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Sequential Steps in Tat *Trans*-Activation of HIV-1 Mediated Through Cellular DNA, RNA, and Protein Binding Factors

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The regulation of HIV expression is controlled by the activity of the Long Terminal Repeat (LTR). *Trans*-activation by the virally encoded Tat protein is one of the main mechanisms of LTR activation. Tat binds to its target, TAR RNA, and cellular proteins that bind the LTR, Tat, or TAR RNA are important components of the *trans*-activation process. We will review the factors that have been characterized for a possible involvement in this mechanism. Whereas LTR binding proteins consist of Sp1 and TBP, a large number of factors that bind TAR RNA have been isolated. We have previously cloned two of them by RNA probe recognition: TRBP and La. We have shown that the in vitro and in vivo binding of TRBP to TAR RNA correlates with a constant expression of the protein during HIV-1 infection. Several proteins that interact with Tat have mainly positive, but some negative, effects on *trans*-activation. Genetic studies have defined that human chromosome 12 encodes a protein that will allow *trans*-activation in rodent cells. The binding and the functional data about these proteins suggest sequential steps for the Tat *trans*-activation mechanism. Each of these intracellular molecular events could be the target for molecular intervention against the virus.

Tat *Trans*-activation HIV Cellular factors RNA binding proteins

CELLULAR and viral proteins control the regulation of HIV-1 and HIV-2 expression. Tat (the *trans*-activator of HIV) activates the expression of genes under the control of the Long Terminal Repeat (LTR) by increasing transcriptional initiation, elongation, or posttranscriptional processes (15,25,34,36,38,73).

Tat acts through an RNA target, TAR (*trans*-activation responsive), located in the R region of the LTR. TAR RNA forms a stable stem-bulge-loop structure required for Tat function (6,8,18). In vitro, Tat bind to the bulge of TAR RNA (17), but this interaction cannot account for the entire *trans*-activation process. Indeed, sequences in the LTR (4,41,65), the structure of TAR (6,8,18), and an appropriate cellular context (30,53) are required in this mechanism.

These data indicate that cellular proteins are important components of the *trans*-activation process. These factors are proteins that bind the LTR, Tat, or TAR RNA. Genetic studies have also indicated that other proteins might play an important role. How these factors modulate Tat-mediated *trans*-activation in not fully understood. We will review here what we know about their activity.

PROTEINS THAT BIND THE HIV LTR

A large number of protein binding sites have been found in HIV-1 and HIV-2 LTR (23,36,

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63,74). Although the role of these sequences is important for transcriptional regulation in the context of the whole integrated virus, *trans*-activation by Tat can be fully reconstituted in cells by a −105 HIV LTR and a reporter gene at +80 (+1 being the transcriptional initiation site) (7). This DNA element in the LTR harbors NFκB and Sp1 binding sites, followed by the TATA box, and the TAR/IST (inducer of short transcript) DNA sequence. On the 5′ end of this region, NFκB are essential motifs that modulate the expression of the LTR primarily at the level of basal rather than Tat-activated transcription (7,27). On its 3′ end, TAR DNA is the binding site for several proteins, some of which repress gene expression but do not affect *trans*-activation (23,55). The IST region overlaps with TAR DNA and induces the synthesis of 60-nucleotide RNAs in some cell lines, but this effect can be mutationally separated from Tat-mediated *trans*-activation (61). In comparison, several studies have demonstrated that two proteins that bind the HIV-1 LTR also bind Tat and are necessary components of the *trans*-activation process: Sp1 and the TATA binding protein (TBP).

Sp1

The HIV-1 enhancer element has three tandem Sp1 binding motifs. These motifs are required for *trans*-activation whether Tat acts in the context of the entire virus, the whole LTR, or a limited LTR (7,28,33,57). Sp1 binding sites are also necessary when Tat is tethered through a heterologous RNA or DNA binding site (4,7,40,41,65–67). A physical interaction between Sp1 transcription factor and Tat has been characterized in vitro and in vivo, and can explain the above functions (35,69). The Sp1–Tat complex could allow a better interaction between Tat and the transcriptional machinery to enhance the initiation of transcription (33).

TBP

The deletion of Sp1 binding sites in the virus generates compensatory mutations in the TATA box, which suggests a functional interaction between these sites (43). Furthermore, a direct interaction between Tat and TBP (or TFIID) has been characterized in vitro and in vivo (42,75). An active Sp1–Tat–TBP complex could be responsible for the high level of transcription from the LTR observed in the presence of Tat.

PROTEINS THAT BIND TAR RNA

Characterization of TAR RNA Binding Proteins

Some mutations in TAR RNA do not affect Tat binding but prevent *trans*-activation, indicating that TAR is the target for functionally important cellular factors. Several cellular proteins that bind TAR RNA (TAR RNA binding proteins) have been identified (22,24). Functional factors are expected to bind the loop, the upper stem, or the bulge of TAR.

