Potentiation of Human Immunodeficiency Virus Type 1 Tat by Human Cellular Proteins

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Received 31 January 1997/Accepted 30 May 1997

The Tat protein is a potent activator of human immunodeficiency virus type 1 transcription. Tat has been shown to act by increasing both transcription initiation and elongation, but a detailed understanding of its interaction with the transcriptional machinery is lacking. With the aim of isolating cellular proteins that interact with Tat and play a role in transactivation, we have reexamined its function in a cell-free transcription assay. Monitoring the appearance of transactivation after addition of purified Tat at intervals to the reaction mix revealed a lag of approximately 10 min before Tat is able to effect transactivation. Incubation of Tat in nuclear or cytoplasmic extracts of human cells was sufficient to eliminate the lag, but nuclear extract from a rodent cell line was inactive. The accelerating effect of the human cell extract could be abrogated by dilution, heat inactivation, or chromatographic depletion. We infer that Tat is potentiated for transactivation through interaction with a protein factor(s) that is specific to human cells.

The lentivirus family contains complex retroviruses encoding a number of accessory proteins that play important regulatory roles in the viral life cycle (6). One of these proteins, Tat, is synthesized early in the viral life cycle and acts to bring about high levels of viral transcription (7). The Tat transactivating protein of human immunodeficiency virus type 1 (HIV-1) is a strong activator of viral transcription that functions by an unprecedented mechanism (11) which is novel among other retroviral subgroups and unique among eukaryotic transcription activators in that it recognizes an RNA element (10). Tat binds to a structured RNA signal, the Tat activation response (TAR) element, which is located near the 5’ end of the nascent transcript. While Tat has been reported to affect the rate of initiation of transcription (13), it exerts its primary influence on transcription elongation (11, 13). Tat is believed to act by modifying the elongating RNA polymerase complex such that transcription complexes are converted from a poorly processive form to a form that elongates efficiently (5, 10).

Although it is clear that Tat functions via interactions with the cellular transcriptional apparatus, the identities and functions of the proteins that it recruits to the TAR element for this purpose remain largely unknown. Several host proteins have been shown to interact directly with Tat. Some of these serve to direct it to regions of the TAR stem-loop structure (19, 23) or are known transcriptional activator proteins that interact with the basal transcriptional machinery (19, 22). Other more recently described activities have been suggested to be directly recruited by Tat to modify the elongating transcription complex; these include a Tat-associated kinase activity (TAK) (9) that binds to a glutathione S-transferase–Tat fusion protein, a 32-kDa Tat-associated protein (TAP) purified from a Tat affinity column (24), and a Tat stimulatory factor (SF) shown to restore transactivation in a reconstituted transcription system (25).

Transactivation by Tat has been recapitulated in vitro in a variety of cell-free transcription systems. In most cases, a pre-incubation step is required for the stimulation of transcription to be observed in vitro (14, 16), but the events occurring during this step are not well defined. To date, most investigations of this question have focused on changes in the cellular transcription extract that take place during preincubation. In this report, we inquire whether Tat undergoes any changes in its activity during preincubation. Our results show that purified Tat is not immediately competent to activate transcription in a runoff transcription assay. The ability to transactivate without a lag is acquired when Tat is incubated in nuclear or cytoplasmic extract of human origin. We propose that a factor(s) in cell extract is required to convert Tat to a form that is transactivation competent.

Pretranscription with Tat leads to increased transcription. The transcriptional activation property of the HIV-1 Tat protein in cell extracts from HeLa cells is dependent on functional Tat and on the TAR element (14, 16) and is due to the increased processivity of complexes initiating at the HIV-1 promoter (10). In our experiments, we used two templates differing in the presence (wild type) or absence (ΔTAR) of a functional TAR element and linearized so as to yield distinct transcripts of 745 and 630 nucleotides (nt), respectively (Fig. 1). Transcription from the TAR mutant template served as an internal control for nonspecific effects; Tat stimulated transcription from this template only slightly or not at all. Our standard in vitro transcription protocol includes a preincubation step which renders the observed stimulation by Tat more evident (14, 16). For example, the degree of transactivation by Tat was greatest when measured during the final 30 min of a 60-min transcription reaction. It has been suggested that this increased responsivity to Tat following the first 30 min of reaction, the so-called presynthesistep, may result from the inactivation of a cellular transcription factor that masks the Tat effect. Alternatively, increased transactivation may be due to a time-dependent process that involves the Tat protein.

