Activation of a Heterologous Promoter by Human Immunodeficiency Virus Type 1 Tat Requires Sp1 and Is Distinct from the Mode of Activation by Acidic Transcriptional Activators

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We have previously shown that the Tat protein of the human immunodeficiency virus type 1 (HIV-1) is a modular transcriptional activator that can be targeted upstream of either a synthetic promoter or the intact HIV promoter to activate transcription. This activation was shown to be largely dependent on the presence of consensus binding sites for the cellular transcription factor Sp1. Since the use of heterologous promoters may provide further insight into Tat-mediated transactivation, we have analyzed the transactivation of the thymidine kinase promoter of herpes simplex virus by Tat and by the acidic transcriptional transactivator VP16. The effects of mutations of defined upstream promoter elements show that Tat transactivation is dependent on Sp1 binding sites in a site-specific manner. In contrast, transactivation by the acidic transactivator VP16 is completely independent of any of the defined promoter elements upstream of the TATA box. These results suggest that Tat and the classically defined modular acidic transcriptional activators have different modes of transactivation. In addition, the substitution of the HIV-1 TATA box for the thymidine kinase TATA box substantially increases Tat transactivation, indicating that Tat transactivation may also ultimately involve TATA box-associated cellular transcription factors.

The Tat protein of the human immunodeficiency virus (HIV) is a powerful activator of viral gene expression and appears to be absolutely required for HIV replication (1, 6, 10, 35). There has been intense interest in the mechanism of Tat transactivation both because of the possibility of using anti-Tat strategies to block HIV replication and because of several unique mechanistic features of Tat transactivation that hold interest for the study of gene regulation. One of the unique mechanistic features of Tat is the use of a downstream targeting mechanism (31). Unlike conventional transcriptional activators, which are targeted upstream of a promoter via DNA-binding sites, Tat is targeted to the HIV promoter by direct binding to the RNA stem-loop structure at the 5' end of the nascent RNA transcripts initiated from the HIV promoter (7, 8, 17, 30, 41). The sole function of this RNA target, termed TAR (transactivating response region), appears to be simply to target Tat to the promoter (4, 34, 36).

It has been difficult to evaluate the mechanism of Tat transactivation on the HIV promoter, because, in addition to this unique RNA-targeting mechanism, the HIV promoter directs the synthesis of high levels of short RNA transcripts (19, 21, 39). Therefore, Tat in principle could be acting by a variety of mechanisms to increase the expression of full-length RNA transcripts. Besides simply increasing transcriptional initiation, Tat could increase the elongational efficiency of transcription or the antitermination of HIV transcripts. Evidence from nuclear run-on assays (19, 21, 22) and from in vitro transcription assays (9, 20, 27) has sometimes indicated that Tat can increase the efficiency of RNA chain elongation from the HIV promoter as opposed to increasing the rate of RNA chain initiation. These results, along with the unusual downstream targeting site of Tat, have prompted the suggestion that

Tat has a unique mechanism of transactivation that does not directly involve transcriptional initiation. The processivity model for the mechanism of Tat transactivation proposes that the HIV promoter is deficient in assembling a transcriptional complex that efficiently elongates RNA and that Tat is in reality a gene-specific processivity factor that promotes the efficient elongation of RNA from the HIV promoter (5, 9).

To test whether Tat could function as a typical transcriptional activator, we previously targeted a Gal-Tat fusion protein upstream of a synthetic promoter via Gal binding sites and were able to show that Tat could transactivate the synthetic promoter and that this activation was dependent on the presence of upstream binding sites for the cellular transcription factor Sp1 (18). Therefore, Tat has a modular domain structure that can be targeted upstream of a promoter like a typical transcriptional activator. Since the synthetic promoter contained no HIV sequences of any kind, Tat could not be acting to specifically relieve an elongational block imposed by the HIV promoter in this system. Our previous results lend support to a Tat mechanism involving stimulation of transcriptional initiation as opposed to a mechanism based solely on elongational processivity (18).