The 68 kDa protein kinase PKR (also called p68, dsI, DAI, P1) is induced by interferon and activated by double-stranded RNA, including TAR. Northwestern studies have shown that its first 91 amino acids bind the stem of TAR RNA (48,59), and recent data indicate that PKR also interacts with Tat (49). SBP is a 140 kDa nuclear protein from HeLa cells that binds the lower part of the TAR stem (58). The cDNA for TRBP has been cloned by RNA probe recognition with TAR RNA. Northwestern analyses indicated that TRBP recognized the upper part of the TAR stem. Gel mobility shift assays with soluble protein show that several or multimeric proteins bind TAR RNA. Furthermore, a 24 amino acid peptide necessary for TRBP binding is sufficient to induce a mobility shift or a TAR probe (19,20). The cDNA for the autoantigen La has been isolated during the same screening. La recognizes U-residues within the overall context of the TAR secondary structure (13).

Although Tat binds to the bulge of TAR, cellular factors also interact with this structure. TRP-2 (a 70–110 kDa family of human proteins) contacts the bulge and displaces Tat from its target, indicating a competition between the two factors (62). A 38 kDa protein called BBP is more ubiquitous, and is another competitive inhibitor of Tat binding to TAR in vitro (3). TARBP-b is also a TAR bulge binding protein; its cDNA has been isolated from a library derived from human Hut-78 cells (56).

The isolation of a functional TAR loop binding protein could elucidate the dramatic decrease in *trans*-activation observed with TAR loop mutants (6,18). One candidate is p68, a protein isolated from HeLa nuclear extracts, that binds TAR with an intact loop (47). TRP-1/TRP-185 is another protein isolated from HeLa nuclear extracts. Its binding to TAR RNA requires the presence of the loop and the bulge, but also intact TAR loop sequences and a 100 kDa cofactor (62,76). RNA polymerase II binds TAR RNA in similar condi-

tions as TRP-185; maximum binding requires an intact TAR and the same cofactor as TRP-185 (78). The autoantigen Ku binds TAR RNA as a heterodimer Ku72/Ku86, but has less affinity for a loop mutant (39). Species-specific assays have characterized a human 83 kDa protein whose binding to the TAR loop sequence correlates with the presence of human chromosome 12 (31).

Some of the genes coding for these proteins have been cloned, allowing further analysis of their structure and function.

Cloning of the cDNAs for TAR RNA Binding Proteins

Some of the proteins that bind TAR RNA were previously cloned and characterized for other functions. For example, the cDNAs coding for PKR, La, and Ku (12,26,50) had been isolated, and the translated products were further studied for their interaction with TAR. For the other proteins, no cDNA was available.

To obtain cDNAs encoding TAR RNA binding proteins, we have developed a new screening technique using RNA probe recognition (20,21). After several screenings of a HeLa expression library, we isolated three independent cDNAs. Two of them encoded different versions of the same protein: TRBP and TRBP2. The third one was the previously described autoantigen La. Figure 1 shows two steps of this cloning, which are the purification of the lambda phage clones (Fig. 1A) and the characterization of the proteins in these clones (Fig. 1B). The diffuse aspect of the La plaques compared to TRBPs predicted a qualitative difference in the protein properties and correlated with a higher solubility of La compared to TRBPs.

TRBP and TRBP2 proteins vary only in their N-terminus (Fig. 2A). Further analysis and sequence comparisons indicated that they belong to the family of double-stranded RNA binding proteins that share a common RNA binding motif (19,68). However, the definition and the extent of this motif varies between the different laboratories and the binding assay. Northwestern binding of mutant proteins, computer homologies, and NMR studies have defined a double-stranded RNA binding domain (dsRBD) of ~70 amino acids (44), whereas peptide binding gel mobility shift and circular dichroism spectra have shown that 24 amino acids of TRBP in this domain are sufficient to bind TAR RNA [(19,44), Erarol et al., in preparation]. At least two proteins of this family, TRBP and PKR, can form homo- or heterodimers [(9,14), Duarte and Gatignol, unpublished data]. It is therefore possible to separate the dsRBD domain between a C-part that will contact the RNA and an N-part that could contribute to the stability of the protein–RNA complex and/or the dimerization of the protein (Fig. 2B). Further studies of the binding complexes with mutant proteins or peptides will define the role of these domains in detail.

