification with primer sets 1-3 (E lane 5) and 1-5 (F lane 5) produces multiple HIV-1-specific products, representing different RNA species arising from alternative splicing events from the primary transcript of genomic length (177, 178). The most abundant product obtained with primers 1 and 3 (indicated by an open arrowhead in E) was cloned and its nucleotide sequence was determined. It corresponds to a processed transcript derived from the usage of the 5' splice site at position 743 and of the 3' splice site at position 5975 (schematically shown in Fig. 4.1 D). This site is the dominant acceptor site used for single spliced transcripts also in different experimental systems (84, 178). The transcript potentially encodes for the Vpu and Env proteins (*env* transcript in Fig. 4.1 D). Similarly, also amplification with primers 1 and 5 in U1 cell RNA produces a major amplification product of 170 bp, deriving from a viral mRNA containing three exons. The first of these exons corresponds to the first HIV-1 exon up to the 5' splice site at position 743, the second exon encompasses the region from the 3' splice site at 5959 and the 5' splice site at 6045, and the last exon contains the fragment downstream of the 3' splice site at position 8377, as schematically indicated in Fig. 4.1 D. The 3' splice site at position 5959 is located between the Tat AUG and the Rev AUG. As a consequence, this transcript potentially encodes for the Rev and Nef proteins (*rev* transcript in Fig. 4.1 D; (84, 177)). The identity of the amplification product was confirmed by the determination of the nucleotide sequence after cloning of the PCR product, and routinely checked by hybridization with the internal *rev* probe (not shown), whose localization is indicated in Fig. 4.1 B.

Construction of a competitor template for HIV-1 DNA and RNA quantification

In order to obtain precise quantitation of the number of copies of HIV-1 proviral DNA and of different classes of HIV-1 transcripts in infected samples, we have developed a procedure based on the competitive PCR and RT-PCR methodology. This procedure is based on the use of a plasmid DNA (pSPLI-II) and of an RNA obtained by *in vitro* transcription from this plasmid, as competitors for DNA and RNA competitive PCR experiments respectively. The use of these competitors also provides the possibility of obtaining independent quantitation, within the same samples, of the abundance of a cellular gene (β-globin) or of a constitutive cellular transcript (β-actin). These measurements can be used as internal standards for the

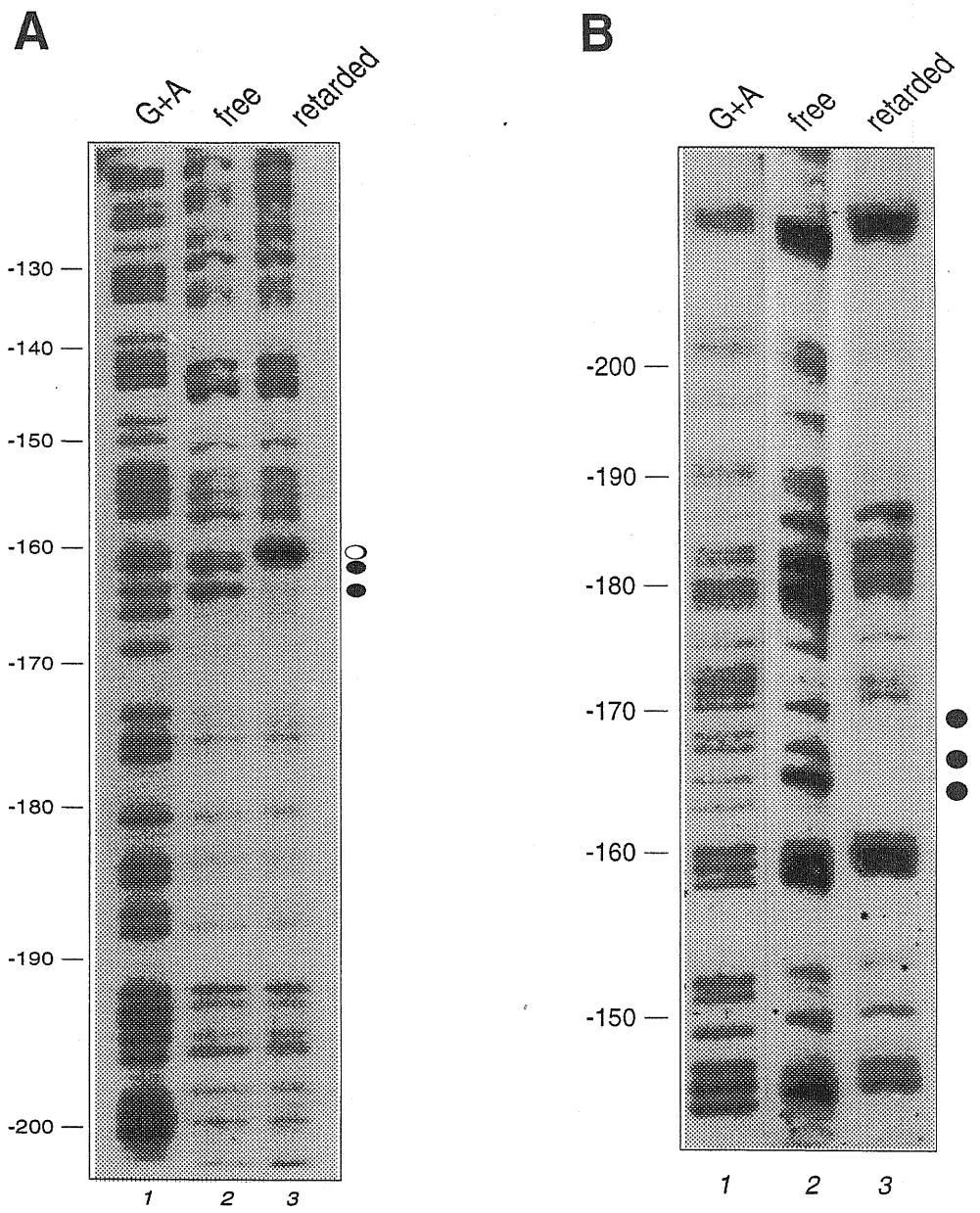


Figure 2.4
Methylation protection assays

A. Methylation protection on the LTR coding strand using recombinant USF[43]. Lane 1: G+A chemical cleavage ladder; lane 2: methylation pattern of the free band recovered from the gel shift assay; lane 3: methylation pattern of the retarded band. The filled and empty circles on the right side indicate, respectively, protected and hypersensitive purines.

B. Methylation protection on the non-coding strand of the LTR using recombinant USF[43]. Lane 1: G+A chemical cleavage ladder; lane 2: methylation pattern of the free band recovered from the gel shift assay; lane 3: methylation pattern of the retarded band. The filled circles on the right side indicate protected purines.