To distinguish between these possibilities, we examined the effect of presynthesis on basal and activated transcription independently. Presynthesis was carried out in the presence or absence of Tat for up to 30 min. Following presynthesis, [α-32P]UTP was added to the reaction mixtures and transcripts were labeled for an additional 30 min (Fig. 2B). As shown in...
ing the missing components together with [α-32P]UTP, and incubation was continued for an additional 30 min. Despite considerable differences in basal transcription levels, Tat stimulated transcription in all cases. The basal transcription level was highest when nuclear extract was preincubated with template DNA but without added nucleotides; it decreased somewhat when only three nucleotides (CTP, GTP, and UTP) were present and decreased further when only ATP or dATP was present (data not shown). The basal transcription level was low when template was omitted from the preincubation mixture, regardless of the presence of nucleotides (data not shown), suggesting that a high basal level of transcription is attributable to the formation of DNA-protein complexes. The degree of stimulation by Tat was lowest when presynthesis was carried out in the presence of CTP, GTP, and UTP, implying that these exogenous nucleotides have an inhibitory effect in the absence of ATP, but it was not otherwise greatly affected by the variations tested. These findings suggested that the critical component is in the nuclear extract itself.

**Time-dependent assembly of Tat-activated transcription complexes.** In vitro preinitiation complexes assemble in a stepwise manner, and some stages have been shown to be rate limiting (18). The formation of transcription complexes activated by Tat may also be a slow process. Therefore, we examined whether the ability of Tat to transactivate is present immediately upon its addition to the reaction mixture or whether it appears with a lag. RNA synthesis was measured during the last 30 min of a 60-min reaction, i.e., after a 30-min presynthesis step. Under these conditions, most runoff transcripts are produced by Tat-activated transcription complexes. As diagrammed in Fig. 3B, presynthesis and RNA labeling were performed identically in all reaction mixtures. Tat was present from the outset or was added at later times during the presynthesis reaction or the labeling step. The results show that maximal transactivation was achieved when Tat was added to the reaction mixture at the outset and was present throughout the 60-min reaction (Fig. 3A; compare lanes 1 and 2). A delay of 10 min led to a slight reduction in transcription (lanes 2 and 3), but transactivation decreased markedly when the addition of Tat was delayed for 20, 30, or 40 min (lanes 4, 5, and 6, respectively). These observations suggested that Tat does not begin to activate transcription immediately upon its addition to the reaction mixture; rather, there is a lag of at least 20 min before the formation of Tat-activated transcription complexes can be detected.