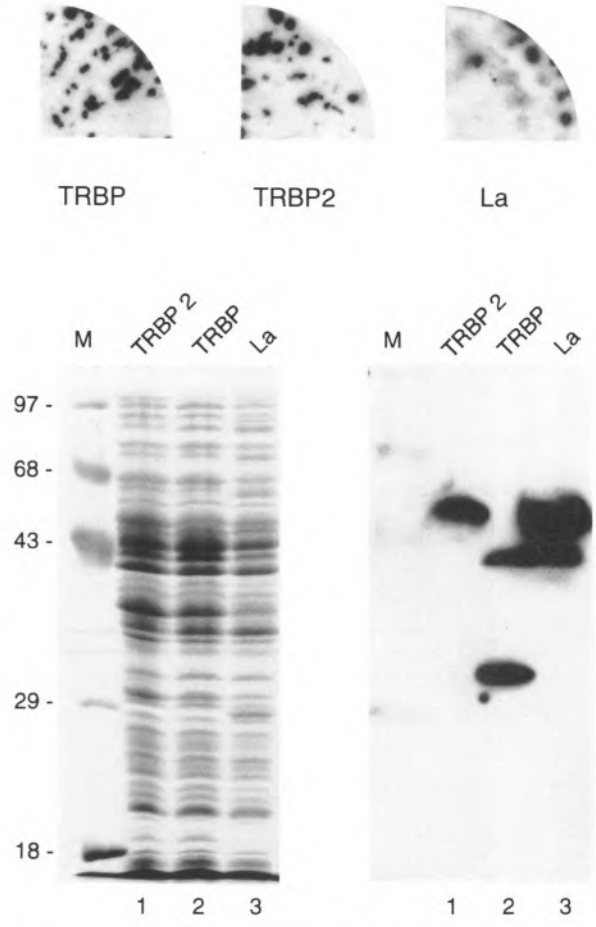


FIG. 1. Molecular cloning of the cDNA coding for three independent TAR RNA binding proteins using [^{32}P]TAR RNA. (A) Isolation of independent lambda phage plaques expressing TRBP, TRBP2, and La. (B) Binding activity of TRBP, TRBP2, and La to TAR RNA. Total cellular extracts were resolved by SDS-gel electrophoresis and stained with Coomassie blue (left). Northwestern analyses (right) were performed as described previously (19,21). The lower migrating band with TRBP is a breakdown product of TRBP that contains the RNA binding domain.

The cDNA for TARBP-b has also been isolated by TAR RNA probe recognition from a cDNA expression library derived from Hut-78 cells (56). Protein purification and microsequencing have been used to clone the cDNA coding for TRP-185