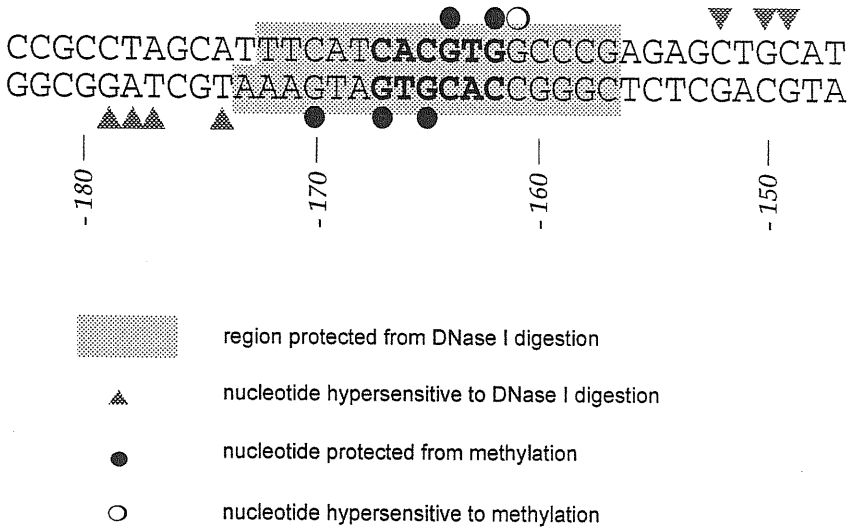


Figure 2.5
Summary of the interactions of USF with the LTR E box obtained by DNase I footprinting and methylation protection experiments.

The nucleotides of the E box motif are typed in bold characters.

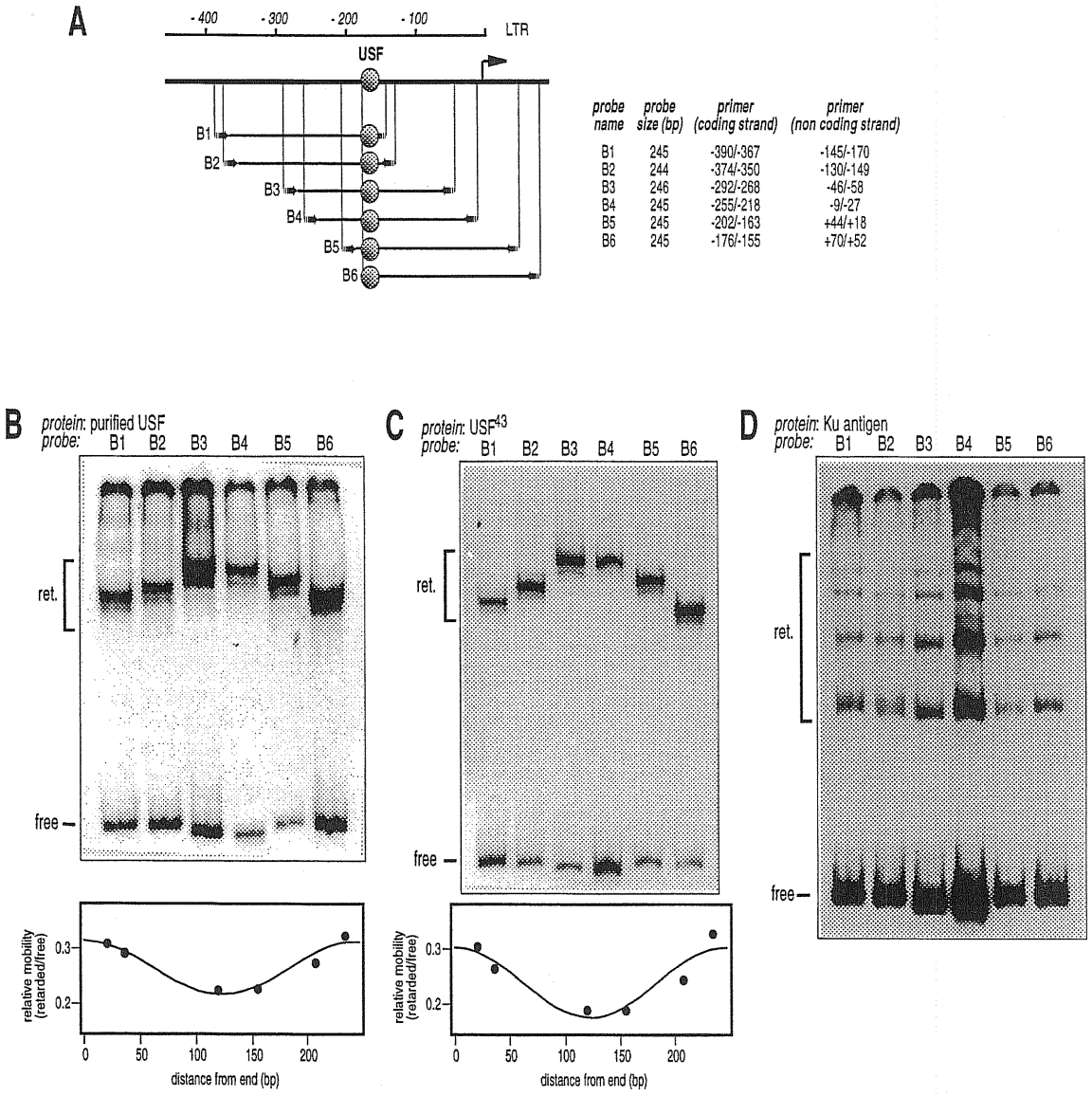


Figure 2.6
Circular permutation assay

A. A set of six probes (B1 to B6) containing the USF site in different position was generated by PCR amplification using the primers localized as indicated in the Table on the right side of the panel (numbering is referred to transcription start site according to sequence hxb2cg of GenBank). The arrow on top indicates transcription start site.

B. Circular permutation assay using probes B1-B6 and purified USF.
The localizations of the free and retarded complexes are indicated. The graph in the lower part of the panel shows the relative mobility of the retarded complexes (distance from the well of the retarded band/distance of the free band) plotted against the distance of the CACGTG box from the end of the fragment.