We recently extended our studies to the HIV promoter and showed that a Gal4-Tat fusion protein targeted via Gal4 DNA-binding sites could also transactivate the HIV type 1 (HIV-1) promoter (16). As with the synthetic promoter, transactivation of the HIV promoter was largely dependent on the presence of three binding sites for the cellular transcription factor Sp1, which functioned synergistically with Tat to activate the promoter; similar experiments on Gal-Tat fusion protein activation of the HIV promoter have also been reported by Southgate and Green (37).

In this study, we have extended our studies on Gal-Tat activation to a well-studied heterologous promoter, the thymi-

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FIG. 1. Map of GTKCAT promoter construct and locations of TK promoter mutations. The TK promoter sequences from nucleotides -105 to +56 of the transcriptional start site were obtained by digesting pBLCAT2 (26) with *Xba*I and *Nco*I and inserting them into the *Xba*I and *Nco*I sites of pG5BCAT (-*Pst*I), generating GTKCAT. GTKCAT has five 17-nucleotide Gal4 binding sites immediately upstream of the TK promoter sequences, followed by the CAT gene. The *Pst*I site of G5BCAT (18) had been destroyed by filling in the *Pst*I site with T4 polymerase. The underlined nucleotides of each of the four previously defined promoter elements (15) were altered as indicated by site-specific mutagenesis (Amersham) and confirmed by sequencing. Double mutant GTK promoter constructs that had all combinations of Sp1 and CAT mutations as well as one triple mutant promoter construct with both Sp1 sites as well as the CAT site altered were generated. Mutations were then transferred into GTKCAT by exchange of *Bam*HI to *Pst*I fragments. In addition, three constructs that had the promoter sequences deleted upstream of the *Eco*RI site (-79) to the *Xba*I site in the multiple cloning site were made. *Eco*RI deletions were made in wild-type GTK as well as in the GTK construct containing the mutated 3' Sp1 site and in the GTK construct containing the HIV TATA box.

dine kinase (TK) promoter of herpes simplex virus, to further analyze the role of upstream promoter elements in Tat transcriptional activation. Detailed mutational analysis of the TK promoter has previously shown that it has four major promoter elements that bind cellular transcription factors important for promoter activity (15). In addition to a TATA box, the TK promoter has two Sp1 binding sites as well as a CAT box which binds the cellular transcription factor CTF.

Figure 1 shows the TK promoter construct (GTK) and the location of the promoter elements. The GTK plasmid construct contains the five Gal4 DNA-binding sites immediately upstream of the TK promoter sequences from -105 to +56 of the transcriptional start site followed by the chloramphenicol acetyltransferase (CAT) gene. These TK promoter sequences include the 5' Sp1 site, the CAT promoter element, and the 3' Sp1 site upstream of the TATA box. The nucleotides of the Sp1 sites and the CTF site that have been defined as important for promoter activity and transcription factor binding (15) are also indicated. Site-specific mutagenesis was used to change the underlined nucleotides in each site. Each of the TK promoter elements was mutated singly and in combination to generate constructs with all possible combinations of double mutations as well as one construct with all three upstream promoter elements, the two Sp1 sites and the CAT box, inactivated. An additional construct was also generated by site-specific mutagenesis that replaced the TK TATA box region with the HIV TATA box region. In addition, another set of constructs that deleted the promoter sequences up-

stream of the *Eco*RI site was made, thus eliminating the 5' Sp1 site as well as the CAT box (Fig. 1).

Each of the TK promoter constructs was cotransfected with pGal-Tat48 which encodes the 147 N-terminal amino acids of the yeast transcriptional activator Gal4 fused to the coding sequences for the N-terminal 48 amino acids of the Tat protein. The N-terminal 147 amino acids of the Gal4 transactivator contain only the DNA-binding domain and lack the activation domain; the 48 N-terminal amino acids of Tat contain the essential transcriptional activation domain of Tat but lack the arginine-rich basic region which is required for binding to TAR RNA. HeLa cells were cotransfected with each of the TK promoter constructs and with either Gal-Tat48 or a negative control construct, Gal4, which expresses only the N-terminal 147 amino acids of Gal4. CAT extracts were prepared 48 h posttransfection.