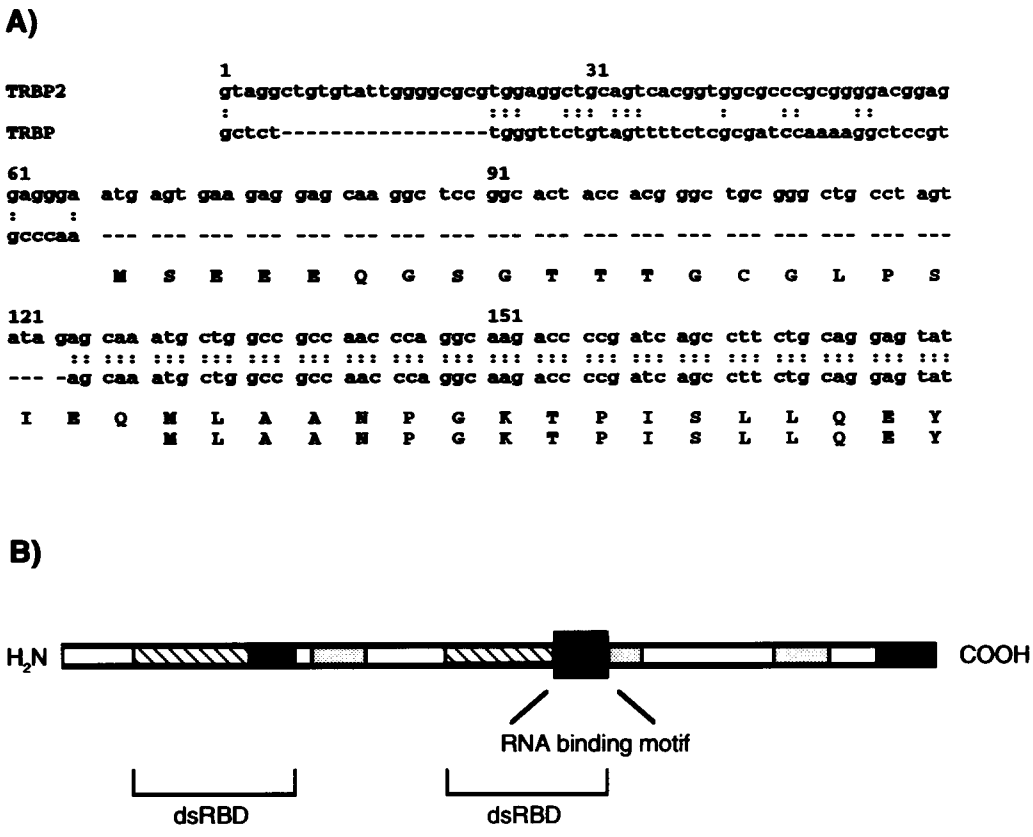


FIG. 2. Protein organization of TRBP and TRBP2. (A) Nucleotide (180 and 109 first nucleotides, respectively) and amino acid sequence differences between TRBP2 and TRBP. Upper lanes are TRBP2 and lower lanes are TRBP. Genbank accession numbers for complete sequences are M60801 (TRBP) and U08998 (TRBP2). (B) Different domains in TRBP. Black boxes are basic domains and gray boxes are acidic domains. dsRBD represents the double-stranded RNA binding domains as defined by computer homologies (44,68). The large black box is the RNA binding motif as defined by protein and peptide binding (19). Hatched boxes are regions that will likely participate to RNA stability and/or dimerization.

(77). Published sequences for other characterized TAR binding proteins are not yet available.

PROTEINS THAT BIND Tat

Trans-dominant inhibition studies suggest that functionally important proteins bind Tat in its core region with an important role for lysine 41 (11). In addition to Sp1 and TBP, other proteins that interact directly with TAT have been identified and cloned. The cDNA for TBP-1 was isolated from an expression library by Tat recognition. It belongs to a family of highly conserved proteins that have various effects on gene expression (51,54,64). A cellular protein kinase, TAK, that associates with Tat and phosphorylates RNA polymerase II could be a bridge between the two proteins (32). TAP was characterized as a Tat associated protein; the cloning of its cDNA has confirmed these properties: TAP binds Tat and TFIIB in vitro and in vivo and is a transcriptional activator in Tat-mediated *trans*-activation of HIV (16, 79,80).

OTHER FUNCTIONALLY IMPORTANT FACTORS

Trans-activation by Tat is very low in rodent cells compared to primate cells; it is absent in insect and yeast cells [(37,60,72), Daviet and Gatignol, unpublished data]. Studies with somatic cell hybrids have indicated that there is a missing factor for Tat *trans*-activation in rodent cells compared to human cells. This factor is functional in cell hybrids when human chromosome 12 is present (30,53). It helps Tat to bind TAR RNA and

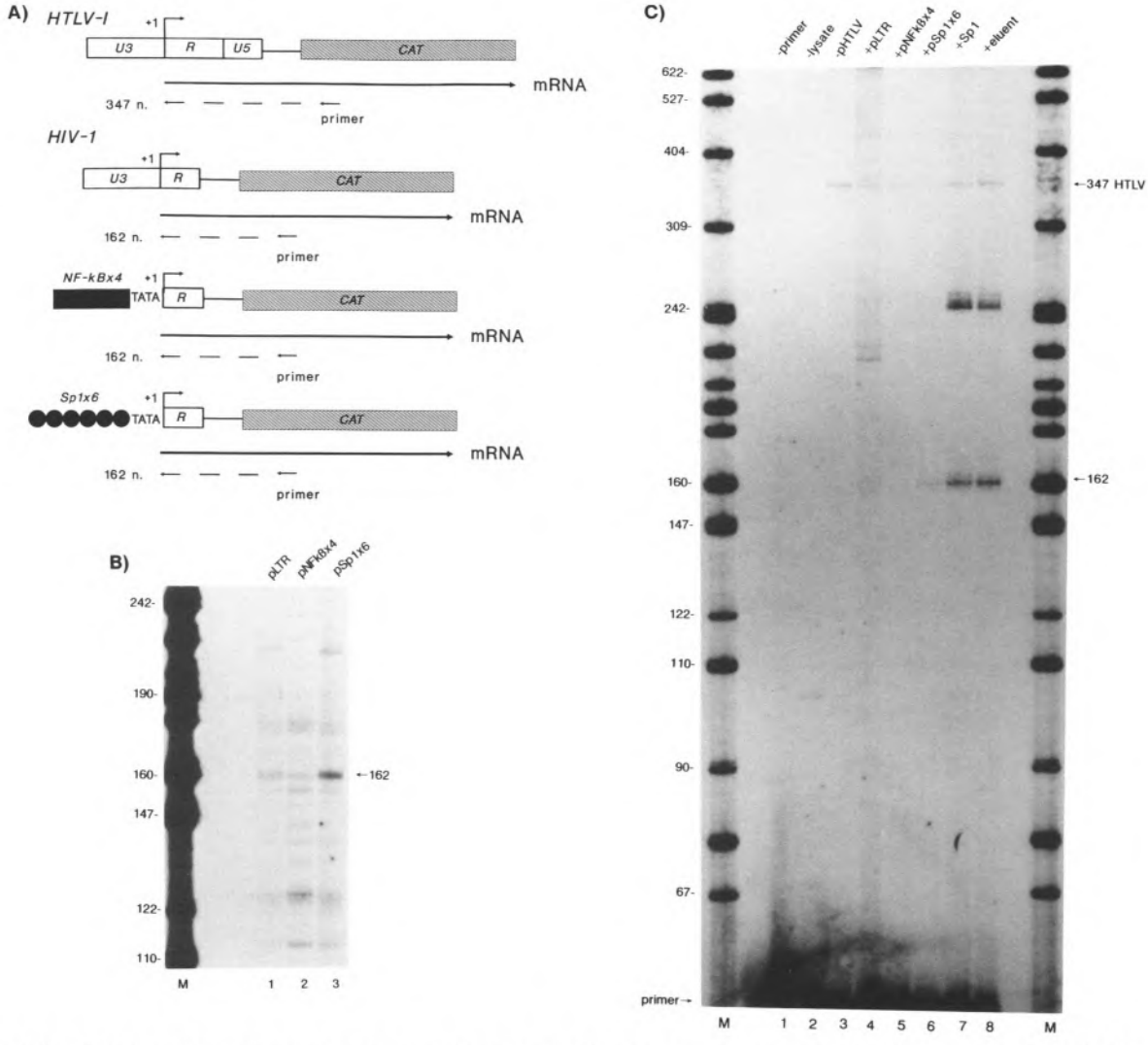
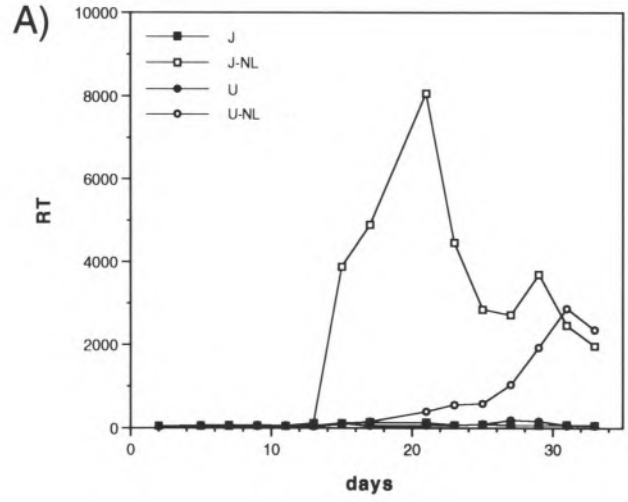