C. Circular permutation assay with recombinant USF^{43}

D. Circular permutation assay with the Ku protein (Csordas et al., 1993)

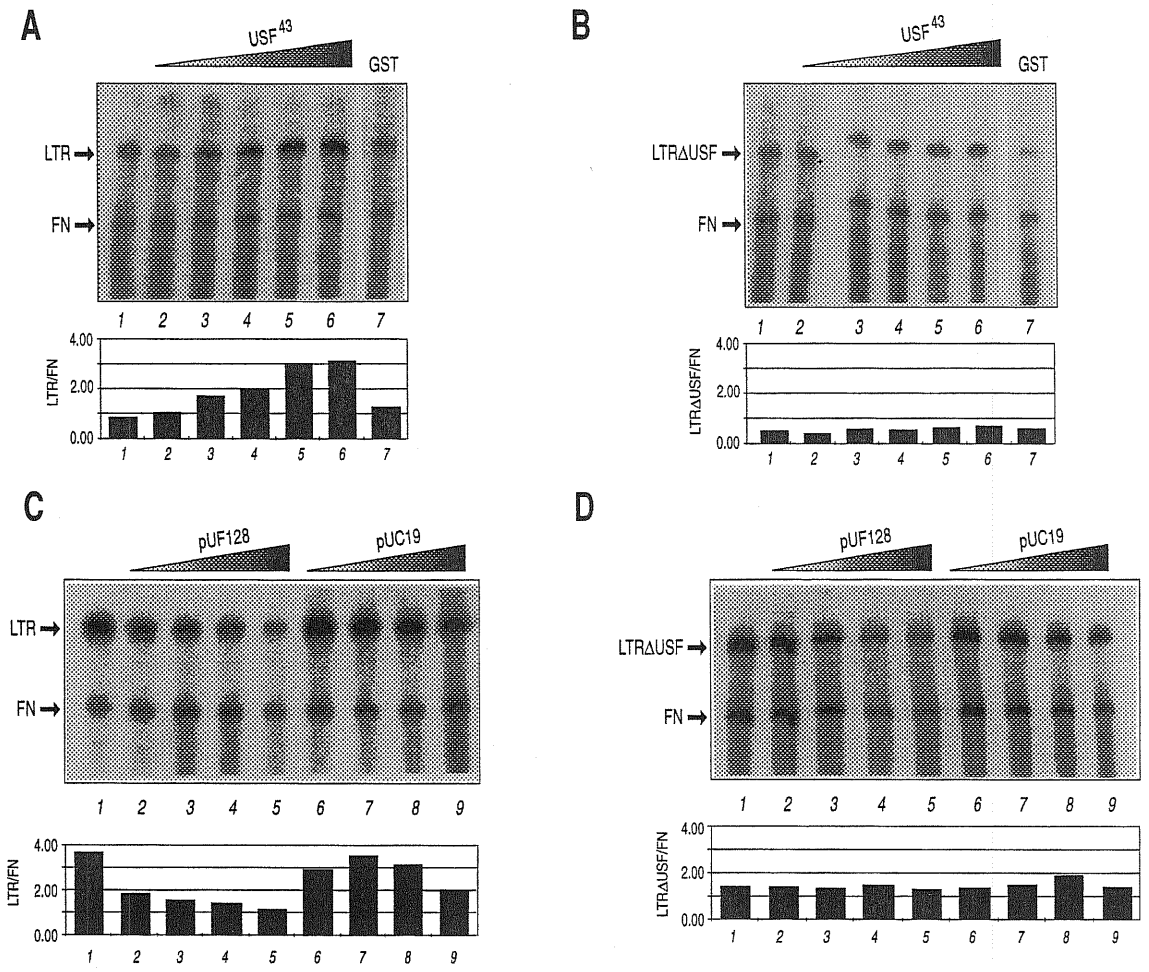


Figure 2.7
In vitro transcription assays

A. Effect of the addition of USF^{43} on the LTR template.
In vitro transcription assays were performed with plasmids pGLA (LTR upstream of a 380 nt G-less cassette) and pFN2 (fibronectin promoter upstream of a 200 nt G-less cassette), and HeLa nuclear extracts.
Lane 1: plasmid templates plus nuclear extract; lanes 2-6: plus the addition of increasing amounts of recombinant GST-USF^{43} (50, 100, 200, 500, 1000 ng, respectively); lane 7: plus the addition of 1000 ng of GST.
The RNA bands corresponding to the two transcripts are indicated.
The graph in the lower part of the panel shows the ratio between the amount of the two transcripts for each experimental point.

B. Effect of the addition of USF^{43} on the LTRΔUSF template.
In vitro transcription assays were performed with plasmids pGLE (LTRΔUSF/G-less cassette) and pFN2, and HeLa nuclear extracts. The amounts of USF^{43} and GST are the same as in panel A.

C. Effect of the addition of E box decoys on the LTR template.
In vitro transcription assays were performed with plasmids pGLA and pFN2, and HeLa nuclear extracts. Lane 1: plasmid templates plus nuclear extract; lanes 2-5: plus the addition of increasing amounts of plasmid pUF128, containing 128 copies of a CACGTG box (250, 500, 750, 1000 ng); lanes 6-9: plus the addition of the same molar excesses of vector pUC19.

D. Effect of the addition of E box decoys on the LTRΔUSF template.
In vitro transcription assays were performed with plasmids pGLE and pFN2, and HeLa nuclear extracts. The amounts of pUF128 and pUC19 are the same as in panel C.

quantification of total extracted genomic DNA and RNA respectively.
The procedure used for the construction of pSPLI-II is shown in Fig. 4.2 and detailed in the Material and Methods section. The plasmid contains an insert where the HIV-1 primers 1, 2II, 3 and 5 (described in the previous section) are arranged in order to produce amplification products which are different in size (and hence easily recognizable by gel electrophoresis) from those deriving from the viral transcripts. This DNA insert (Fig. 4.2 A) was obtained by the recombinant PCR technology (92, 94) starting from the PCR product obtained by amplification with primers 1 and 2II. By two subsequent re-amplifications of this product, the sequences of primer 5 and primer 3 were inserted on the lower strand, in the middle portion and at the 5' end respectively (Fig. 4.2 A). Plasmid pPSLI-II additionally contains the competitor fragments for β-actin mRNA and β-globin gene amplification (Fig. 4.2 panels B and C). These competitor segments have a perfect match to the respective target sequences, except for the addition of 20 extra bp in the middle, to allow resolution by gel electrophoresis after co-amplification with their targets. The construction of these competitor fragments has been already described elsewhere (48, 81, 82). The plasmid also contains a 18-A stretch positioned 3' of the β-actin competitor on the coding strand. Consequently, *in vitro* run off transcription using this plasmid linearized at the *Kpn* I site produces an RNA molecule (Fig. 4.2 D), which can be easily purified from the plasmid template by oligo-dT affinity chromatography, and can be used in competition assays.

Plasmid pSPLI-II can be directly used in competitive PCR experiments to quantify the copy number of proviral DNA with respect to β-globin gene DNA in DNA samples, while its *in vitro* transcription product can be used as competitor in RT-PCR experiments on RNA samples for the quantification of unspliced, single-spliced and multi-spliced viral mRNAs with respect to the β-actin cellular mRNA. An example of these competitive amplifications is shown in Fig. 4.3 on DNA samples from HIV-1 infected peripheral blood lymphocytes (panels A and B) and RNA samples from uninduced U1 cells (panels C to F). The experiments were carried out by mixing the indicated quantities of competitor plasmid (panels A and B) or competitor RNA (panels C-F) to a fixed quantity of cellular DNA or RNA respectively, before PCR or RT-PCR with the indicated pairs of primers. After amplification, the samples were resolved by polyacrylamide gel electrophoresis, the gels were stained with ethidium bromide, and the intensity of each band (competitor and HIV-1 target) was detected by densitometric scanning. For the amplifications with primer sets 1-3 and 1-5, the prevalent *env* and *rev* transcripts (see above) were chosen as representative for the quantification of the single-spliced and multi-spliced transcript classes respectively. It should be observed that all the HIV-specific bands, detected in the absence of competitor, compete for amplification when the competitor is added (see Fig. 4.3 F), as expected. According to the principles of competitive PCR (48), the ratio between competitor and target amplification products (indicated on the bottom of each gel) is linearly dependent on the amount of input competitor added to the reaction (graphs in Fig. 4.3). Therefore, the number of molecules of competitor corresponding to a 1:1 ratio between the two products exactly indicates the number of molecules of target initially present in the reaction. This value is presented at the bottom of Fig. 4.3 for each of the reported amplifications. The described competitive procedures were applied to two different experimental systems, in order to quantitatively determine the kinetics of activation of HIV-1 expression.