To determine whether the omission of nucleotides from the presynthesis reaction affects the outcome, reaction mixtures were preincubated in the absence or presence of one or more nucleoside triphosphates (NTPs) for 30 min. Moreover, the DNA templates were either included in or omitted from the presynthesis step for each of the various NTP presynthesis conditions. The reaction mixture was then completed by adding the missing components together with [α-32P]UTP, and incubation was continued for an additional 30 min. Despite considerable differences in basal transcription levels, Tat stimulated transcription in all cases. The basal transcription level was highest when nuclear extract was preincubated with template DNA but without added nucleotides; it decreased somewhat when only three nucleotides (CTP, GTP, and UTP) were present and decreased further when only ATP or dATP was present (data not shown). The basal transcription level was low when template was omitted from the preincubation mixture, regardless of the presence of nucleotides (data not shown), suggesting that a high basal level of transcription is attributable to the formation of DNA-protein complexes. The degree of stimulation by Tat was lowest when presynthesis was carried out in the presence of CTP, GTP, and UTP, implying that these exogenous nucleotides have an inhibitory effect in the absence of ATP, but it was not otherwise greatly affected by the variations tested. These findings suggested that the critical component is in the nuclear extract itself.
Preincubation of Tat with nuclear extract is sufficient for Tat potentiation. From the foregoing, it seemed likely that the lag observed in Fig. 3 represents the time needed for Tat to renature or to establish an active conformation or complex. To address this possibility, we tested the ability of Tat to activate transcription when preincubated separately from the transcription reaction mixture. In the experiment shown in Fig. 4, Tat was preincubated for 0 to 30 min at 30°C with nuclear extract or in Tat dilution buffer (10 mM Tris [pH 7.9], 50 mM KCl, 1 mM EDTA, 5 mM dithiothreitol) prior to its addition to the presynthesis reaction mixture. To minimize the formation of Tat-activated complexes during the labeling period, the labeling time was reduced to 10 min for this experiment. The results showed that, as expected, Tat elicited strong transactivation when preincubated with the transcription reaction mix for 30 min (Fig. 4B, lanes 1 and 2). Transactivation was weakened slightly when Tat was preincubated with buffer for 10 or 20 min and then added to the reaction mixture for the balance of the 30 min of presynthesis (Fig. 4B; compare lanes 3 and 4 and 5 and 6), and transactivation all but disappeared when Tat was preincubated with buffer for the entire presynthesis period (lanes 7 and 8). In contrast, incubation of Tat in nuclear extract for various lengths of time before addition to the presynthesis reaction elicited strong transactivation in all cases (Fig. 4C). Thus, Tat becomes more potent for transactivation when it is incubated in nuclear extract, but not when it is incubated in buffer alone, indicating that this potentiation effect reflects an essential process involving a component(s) of the nuclear extract.

Potentiation may require an interaction between Tat and a cellular protein(s). These observations argue that potentiation is unlikely to be a consequence of Tat refolding spontaneously in the appropriate environment. Rather, preincubation with nuclear extract is probably required for the interaction of Tat with a cellular protein(s). To obtain evidence that potentiation of Tat depends on specific protein-protein interactions, we asked whether a component of nuclear extract that is required for Tat potentiation can be inactivated by heating. For this purpose, aliquots of nuclear extract were held for 5 min at different temperatures, cooled, and assayed with Tat as diagrammed in Fig. 5B. The results show that heating nuclear extract at 42°C did not affect the level of transactivation (Fig. 5A, lanes 3 and 4); however, heating at 50, 55, and 65°C steadily diminished the Tat response (lanes 5 to 10), which was abolished by heating the extract to 100°C (lanes 11 and 12). These observations imply the involvement of a cellular protein or proteins, at least one of which must be present in its native conformation to allow Tat potentiation. Furthermore, the potentiation effect was sensitive to dilution of the extract, as

FIG. 3. Time course of Tat addition. Nuclear extract (NE), DNA templates, and NTPs were incubated together for 30 min, and then [α-32P]UTP was added and transcription was continued for 30 min. The reaction products are shown (A). Tat (75 ng) was added at intervals during the course of the presynthesis reaction (B).
demonstrated in Fig. 5C. Tat was incubated in decreasing concentrations of nuclear extract diluted with Dignam buffer D. The potentiation effect decreased as the concentration of nuclear extract in which Tat was incubated was reduced (Fig. 5C, even-numbered lanes), such that the potentiation effect was largely eliminated when the extract was diluted threefold (lanes 9 and 10). No potentiation was observed with buffer D alone, with a solution of bovine serum albumin, or with fractions enriched for TFIID (data not shown); therefore, we conclude that potentiation requires the interaction of Tat with one or more specific proteins in the HeLa nuclear extract.