FIG. 3. Sp1 eluted from a Tat matrix is functional in vitro. (A) Schematic representations of plasmid constructs used for in vitro transcription in *Drosophila* embryo nuclear extract. The HTLV-I CAT construct (10) was used as an internal control for each of the in vitro transcription reactions. The HIV-1 LTR CAT constructs include pLTR (containing all of U3 and R to +80), pNFκB × 4 (four copies of NFκB binding motif inserted upstream of the HIV-1 TATA element), and pSP1 × 6 (six copies of Sp1 motifs inserted upstream of TATA). All plasmids were tested in supercoiled forms; in vitro transcripts were assayed using primer extension of a 31-mer oligonucleotide complementary to the CAT gene (5). (B) Basal activities of pLTR (lane 1), pNFκB × 4 (lane 2), pSp1 × 6 (lane 3) in *Drosophila* embryo extract. Supercoiled template (2 μg) was used in each assay. Under these conditions of template excess, correctly initiated (162 nucleotide) and aberrantly initiated products can be seen. (C) Augmentation of Sp1-dependent transcription using eluant from Tat-bead (35). Control template (pHTLV, 0.5 μg) and 0.5 μ of test plasmid (as indicated) in supercoiled form was used in each reaction. In lanes 7 and 8, the test plasmid was pSp1 × 6 except that 2 fpu of purified Sp1 (Promega; lane 7) and 1 μl of a Tat-bead eluant (lane 8) were added, respectively. The 162 nucleotide band represents authentically initiated transcript; the 347 nculeotide band represents the internal control transcript from pHTLV. Phosphoimaging quantitation indicated that the 162 nucleotide band is augmented by approximately 6× (lane 7) and 7× (lane 8) from that in lane 6. The 347 nucleotide signal varied by 2–3× between each of the lanes. We do not know the identity of the 250 nucleotide band in lanes 7 and 8.