Pattern of transcriptional activation of HIV-1 in stimulated U1 cells.
The pattern of transcriptional activation of HIV-1 was monitored in the promonocytic U1 cell line, containing two HIV-1 proviral DNA copies (64, 65). In this cell line, viral expression is almost undetectable in basal conditions by Northern hybridization (45, 132), but it is highly inducible by a variety of stimuli triggering cellular activation, such as cytokines, antibodies, or phorbol esters (112, 154). Due to transcriptional silence and prompt inducibility of the provirus, this cell line has been considered an *in vitro* experimental model for viral latency (156).

Precise quantitation of the kinetics of mRNA accumulation after activation of provirus expression was obtained by competitive RT-PCR on RNA samples obtained at few hours after stimulation with PMA (Fig. 4.4). Before treatment, a limited quantity of multi-spliced transcripts is detectable, (one molecule of the *rev* transcript every 100 molecules of β-actin mRNA, Fig. 4.4); the single-spliced and unspliced mRNAs are 50 and more than 650 times less represented. During the first hours after addition of PMA, there is a considerable increase in the levels of all these transcripts. Remarkably, while this increase reaches a maximum of 9-fold for the short transcripts,

peaking at one hour after stimulation, the single-spliced and unspliced transcript classes increase 250- and 20,000-fold respectively, the latter representing the majority of transcripts at 6 hours after stimulation. Thus, during the first hour after stimulation of U1 cells, there is a clear switch in the ratio between spliced and unspliced viral mRNAs.

Viral expression in experimentally infected peripheral blood lymphocytes

The pattern of viral expression was also studied after infection of peripheral blood lymphocytes from normal individuals. These experiments were carried out by measuring the accumulation of viral p24 antigen in the culture supernatant, and of proviral DNA and unspliced or multi-spliced RNAs in the cells. Despite the expected variability in the efficiency of infection among the different lymphocyte samples, a common transcriptional pattern could be detected, as shown in the representative experiment of presented in Fig. 4.5. In this experiment, the p24 antigen started to be detected in the culture medium at day 3 post-infection, and at this time the ratio between proviral DNA and cellular genomes, as measured by the β-globin gene copy number, was ~2.5 fold (it should be considered that the measurement of the number of HIV-1 DNA copies with primers 1-2II includes both unintegrated and integrated provirus). The pattern of viral transcripts was monitored during the first days of infection by means of competitive RT-PCR. As reported in Fig. 4.5, only a very low amount of HIV-1 mRNAs of genomic length was detectable at 1 hour after infection; these transcripts most probably represent the input genomic RNAs released into the cells by the virions. Contrary to other kinetic studies (5), no spliced mRNA products could be detected at this time. At day 3 after infection, multispliced and unspliced transcripts were equally abundant, and were 20-fold less represented than the β-actin mRNA. At day 7 after infection, while the levels of multispliced transcripts still remained almost unchanged, there was a marked increase in the quantity of unspliced transcripts, that at that time was one-fourth of that of the β-actin mRNA. Thus, with remarkable similarity to the data obtained in activated U1 cells, also during the acute infection of normal lymphocytes it appears that the production of infectious virus is accompanied by a specific increase in the levels of the unspliced class of transcripts.

DISCUSSION

Competitive PCR and RT-PCR for the quantitation of HIV-1 nucleic acids

The competitive PCR techniques are particularly advantageous for the quantification of low-abundance DNAs and RNAs, since they couple the exquisite sensitivity of PCR to the possibility of obtaining precise measurement of the concentration of the target molecules (48, 183). The application of these techniques to HIV-1 nucleic acid quantitation in clinical samples has recently offered new insights into the pathogenetic mechanisms of disease progression (9, 133, 152) and has provided a surrogate marker for monitoring the efficacy of antiretroviral therapies (10, 93, 205).

In this work, we address the problem of developing a strategy for the quantification of viral DNA and of different viral mRNAs by competitive PCR through the use of a single competitor, that also offers the possibility of quantification of a reference cellular gene (β-globin, for genome copy number determination) and of a reference cellular transcript (β-actin, as a standard for total cellular RNA quantification). The possibility of the last two determinations appears to be important, since the efficiency of extraction of viral nucleic acids from clinical samples is often very variable. Furthermore, since a single DNA or RNA competitor preparation can be used for all DNA or RNA determinations respectively, the individual competitive PCR results are directly comparable to one another, thus avoiding the problem of the exact determination of the competitor concentration.

According to the principles of competitive PCR and since the ratio of the competitor to target amplification products is predictably related to the quantity of input competitor RNA or DNA, a single determination should be sufficient for the correct determination of the target copy number. In our routine work, we usually perform at least three determinations with the same sample, using 10-fold scalar dilutions of competitor. If a more precise quantitation is further needed, additional amplifications are carried out with 2- or 4-fold dilutions of competitor in the range of concentrations where equivalence between the two products had been detected. As a matter of fact, in the representative quantitative PCR experiments shown in Fig. 4.3, different scalar quantities of competitor were used, ranging from 2-fold to 10-fold dilutions. In any case, the detected experimental points were fitted by a straight line, thus allowing the precise determination of

the concentration of the target HIV-1 nucleic acids, independently of the total number of reactions in which the ratio between competitor and target products could be actually measured.

Quantitative kinetics of production of HIV-1 transcripts

The developed quantitative procedure appears particularly suitable for the determination of HIV-1 transcript classes in biological samples. This problem was quantitatively addressed in two experimental systems, represented by the monocytic U1 cell line after activation of proviral expression by phorbol esters and by experimentally infected primary lymphocytes. Despite the remarkable biological differences between these two systems and the different kinetics of viral gene expression, it appears that in both cases the transition to the productive stage of infection is concomitant mostly with an increase in the levels of the unspliced transcripts, in accordance with similar qualitative observations that we and others have already presented (45, 132).

In particular, the *rev* transcript (i.e. the multispliced mRNA using the splice acceptor site at position 5959, 9 nt upstream of the *rev* AUG (4, 177)) is the most abundant viral mRNA in unstimulated U1 cells, being over 600-fold more represented than the unspliced mRNA. At 6 hours after PMA stimulation, there is only a 5-fold increase in the levels of this transcript, in contrast with the $2x10^5$-fold increase in the genomic messenger. It has been proposed that the low levels of viral expression in unstimulated U1 cells is related to the non permissive intracellular milieu of these cells (31). The already high levels of the *rev* transcript found also in the uninduced state fit with the notion that latency in these cells is not due to inadequate Rev production (2, 26, 50). Several groups have indeed shown that proviral transcription can be rescued in these cells by exogenously added Tat protein (26) or *tat*-expressing vectors (2, 50).

Independently of the actual identity of the different transcripts within the multi- and single-spliced classes, and of the molecular mechanisms involved in their regulation, the data obtained in the two different experimental systems examined in this work suggest that the state of productive infection can be linked at the cellular level to the ratio between spliced and unspliced HIV-1 mRNAs. If such a relationship were also found in HIV-1 infected individuals, this might in turn represent an important marker for the clinical prediction of development of the disease. The quantitative procedure developed in this work hopefully will offer a useful tool to address this problem.

ACKNOWLEDGMENTS

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The authors are indebted to Prof. Arturo Falaschi for the continuous support and encouragement, and are grateful to Miss Barbara Boziglav for the excellent technical assistance.