Figure 2 shows the CAT activity of the extracts. The basal activity of the GTK promoter is substantially higher than the activity of either the synthetic promoter (18) or the HIV-1 promoter (16) under similar assay conditions. Gal-Tat48 activated CAT expression of the GTK construct approximately 13-fold over that of the Gal4 control (Fig. 2). Mutation of the CAT enhancer reduced CAT basal activity 5-fold from 5.25 to 1.16% acetylation but did not reduce Tat transactivation (13.4- versus 16.9-fold activation). Likewise, mutation of the 3' Sp1 site reduced basal CAT activity 2-fold (5.25 versus 2.6% acetylation) but did not substantially reduce Tat transactivation (11.7-fold activation). However, mutation of the 5' Sp1 site, while it did not reduce basal promoter activity (1.18% acetylation) any more severely than the CAT mutation, reduced Gal-Tat transactivation almost completely. There was less than a twofold activation (Fig. 2). Elimination of both Sp1 sites completely abolished transactivation by Gal-Tat, although basal activity is still readily detectable (0.25% acetylation) (Fig. 2), indicating that the mutated promoter is not totally inactive.

Therefore, although mutation of either the CAT box or the 3' Sp1 site can substantially reduce the basal activity of the TK promoter, the reduction in basal promoter activity in itself is not sufficient to affect transactivation by Tat. These data indicate that the role of an upstream Sp1 site is not simply to increase the basal level of promoter as a nonspecific requirement for Tat activation but to serve in a more specific role. The greater contribution of the 5' Sp1 site to Tat transactivation over the 3' Sp1 site correlates with the greater affinity of this site for Sp1 protein binding in vitro (15).

Mutation of both the CAT site and the 3' Sp1 site only reduced transactivation by one-half (6.2-fold activation) (Fig. 2), and mutation of both the CAT site and the 5' Sp1 site did not further reduce the already low activity of the promoter with the mutated 5' Sp1 site further. As expected, mutation of all three sites completely abolished Gal-Tat transactivation.

The role of Sp1 in Tat transactivation is specific beyond the general contribution of Sp1 to the basal activity of a promoter. Neither in our previous studies (16, 18) nor in the published work of others (2, 13, 37, 42) has it been clear that Sp1 can serve in a more specific role in Tat transactivation. For instance, although we have previously shown that deletion of the Sp1 sites from a defined synthetic promoter (18) or from the intact HIV-1 promoter (16) drastically reduces Tat transactivation, it was not clear whether Sp1 was required only for the activation of the basal activity of a promoter to some predetermined level. Our mutational analysis of the TK promoter shows that mutation of a specific Sp1 binding site drastically reduces Tat transactivation, while mutations of the binding sites of other transcription factors that even more drastically reduce basal activity of the promoter have no effect

FIG. 2. CAT activity of mutant TK promoter constructs. Four micrograms of each of the GTK promoter constructs was cotransfected with either 2 μ g of Gal-Tat48 (+) or the control Gal4 plasmid (-) into HeLa cells (100-mm-diameter plates) via CaPO_4 -DNA precipitation. Cell extracts were assayed for CAT activity 48 h posttransfection (12). Promoter element designations indicate the sites deleted in each GTK construct or, in the case of HIV TATA, the presence of the HIV TATA box in place of the TK TATA box.

on transactivation by Tat. The Sp1 sites of the TK promoter appear to be responsible for Tat responsiveness in a site-specific manner.