could be a Tat binding protein, a TAR loop binding protein, an intermediate of those, or a protein that has all these functions (2,11,29,46,52,70). Considering the number of missing steps in this process, it is unlikely that a single protein will support all these functions. It is more conceivable that human chromosome 12 will encode a protein, which is the first step for intracellular molecular events that will lead to the *trans*-activation of HIV-1 by Tat. The preliminary analysis of the function of the characterized proteins supports this view.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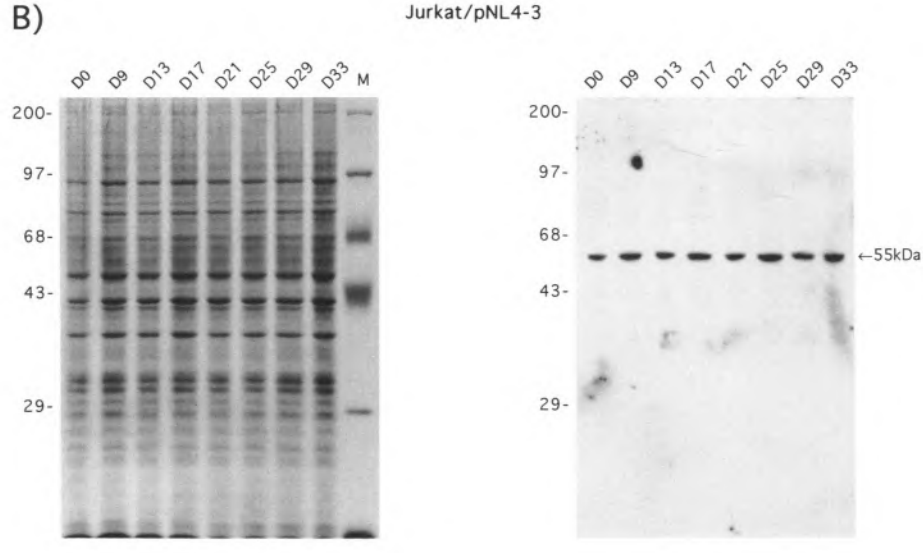
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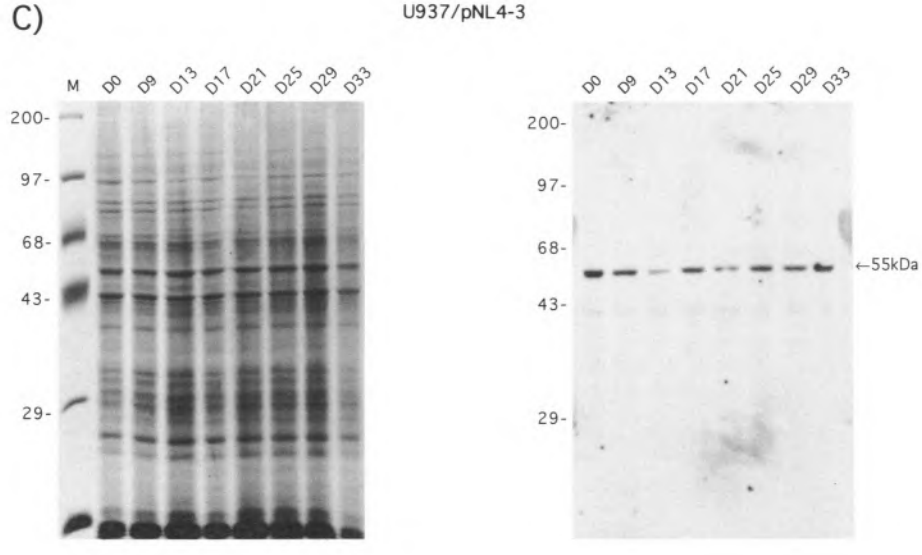
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FUNCTION OF THESE PROTEINS

Criteria have been defined for the "missing factor" in *trans*-activation by Tat. It should: 1) be present in primate but not in rodent and nonmammalian cells; 2) have its gene mapped on human chromosome 12; 3) be a TAR loop binding protein; 4) be a Tat binding protein; 5) help Tat to bind with TAR; 6) have a functional positive effect on *trans*-activation. Until now, all these criteria have not been found in a single protein, but are distributed in some of the described factors. Based on their function, we can separate the potential inhibitors of gene expression from the activators, and from those with mixed or no function defined.

Negatively Acting Proteins

Among the proteins described, PKR is probably the most potent inhibitor of HIV expression. It has been shown to inhibit the replication of HIV-1 (Jeang, unpublished data). However, its function occurs through phosphorylation and translational effects and is unlikely to have a direct role in *trans*-activation by Tat. BBP is a bulge binding protein and a competitive inhibitor of Tat binding. It could prevent Tat function by this way but this mechanism awaits direct evidence (3).

Positively Acting Proteins

Proteins that belong to the basic transcriptional machinery have already a proven function. The involvement of Sp1, TBP, and RNA polymerase II in *trans*-activation has become evident by their in vitro and in vivo binding to Tat or TAR. The interaction between Sp1 and Tat maintains Sp1 function as a *trans*-activator. Indeed, Sp1 eluted from a Tat-bound column can activate the transcription from an HIV promoter with Sp1 binding sites (Fig. 3). In addition, Sp1 has been mapped to human chromosome 12. TBP interacts in vitro and in vivo with the cysteine and the core region of Tat. The overexpression of TBP in the presence of Tat enhances HIV expression, indicating a functional interaction between the two proteins. The importance of the position of Sp1 binding sites and the sequence of TATA motifs in Tat *trans*-activation and virus infectivity have shown a real functional role for these proteins (7,33,35, 42,43,75).