	Name	Sequence and homologies	Isolate	File (nucleotides)
HIV-1	Primer 1	5'GGCTTGCTGAAGCGCGCACGGCAAGAGG3'		
		----------	LAI	*hivlai* (246-273)
		----------	MN	*hivmn* (696-723)
		----------A--------	SF2	*hivsf2* (701-728)
		----------G-T--A---A--------	MAL	*hivmal* (263-290)
		----------	ELI	*hiveli* (246-273)
		----------	Z2	*hivz2z6* (700-727)
		----------	JRFL	*hivjrfl* (23-50)
		----------	NY5	*hivny5cg* (700-727)
		----------A--------	NDK	*hivndk* (244-271)
		----------A--------	CAM1	*hivcam1* (702-729)
		----------G----------	RF	*hivrf* (214-241)
	Primer 2II	5'CGTTCTAGCTCCCTGCTTGCCCATAC3'		
		----------	LAI	*hivlai* (463-438)
		----------	MN	*hivmn* (914-889)
		----------	SF2	*hivsf2* (918-893)
		-T---C----------	MAL	*hivmal* (477-452)
		----------	ELI	*hiveli* (464-438)
		----------	Z2	*hivz2z6* (917-892)
		----------	JRFL	*hivjrfl* (240-215)
		--------T----------	NY5	*hivny5cg* (917-892)
		----------T	NDK	*hivndk* (461-436)
		----------	CAM1	*hivcam1* (919-894)
		----------	RF	*hivrf* (431-406)
	Primer 3	5'GTAGCACTACAGATCAT3'		
		----------	LAI	*hivlai* (5894-5878)
		----------	MN	*hivmn* (6327-6311)
		----------	SF2	*hivsf2* (6321-6305)
		-----C-C--------A	MAL	*hivmal* (8733-8717)
		-C----------G----	ELI	*hiveli* (5858-5842)
		-C-TT-------C----	Z2	*hivz2z6* (6315-6299)
		AC----------	JRFL	*hivjrfl* (5629-5613)
		-C----------	NY5	*hivny5cg* (6268-6252)
		-C----------G----	NDK	*hivndk* (5847-5831)
		-C----------	CAM1	*hivcam1* (6314-6298)
		TC----T----------	BAL	*hivbal1* (737-721)
		-C----------	RF	*hivrf* (5831-5815)
	Primer 5	5'TCTTCTATTCCTTCGGGCCTGT3'		
		----------	LAI	*hivlai* (8021-8000)
		-----G----------	MN	*hivmn* (8445-8424)
		-----G-----G----------	SF2	*hivsf2* (8433-8412)
		----------	MAL	*hivmal* (8011-7990)
		------G----------	ELI	*hiveli* (7964-7943)
		----------	Z2	*hivz2z6* (8421-8400)
		-----G----------	JRFL	*hivjrfl* (7717-7696)
		-----G----------	NY5	*hivny5cg* (8365-8344)
		---------T----------	NDK	*hivndk* (7932-7911)
		-----G----------	CAM1	*hivcam1* (8429-8408)
		-----G------C---------	BAL	*hivbal1* (2846-2825)
		C----G----------	RF	*hivrf* (7973-7952)
β-actin	BA1	5'CATGTGCAAGGCCGGCTTCG3'		*hsac07* (86-105)
	BA4	5'GAAGGTGTGGTGCCAGATTT3'		*hsac07* (311-292)
β-globin	PCO3	5'ACACAACTGTGTTCACTAGC3'		*humhbb* (62148-62167)
	PCO4	5'CAACAACATCCACGTTCACC3'		*humhbb* (62257-62238)

Table 4.I
Name, sequence, localization and homologies of the oligonucleotide primers used in this work. Numbering refers to the indicated files of the LANL data bank of AIDS related nucleic acid sequences

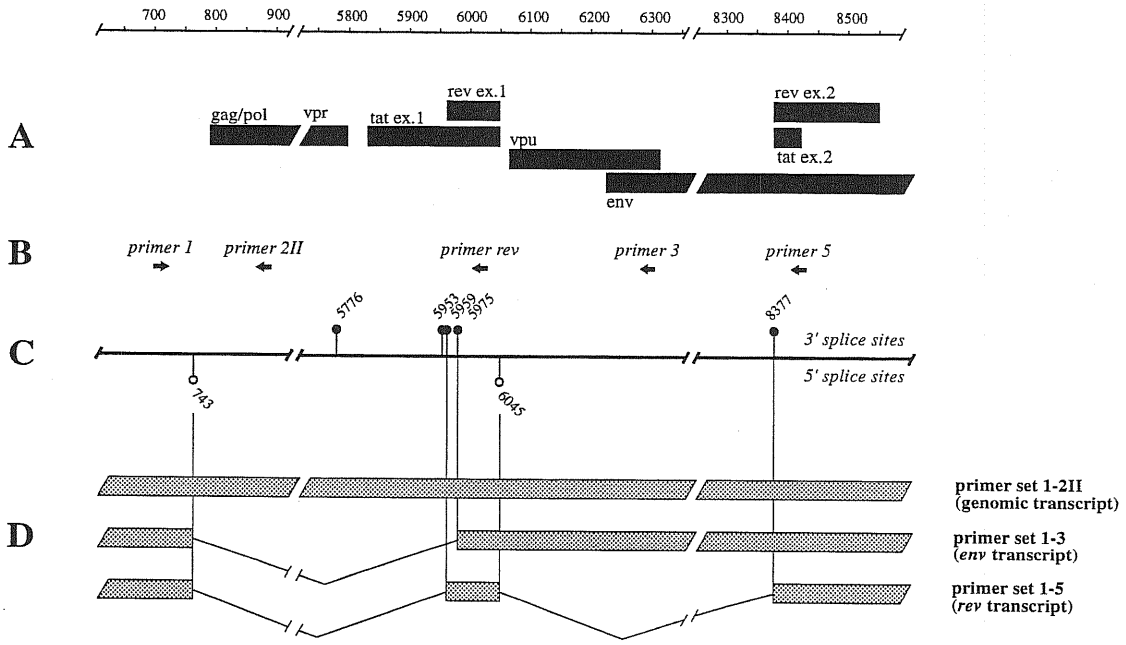

Figure 4.1
Localization of primers on the HIV-1 genome and transcripts

A. Schematic representation of the coding regions of HIV-1 genes (ex.: coding exon); numbering refers to file hxb2cg of the LANL data bank of AIDS related nucleic acid sequences.