Substitution of the HIV TATA box region for the TK TATA box region reduced basal promoter activity only slightly but resulted in a substantial increase in Gal-Tat48 transactivation (Fig. 2). Under the assay conditions used (Fig. 2), transactivation by Gal-Tat resulted in nearly complete acetylation of the chloramphenicol in these CAT assays. Therefore, additional assays were performed to evaluate the relative activities of the TK TATA and the HIV TATA regions in the linear range of CAT activity (Fig. 3A). Transfection assays containing 1/10 the amount of Gal4 and Gal-Tat DNAs used in the previous assays resulted in a 14-fold activation of CAT expression with the TK TATA and a 131-fold activation (i.e., a 9-fold increase) with the HIV TATA region (Fig. 3A). Therefore, the HIV TATA box region also appears to be involved in Gal-Tat transactivation of transcription and suggests that additional cellular factors might be complexing with this region to increase Tat responsiveness. A recent report by Olsen and Rosen (28) also found that the HIV TATA box was 3- to 10-fold more responsive to Tat than were a number of other TATA box regions.

To determine whether Gal-Tat transactivation of the GTK promoter constructs results in an increase in the steady-state levels of RNA correctly initiated from the TK promoter, S1 nuclease protection assays were performed. Total cellular RNA was isolated at 48 h posttransfection from cultures transfected as for Fig. 3A and hybridized to a uniformly labeled antisense RNA probe generated from SP72TK linearized with *Xba*I. SP72TK contains GTKCAT sequences from the *Xba*I site (-105) to the *Pvu*II site (+218) cloned into the *Pvu*II and *Xba*I sites of the SP72 expression vector (Promega). SP6 RNA polymerase generates a 323-base antisense probe from SP72TK linearized with *Xba*I, and RNA correctly initiated from the TK promoter should protect a probe fragment of approximately 218 bases. RNA isolation and S1 nuclease assay conditions were performed as described previously (16). Gal-Tat transactivation of the GTK construct resulted in a substantial increase in the steady-state levels of RNA correctly initiated

from the TK promoter, as evidenced by the increase in the level of protection of the 218-base probe fragment over that of the Gal4 control. Gal-Tat transactivation of the GTK promoter construct containing the HIV TATA box region increased the level of protection of the 218-base probe fragment over that of the Gal4 control even more. The 218-base probe fragment was the major S1 nuclease digestion product in these assays; therefore, with either the HIV or TK TATA regions, RNA is correctly initiated from the promoter by Gal-Tat, and the steady-state levels of TK RNA correlate with CAT activation.

To determine whether the preferential dependency of Tat transactivation for the 5' Sp1 site was a position-dependent feature resulting from the proximity of the 5' Sp1 site to the Gal4 binding sites, additional constructs that deleted the 5' Sp1 site and CAT box and placed the Gal4 binding sites next to the 3' Sp1 site were made (Fig. 1). These constructs were made by deleting from the *Eco*RI site in the TK promoter to the *Xba*I site in the multiple cloning site immediately downstream of the Gal binding sites. The construct $\text{GTK}\Delta\text{Eco}$ deletes the sequences upstream of the *Eco*RI site in the wild-type TK promoter, 3' Sp1 ΔEco deletes the upstream *Eco*RI sequences from the GTK promoter construct containing the mutated 3' Sp1 site, and HIVTATA ΔEco deletes the upstream *Eco*RI sequences from the GTK promoter construct containing the HIV TATA box.

Each of these ΔEco TK promoter constructs was cotransfected into HeLa cells with either Gal-Tat48 or the control Gal4 plasmids, and the CAT activity was compared with the activity of the parental TK promoter constructs, GTK or HIVTATA, cotransfected with either Gal-Tat48 or Gal4 DNA. $\text{GTK}\Delta\text{Eco}$, which contains only one (3' Sp1) of the three upstream factor binding sites, was only transactivated 2.3-fold by Gal-Tat48 compared with 18.5-fold for the parental GTK plasmid (Fig. 4), indicating that positioning Gal-Tat48 next to the 3' Sp1 site does not increase Tat transactivation. As expected, the 3' Sp1 ΔEco construct, which also has a mutated 3' Sp1 site in addition to the deletion of the CAT box and 5' Sp1 site, was not transactivated by Gal-Tat48. The HIVTATA ΔEco construct was also only very weakly transac-