The positive effect of TRBP in *trans*-activation has been shown by cotransfection experiments. In vivo, TRBP *trans*-activates HIV-1 LTR and acts in synergy with Tat function. In Jurkat and U937 cells infected by HIV-1 (pNL4-3) (1), its expression remains constant during viral kinetics, indicating that it coexists with TAR RNA (Fig. 4). To correlate its in vitro binding to TAR RNA and its cellular expression, we have shown by coimmunoprecipitation that TRBP and TAR interact during HIV infection. Recently, TRBP gene has been mapped to human chromosome 12 and the protein has different sizes in rodent compared to primate cells (19,20,45). Therefore, it is a potential candidate as a factor that modifies TAR structure in a manner that could allow another protein to bind TAR.

TAP binds Tat core region in vitro and in vivo; this binding is dependent on K41 and F38, two amino acids required for Tat function. TAP also contacts the transcriptional machinery through TFIIB. It possesses a strong transcriptional activation domain and it synergizes Tat *trans*-activation. Its gene is not on human chromosome 12 and it is likely to act at a downstream step in which Tat will recruit TAP to the promoter. Tat, TAP, and TFIIB might act in synergy to activate transcription from the HIV-1 LTR (16,79,80).

In vitro, La releases a translation block created by TAR. Because it binds TAR in vivo during viral infection, this activity probably exists inside the cell but cannot be detected because of the intracellular amount of the protein (13,71). TARBP-b *trans*-activates the HIV LTR and contributes positively to Tat *trans*-activation, whereas TRP-2 moderately activates transcription. How these effects are related to their bulge binding and the competition with Tat observed for TRP-2 remains to be determined (56,62).

Proteins Whose Function Remains to be Determined

The binding sites of SBP correlate with the corresponding IST DNA sequence. It could be in-

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FIG. 4. TRBP is expressed at constant level during HIV infection. (A) Viral kinetics of pNL4-3 in Jurkat and U937 cell lines monitored by reverse transcriptase (RT) assay (57). Cell supernatants containing 2.5×10^6 cpm of RT activity were used to infect 10^7 Jurkat or U937 cells in 25 ml RPMI. Viral production was measured every 2 days in the cellular supernatant by RT assay. (B, C) Characterization of TRBP during HIV infection of Jurkat (B) or U937 (C) cells. Medium (7 ml) and cells were taken out every 4 days and centrifuged, the cell pellets were heated in SDS lysis buffer, and a similar amount was deposited on two identical gels. After migration, one gel was stained with Coomassie blue (left panels) and the second was transferred for Western analysis (right panels) as previously described (45). Numbers on top of the gels indicate the days after infection. M represents molecular weight markers. TRBP appears as a 55 kDa band as indicated. Small variations at D13 and D21 in U937 cells do not seem to be significant.