B. Localization of primers used in this work.

C. Localization of 3' splice sites (upper part, filled circles) and 5' splice sites (lower part, empty circles) on the HIV-1 genome

D. Major mRNA species detected by RT-PCR amplification using the indicated pairs of primers.

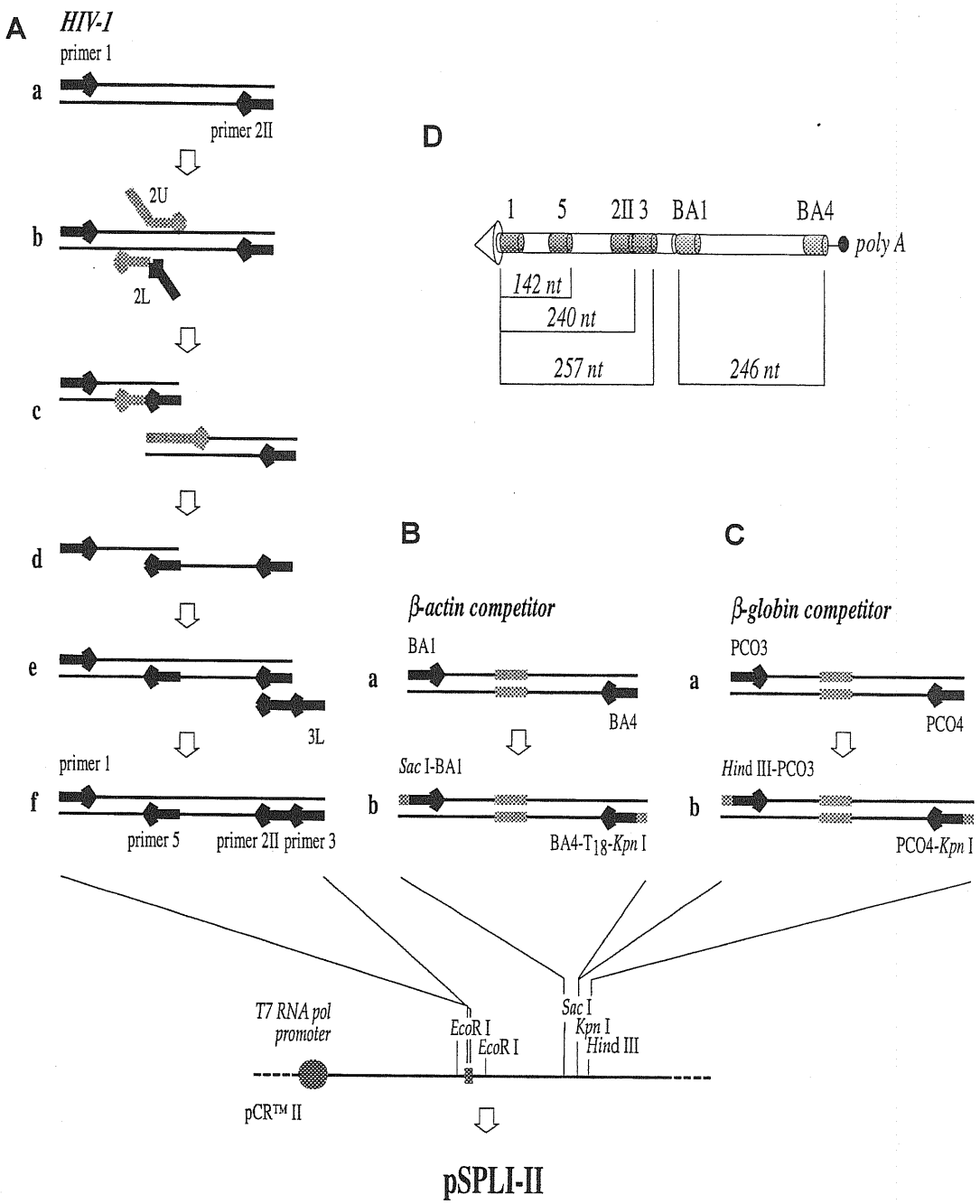

Figure 4.2
Flow chart for the construction of plasmid pSPLI-II

A. Construction of the competitor fragment for HIV-1 DNA and RNA amplification. The black arrows indicate HIV-1-specific primers, the gray arrows primers used for the construction of the recombinant PCR product. A detailed description of the procedure is presented in the Materials and Methods section.

B. Construction of the competitor for β-actin amplification

C. Construction of the competitor for β-globin amplification

D. RNA product obtained by in vitro run-off transcription from plasmid pSPLI-II, linearized at the Kpn I site. The localization of primer sequences and the expected size of RT-PCR products are shown.

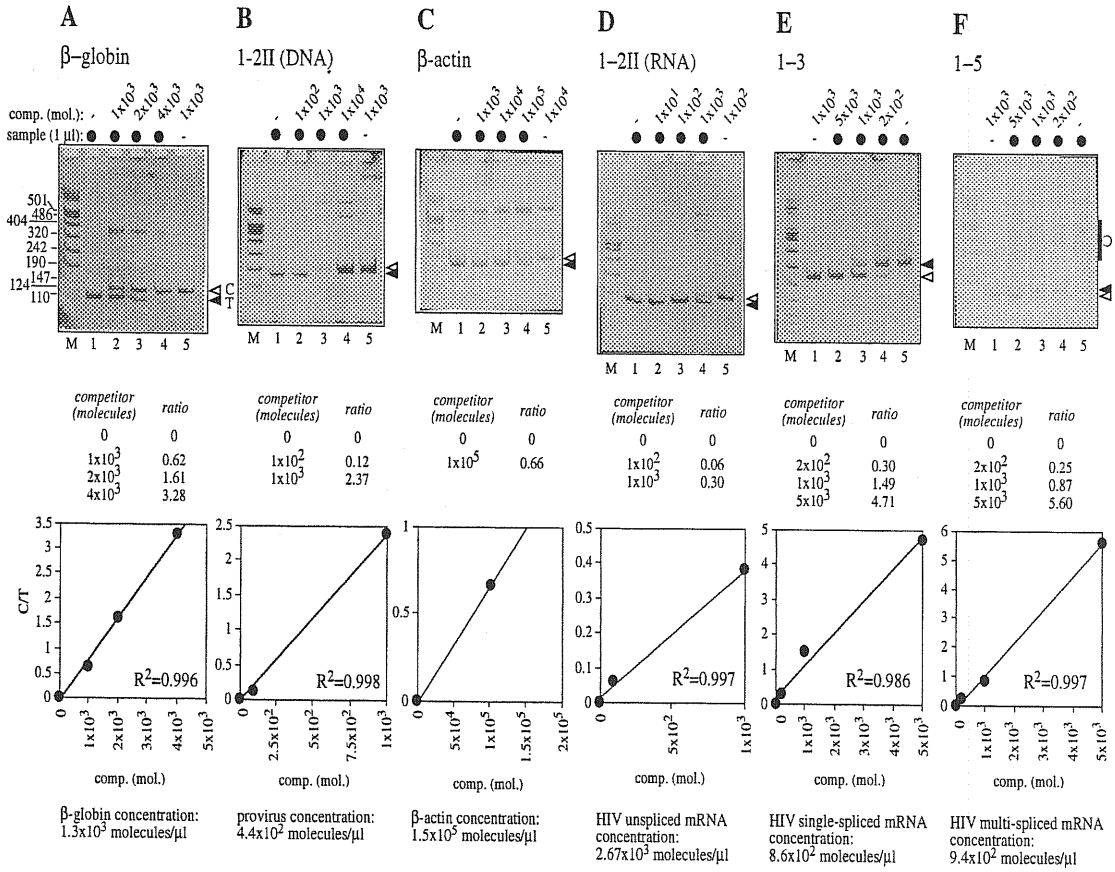

Figure 4.3
Competitive PCR and RT-PCR amplifications

Competitive experiments using the indicated set of primers were performed on DNA (panels A and B) and RNA (panels C to F) extracted from peripheral blood lymphocytes infected with HIV-1. For each experiment, a fixed amount (1 µl) of appropriately diluted DNA or RNA preparation was amplified with the number of competitor molecules indicated on top of each gel. After amplification, the PCR products were resolved by polyacrylamide gel electrophoresis, stained with ethidium bromide, quantified and photographed. At the bottom of each gel, the ratio between the two amplification products for the competitor (C) and the target in the sample (T) are reported for the lanes where both products were detectable. Further at the bottom, these ratios are plotted against the amount of competitor initially added to the reaction and the experimental points are fitted by a linear function, whose correlation coefficient is reported (with the exception of panel C, where a single measurement had been obtained). Below the graphs, the concentration of each target in the sample is shown, as estimated according to the equation of the line and considering the value of 1 on the Y axis as the point where the number of molecules of competitor is equal to the number of molecules of target.
For the experiments with primers 1-3 and 1-5, where multiple amplification products were detected from HIV-1 spliced mRNAs - such as in panel F, indicated by a bar and an empty circle on right side of the gel -), the filled arrow indicates the position of the band chosen as representative for quantification. See text for further discussion.
(comp.: competitor; mol.: molecules; M: molecular weight marker; open arrow: amplification product from the competitor; filled arrow: amplification product for the target).