**Tat potentiation occurs in nuclear and cytoplasmic extracts of human but not rodent origin.** The specificity of the potentiation effect was assessed by replacing the nuclear extract with other protein extracts in the Tat preincubation assay. Preincubation with the postribosomal supernatant fraction of a HeLa cell cytoplasmic extract (S-100) was as effective as preincubation with the nuclear extract (Fig. 6A, lane 4). Both nuclear and cytoplasmic extracts from the thymocyte-derived Jurkat cell line were similarly able to support potentiation at a concentration of 6 mg/ml (lanes 8 and 10), as was S-100 extract from the embryonic kidney cell line 293 (lane 12). On the other hand, nuclear extract from a Chinese hamster ovary (CHO) cell line was not essentially ineffective for Tat potentiation (1.6-fold, compared to 9.8-fold for HeLa S-100, 7.1-fold for 293 cell S-100, and 1.0-fold for buffer), implying that the potentiation effect is species specific. Previous reports have demonstrated the inability of Tat to transactivate efficiently in rodent cells (1, 8). Mixing experiments indicate that the CHO nuclear extract does not inhibit potentiation by human extracts, implying that the required factor(s) is not present in these cells. Preincubation with nuclear extract that had been depleted of the majority of transcription factors by passage through cellulose and heparin agarose columns also failed to potentiate Tat (data not shown). Potentiation could be due to formation of a complex between Tat and a host cell protein(s) or to covalent modification of Tat.

We deduce the existence of a rate-limiting step that involves Tat’s interaction with some factor that is not present in the basal transcription complex. The interaction results in the conversion of Tat to an activated or potentiated form. Such modification might be noncovalent; for example, Tat may undergo refolding catalyzed by chaperone proteins. Although there is no firm evidence for this possibility, it should be taken seriously in view of the abundance of cysteine residues in the molecule and the harsh treatments employed in its purification. Alternatively, Tat may be subject to posttranslational modifications,
and preincubation may be required for this process to occur. No such modification has been characterized for Tat to date, although Tat can be phosphorylated by cellular kinases in vitro (4, 9, 17). However, the functional significance of Tat phosphorylation remains undetermined. Perhaps most attractive, Tat may associate with a cellular protein in a rate-limiting fashion to generate a complex that could influence the degree of transcription activation. Recently, several cellular proteins have been described and isolated based on their interaction with Tat and their ability to confer transcriptional properties when assayed together with Tat. Preincubation might be required for the assembly of Tat with a cellular cofactor which either participates in the recognition of TAR (15) or mediates the interaction with basal transcription factors to influence the efficiency of transcription elongation (2, 3, 9) or initiation (22). Is the factor responsible for Tat potentiation in our assay equivalent to any of the factors reported in the literature to date? Based on our inability to deplete cellular extract of a potentiation factor(s) using affinity chromatography (data not shown), it probably does not bind directly or with high affinity to Tat. By this criterion, it is distinct from the cellular factor isolated by Sun˜e and Garcia-Blanco (22) and that isolated by Yu et al. (24) (TAP and p32, respectively). More directly, incubation of Tat with p32 (provided by A. Krainer) under various conditions did not give rise to transcription activation (data not shown). Furthermore, Tat was not potentiated in CHO extracts, although TAP mRNA is detected in CHO cells (24). Preliminary chromatographic data indicate that fractions enriched for TAK activity (9) are not able to potentiate Tat (data not shown), implying that the potentiation factor is distinct from TAK. The transcription elongation factor TFIIIS has been shown to act synergistically with Tat (12). Incubation of Tat in a HeLa fraction enriched for TFIIIS likewise failed to potentiate Tat (data not shown). Other possibilities include the transcription factors elongin (SII) and the product of the human ELL gene, which have been shown to elevate transcription elongation rates (3, 20). Target templates for these factors in cells have yet to be determined, and it is not known whether they associate with Tat-activated transcription complexes. Our activity also appears to be distinct from the stimulatory factor SF described by Zhou and Sharp (26) based on its chromatographic behavior (data not shown). Further work is under way to characterize this activity and its mode of action.

These experiments were begun during a sabbatical (M.B.M.) in the laboratory of the late Yosef Aloni at the Weizmann Institute. We thank I. Haviv, Y. Shaul, A. Krainer, and T. Pe’ery for providing purified and partially purified proteins. The work was supported by NIH grant AI31802 and ACS grant SG-199. M.B.M. was a Meyerhoff Visiting Professor and Fellow of the J. S. Guggenheim Foundation.

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