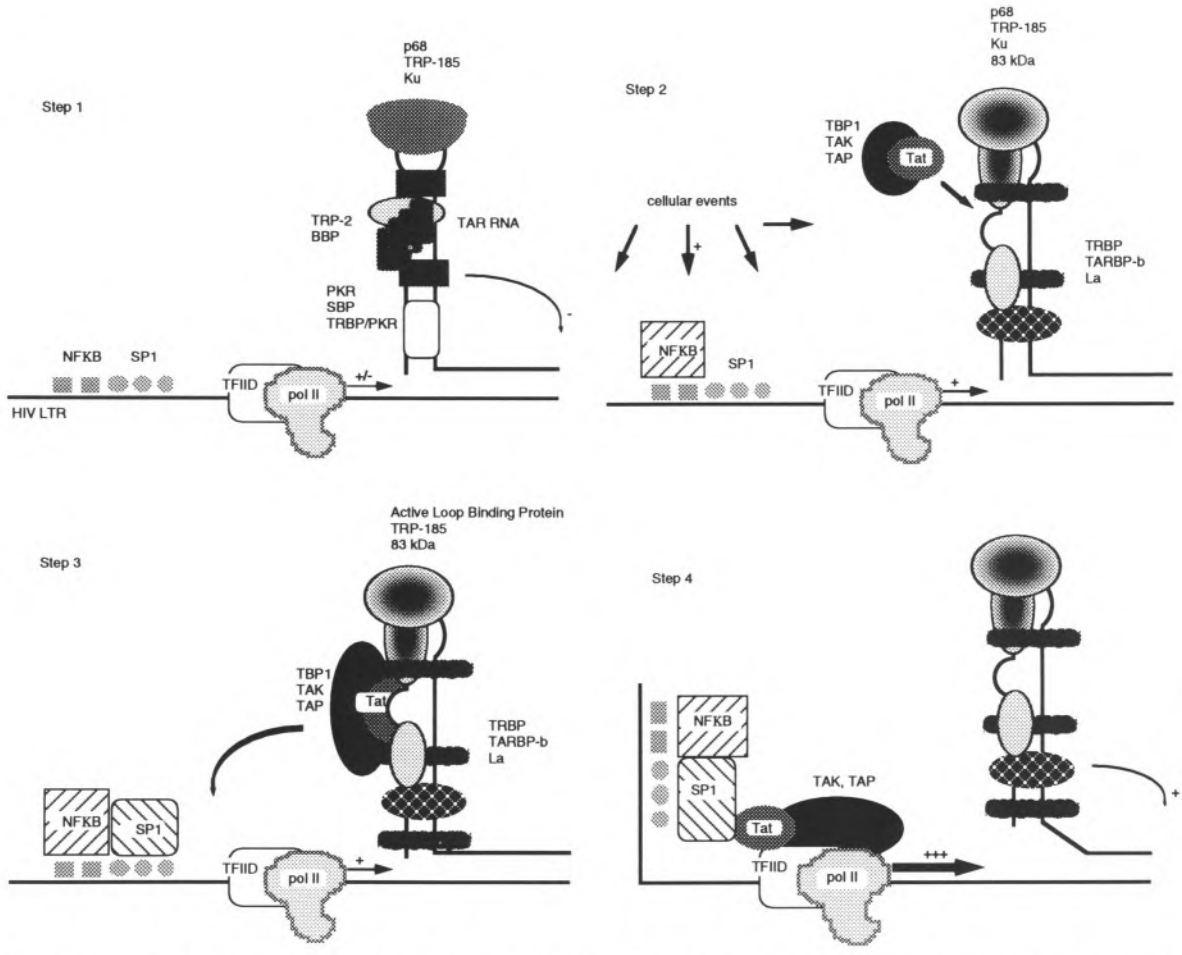

FIG. 5. Sequential mechanism for the *trans*-activation of HIV-1 by Tat. Each step is as described in the text. Proteins mentioned are potential candidates for the position and the function described at the indicated step.

volved in the production or the stabilization of short transcripts, which are independent of Tat activity (58,61). Ku and p68 have been proposed for loop binding proteins, but the correlation between their binding and Tat *trans*-activation with TAR loop mutants awaits further studies. Indeed, two mutants that strongly bind p68 are inactive in *trans*-activation (6,18,39,47). TRP-1/TRP-185 binding to the TAR loop correlates with the activity of TAR mutants. It activates positively the HIV LTR in vitro in the absence or presence of Tat, but it competes with Tat for binding. The recent cloning of its cDNA has confirmed the binding properties of TRP-185. However, its gene is not on human chromosome 12 and its function is not yet elucidated (62,76,77). A recent finding shows that a 83 kDa protein has a loop binding activity correlated to the presence of human chromosome 12. Further characterization should define its functional role and indicate if it could be the "missing factor" in rodent cells (30,31,53).

TBP-1 and MMS1 belong to a family of proteins that have positive or negative effects on *trans*-activation. The mechanism of this activity awaits a functional correlation with its binding to Tat (51,54,64). TAK phosphorylates RNA polymerase II and this activity is linked to Tat binding. The correlation between the loss of this function and Tat mutations suggests an implication in *trans*-activation (22).

PERSPECTIVES

The cellular factors in Tat-mediated *trans*-activation have become increasingly important with the identification of a large number of proteins involved in this process. One major question that arises is how they will bind and function on the same target. If some proteins still require more characterization to definitively include them in the *trans*-activation process, it seems obvious that

those involved will not exert their function all together but rather in a sequential manner. We therefore propose a *trans*-activation mechanism in four steps (Fig. 5).

1. Inactivated LTR with low basal level of RNA production: the LTR binding factors will assemble in a manner that allows only low level of transcription. The TAR RNA binding factors that compete with Tat could prevent the binding of the low level of Tat product (i.e., BBP, TRP-2, TRP-1/TRP-185, and perhaps SBP, PKR, or PKR/TRBP heterodimers).
2. Cellular signaling, possibly via NFκB, will increase the basal level of transcription, and therefore the production of Tat that will trigger the *trans*-activation mechanism. Tat (alone or with a binding factor) will associate with TAR RNA. Molecular events and Tat will modify the nature of TAR binding factors so that "helper proteins" such as TRBP (monomers or homodimers), TARBP-b, and a positively acting loop binding protein will reach their target. In this context, Tat will be stabilized on the nascent TAR RNA.
3. Tat will then be transferred from TAR to the transcriptional machinery via "bridging" Tat binding proteins such as TAP or TAK.
4. TBP, TFIIB, and RNA polymerase II will form an activated transcription complex that will mediate an activation by Sp1, TAP, Tat, and other possible transcriptional activators.

The summary presented here provides the basis for the elucidation of the intracellular molecular steps of Tat-mediated *trans*-activation of HIV-1.

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