FIG. 3. Comparison of Gal-Tat activation of promoter constructs containing either the TK or the HIV TATA box regions and determination of steady-state RNA levels. (A) Four micrograms of either GTK or GTK(HIVTATA) CAT reporter constructs was cotransfected with either 0.2 μ g of Gal-Tat48 (+) or Gal4 (-) into HeLa cells. CAT activity was measured 48 h posttransfection. (B) HeLa cell cultures were transfected as for panel A, and 48 h posttransfection, total cellular RNA was isolated and hybridized, as previously described (16), to a radiolabeled antisense RNA probe spanning the TK promoter. S1 nuclease protection assays were all performed as previously described (16). Arrows indicate the position of the undigested probe and the position of the major S1 nuclease digestion product at 218 bases.

tivated by Gal-Tat48 compared with that of the parental construct HIVTATA (6.9-fold activation versus 141.8-fold, or less than 5%), indicating that transactivation of the HIV-1 TATA box by Tat still requires the 5' Sp1 site for substantial amounts of activity.

Our previous studies have shown that Gal-Tat transactivation of the HIV promoter containing five upstream Gal4 binding sites is dependent on the presence of the Sp1 sites; although deletion of all three of the Sp1 sites of the HIV promoter did not completely eliminate Tat transactivation (18). These results are also in agreement with previous reports that have indicated that the presence of Sp1 binding sites appears to be particularly important for high levels of Tat

transactivation of the HIV-1 long terminal repeat (2, 3, 13, 42). Our present results show that when Gal-Tat is targeted like a typical transcriptional activator upstream of the complex heterologous TK promoter, Sp1 binding sites are absolutely required for activity (Fig. 2). However, several studies have suggested that Tat can transactivate promoters lacking binding sites for Sp1. In one report it was shown that Tat could activate a number of heterologous promoters in transient expression assays when the HIV TAR sequences were placed downstream of the promoters (30). Several of these promoters lacked binding sites for Sp1. In another recent report, Gal-Tat targeted upstream of the HIV core promoter could transactivate the promoter in the absence of Sp1 binding sites, although to only a very low level (37). In that same report it was also shown that native Tat could transactivate the HIV core promoter containing only the TATA box region and TAR sequences if a variety of different chimeric Gal4 transactivators were targeted upstream of the promoter, although none of the other chimeric transactivators had nearly the stimulatory effect of Gal-Sp1 (37).

Both of the above reports used promoter constructs containing HIV promoter sequences. A number of studies using nuclear run-on assays (19, 21, 22) and in vitro transcription assays (9, 20, 27) have indicated that Tat transactivation can increase the efficiency of transcriptional elongation from the HIV promoter as well as increasing the rates of transcriptional initiation. These results have prompted the suggestion that unknown elements of the HIV promoter render the promoter incapable of efficiently elongating RNA and that Tat functions to alleviate this lesion. In our studies, neither the synthetic promoter nor the TK promoter contains HIV sequences, and Sp1 sites are shown to be required. Therefore, one possible explanation of these results is that Tat has two independent functions. First, Tat can function as a modular transcriptional activator in cooperation with the cellular transcription factor Sp1 to increase the rate of transcriptional initiation from a defined promoter. Second, Tat can also act as a processivity factor when assayed on the HIV promoter or with constructs containing HIV promoter sequences. However, the possibility that intrinsic rates of transcriptional elongation from a variety of promoters can be altered by transcriptional activators to a degree that is not yet fully appreciated also exists. This view is compatible with the observation that the *Drosophila* hsp70 promoter displays a block to efficient transcriptional elongation that can be overcome under some assay conditions (32). Tat may ultimately prove to have a processivity function shared by a number of other transcriptional activators which can increase transcriptional elongation from a variety of promoters.