time after PMA addition (hours)	unspliced mRNAs (molecules)	single-spliced mRNAs (molecules)	multi-spliced mRNAs (molecules)	β-actin (molecules)	multi-spliced /β-actin ($x10^3$)	single-spliced /β-actin ($x10^3$)	unspliced /β-actin ($x10^3$)
0	1.5×10^2	2×10^3	1×10^5	1×10^7	10	0.2	0.015
1	2×10^3	1.5×10^3	9×10^4	1×10^6	90	1.5	2
2	2.5×10^4	2×10^4	1×10^5	3×10^6	33	6.7	8
6	3×10^6	5×10^5	5×10^5	1×10^7	50	50	300

Figure 4.4
Competitive RT-PCR for the quantification of transcript classes in PMA-activated U1 cells

The number of molecules for the indicated mRNA species are reported for RNA samples extracted at different times after PMA stimulation of U1 cells. The bars on the right side show the number of HIV-1 transcripts after standardization for the number of molecules of b-actin mRNA in the same sample.

time after infection	unspliced mRNAs (molecules)	multi-spliced mRNAs (molecules)	β-actin (molecules)	multi-spliced /β-actin ($x10^3$)	unspliced /β-actin ($x10^3$)
0	0	0	2×10^9	0	0
1 hour	2×10^6	0	6×10^9	0	0.33
3 days	4×10^7	4×10^7	8×10^8	50	50
7 days	1.4×10^7	3.5×10^6	6×10^7	58.3	233.3

Figure 4.5
Quantification of transcript classes in infected lymphocytes

The number of molecules for the indicated mRNA species are reported for RNA samples extracted at different times after infection of lymphocytes with HIV-1_{IIIB}.

APPENDIX

HIV-1 GENE EXPRESSION AND CELL PROLIFERATION

INTRODUCTION

In HIV-1 infected patients, the absolute level of viremia fluctuates during the different stages of the infection. However, active virus replication clearly persists throughout the disease course, with low or undetectable levels of viremia correlating with the asymptomatic phase of the infection (for a recent review, see ref. (62)). In spite of chronically persistent viral replication, HIV-1 positive individuals also harbour cells carrying latent genomes. Such latency is likely associated with the fact that most CD4+ cells are resting *in vivo*. Resting CD4+ lymphocytes are susceptible of infection by HIV-1, but integration cannot occur in cells that are in the G0 phase of the cell cycle. Extra-chromosomal replicative intermediates remain unintegrated in the cell for a limited period of hours to days and viral integration and replication do not occur unless the cell is subsequently activated (189, 211). Given the short half life of the unintegrated genome compared to the odds of a resting T cell to become activated, this pre-integration latency is likely to be a rare event *in vivo*.

On the contrary, the major pathway to productive infection probably involves direct infection of activated CD4+ T cells. These cells are highly permissive for viral replication and rapidly begin to produce virus. Although the half life of infected activated lymphocytes is very short due to viral cytopathic effects and/or host immune response, a fraction of these cells may survive long enough to revert to a resting memory state. Post-integration latency represents therefore an important mechanism of viral persistence in the organism resulting from the integrated nature of the latent provirus and the long life-span of the memory cells harbouring the latent provirus.

I applied the competitive PCR-based system described in this chapter to study an *in vitro* model of HIV-1 latency and reactivation. In particular, I investigated how the activation state of the infected cells influences viral gene expression and the significance of viral latency at a quantitative level.

RESULTS

Peripheral blood lymphocytes were isolated, stimulated by PHA, and infected according to the described procedure. As shown in Fig. 4.6, PHA-treated lymphocytes remained in an active state for about two weeks in the presence of IL-2 before returning to a resting state (upper graph).

A subsequent addition of PHA to the medium in the presence of the appropriate antigen presenting cells (day 13) rapidly prompted the cells for a new activation cycle. As expected, the level of HIV-1 genomic transcripts, representing the input RNA from the virions, rapidly declined after infection as they became reverse-transcribed into DNA (quantitation of viral DNA not shown). Regulatory RNA molecules appeared a few hours after infection, followed with some delay by genomic transcripts. Viral transcription was maximal 48 hours after the infected cells reached a peak of activation, only to become virtually silent shortly after the cells returned to the quiescent state. The next cell stimulation promptly resulted in a new peak of viral transcription until the cells stopped duplicating. Interestingly, the order and time of appearance of genomic and regulatory transcripts closely resembled the pattern observed during the previous cycle.

DISCUSSION AND CURRENT WORK

These observations reinforce the notion that in HIV-1 infected cells the integrated provirus is strictly dependent on the cellular environment for its gene expression. In the absence of activation-dependent host transcription factors and sufficient levels of viral positive regulatory proteins such as Tat and Rev, the integrated provirus in memory cells remains in a state of true transcriptional latency until the cell is reactivated by antigen.

An appealing possibility is that cellular activation results in a major chromatin rearrangement ultimately affecting also the LTR promoter and causing a release of the nucleosome-induced inhibition of transcription. I am currently investigating this hypothesis by exploiting the quantitative chromatin immunoprecipitation assay described in Chapter 3. After purification of oligo-nucleosomal particles, nucleosome-bound LTR molecules are immunoprecipitated using anti-sera raised against acetylated and non-acetylated histones. Immunoprecipitated DNA is then quantitated using LTR-specific primes to determine whether the actively transcribing LTR sequence is predominantly associated with acetylated histones and if this association is consequent to CD4+ cell

activation. If this is the case, such results will provide the evidence that transcriptional silencing imposed by the chromatin scaffold is an important determinant of the activation state of the virus.

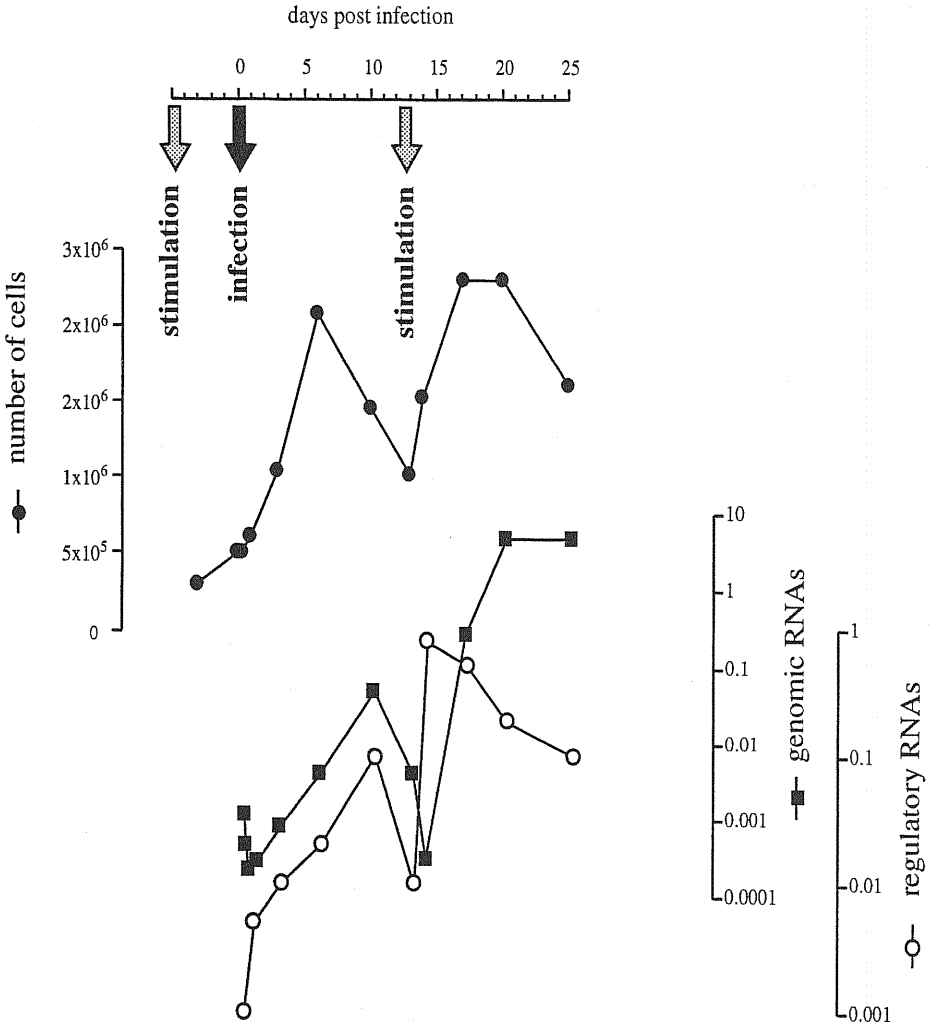

Figure 4.6
Kinetics of HIV-1 expression in infected human peripheral blood lymphocytes

Chapter 5

CONCLUSIONS

Elucidation of the mechanisms regulating HIV-1 gene expression is of crucial importance for the understanding of progression from the asymptomatic phase of the infection to overt AIDS. The level of circulating viral RNA is now commonly considered and diagnostically exploited as a prognostic marker of disease progression, a reduction in viral load being generally concomitant with remission and improved prognosis of the patient. With the advent of combination therapy, it is possible to achieve a durable control of HIV-1 replication in infected individuals. When *de novo* infection is inhibited by drug treatment, cell-free virions are rapidly cleared from all body districts as productively infected lymphocytes die after a short life span. However, complete eradication of the virus from the patient is still beyond medical reach, as infectious virus is able to persist in a post-integration latent form in resting lymphocytes in spite of prolonged therapy. Conventional antiretroviral agents are ineffective while the targeted steps in the virus life cycle are suspended, and latently infected cells will escape immune recognition and clearance while viral antigen are not expressed. This latent reservoir of infection-competent virus is a major hurdle to virus eradication, as the transcriptionally silent virus can become activated following immune activation of the infected cell.

The research work presented in this thesis covers several critical aspects and factors implicated in the modulation of HIV-1 transcriptional activation.

Chapter 2.

The role played by cellular factors in the regulation of the viral promoter has been investigated, focusing on the function and properties of transcription factors USF. Cellular proteins binding to the LTR are responsible for basal promoter activity when the levels of virus-encoded transactivator Tat protein are still sub-optimal. The relevance of this observation in the contest of viral latency is best understood considering that these conditions occur during the silent phase of the infection. Moreover, cellular transcription factors may also influence HIV-1 transcription at a higher level, in that the presence of cellular proteins constitutively bound to the viral promoter may dictate the pattern of nucleosome positioning on the LTR. Nucleosomes act as general inhibitors of gene expression and active transcription requires alleviation of this suppressive effect by acetylation of histones by histone-specific acetylating enzymes.

Chapter 3.

It has been herein demonstrated that histone-specific acetyltransferases are also involved in activation of the HIV-1 promoter and an important novel function of Tat is reported. Tat binds and specifically recruits two histone acetyltransferase proteins, p300 and CBP, to the chromatin-bound HIV-1 promoter for viral transactivation. Although p300 and CBP are transcriptional coactivators of many cellular and viral proteins, this is the first demonstration that they can also be recruited by an RNA-targeted transactivator such as HIV-1 Tat. This finding also has important implications for the understanding of the pathogenetic mechanisms of HIV disease. In HIV-infected patients, a large number of cells harbor proviral DNA molecules which are transcriptionally inactive. In these latently infected cells, the Tat-mediated recruitment of HATs to the viral promoter is likely to represent a critical step in viral reactivation. As such, the interaction of Tat with p300 and CBP also represents a potential target for the development of novel therapeutic strategies.

Chapter 4.

Accurate monitoring of HIV-1 gene expression requires precise quantification of viral nucleic acids in infected samples. Not only does the overall viral load correlate with the clinical stage of the disease, but the appearance of viral transcripts of the three different classes reflects the phase of the infection. A predominance of multi-spliced transcripts, coding for Tat and other HIV-1 regulatory proteins, is a hallmark of the early phase of transcriptional activation, whereas single-spliced and unspliced transcripts appear subsequently and correlate with the advanced phase of the infection. Part of this thesis work dealt with the development of a sensitive competitive PCR-based system that allows the precise quantification of all species of HIV-1 RNA and DNA and, importantly, the normalization of these values to control cellular nucleic acids. Moreover, through the application of this quantitation procedure to two *in vitro* models of infection, it has been proved on a quantitative basis that the dynamics of HIV-1 activation follow the same temporally dependent pattern in spite of the

biological differences between the two models.

Appendix.

The same quantitative technique was used to investigate the significance of viral latency at the molecular level in the context of lymphocyte activation. Viral activation is shown to be strikingly correlated with the proliferative state of the infected cells, as HIV-1 gene expression is virtually shut off by entrance of the infected cells in the non-proliferative phase. However, the silent provirus becomes rapidly activated upon a subsequent cellular stimulation. These data reinforce the notion that in long-lived resting lymphocytes HIV-1 remains in a state of true transcriptional latency until activation by specific antigenic stimulation takes place.

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X


WELCOME TO THE "SCIENTIST ANTI-DEFAMATION LEAGUE" WEEKLY MEETING.
TONIGHT'S TOPIC IS THE STEREOTYPE THAT WE SCIENTISTS HAVE NO SOCIAL LIVES...
BUT FIRST...
IS SATURDAY NIGHT OKAY FOR OUR NEXT MEETING?
I'M FREE
NO PROBLEM
WIDE OPEN
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