The best-studied modular transcriptional activators are the acidic activators. Preliminary results indicate that acidic activators function to increase the rate of initiation of transcription by either recruiting TFIIB to the preinitiation complex (24) or promoting TFIIA function (40). A distinguishing feature of these transactivators is their ability to strongly activate transcription when targeted just upstream of a minimal promoter containing only a TATA box (23). This is consistent with the fact that acidic activators like VP16 can directly bind to TFIIB (25) and TFIID (14) and activate transcription, probably in concert with protein cofactors associated with TFIID that are necessary for activation but not basal transcription (11). However, these transactivators can also activate transcription synergistically in conjunction with other promoter elements when they are located at longer distances from promoters (23).

Since we have shown that Tat can function upstream of a

FIG. 4. Gal-Tat activation of the ΔEco TK promoter constructs. The GTK promoter constructs containing the *Eco*RI deletions [GTKΔEco, GTK(Δ3'-Sp1)ΔEco, and GTK(HIVTATA)ΔEco] and the parental promoter constructs GTK and GTK(HIVTATA) were cotransfected with 2 μg of either Gal-Tat48 (+) or Gal4 (-) as for Fig. 2.

minimal TATA box promoter supplemented with Sp1 binding sites as well as upstream of a heterologous promoter containing SP1 binding sites, these results show that Tat can activate transcription independently of any HIV-specific sequences. To determine whether acidic transcriptional transactivators typified by the herpes simplex virus VP16 transactivator have a similar requirement for upstream promoter elements in the TK promoter, we cotransfected each of the TK promoter constructs with a Gal-VP16 expression vector or the Gal4 control. The Gal-VP16 transactivator has the N-terminal 147

amino acids of Gal4 linked to the C-terminal activation domain of VP16 (33).

The level of transactivation by Gal-VP16 was more than an order of magnitude greater than that with Gal-Tat on the TK promoter. Mutation of each of the three upstream promoter elements of the TK promoter had no significant effect on transactivation by Gal-VP16 (Fig. 5). Even double promoter mutants showed no reduction in promoter activity. Elimination of all three upstream sites still had no effect on transactivation by Gal-VP16. Transactivation by the acidic transactivator

FIG. 5. Gal-VP16 activation of the TK promoter constructs. Each of the GTK promoter constructs was cotransfected with either 0.2 μg of Gal-VP16 or the Gal4 control DNA as for Fig. 2.

Gal-VP16 is completely independent of any of the defined promoter elements upstream of the TATA box. In addition, Gal-VP16 appeared to be equally active on TK promoter containing the TK TATA box or the TATA box region from the HIV-1 promoter.

Unlike Gal-VP16, Gal-Tat cannot activate the TK promoter if Sp1 sites are absent. It seems unlikely that the different requirements for upstream promoter sites between Tat and VP16 can be attributed to positioning effects or to the fact that VP16 is simply a stronger transactivator than Tat. Substitution of the HIV-1 TATA box region for the TK TATA box increased Gal-Tat transactivation seven- to ninefold (Fig. 3A); however, this did not reduce the dependency of Gal-Tat transactivation on the 5' Sp1 site. Deleting the 5' Sp1 and CAT sites and repositioning the Gal4 binding site next to the 3' Sp1 site reduced Tat transactivation to below 5% of the control level (Fig. 4). This is very similar to the effect of mutating the 5' Sp1 and CAT sites in the promoter containing the TK TATA box (Fig. 2). In other experiments, with five Gal4 binding sites upstream of the HIV-1 long terminal repeat, Gal-Sp1 strongly synergized with Tat, while Gal-VP16 showed only an apparent additive effect with Tat (16). This result also suggests that Tat and Gal-Sp1 might be acting on the same pathway or rate-limiting reaction, whereas Tat and Gal-VP16 might be acting independently.

Two previous reports (37, 38) have suggested that Tat and VP16 act on the same step in transcriptional activation and that Tat and VP16 might work via the same mechanism (38). These proposals are based on the observations that both Tat and VP16 can be targeted upstream as well as downstream of a promoter to activate transcription and that under presumably saturating levels of expression of VP16, Tat cannot augment transactivation by VP16. Our results show that Gal-Tat and Gal-VP16 have strikingly different requirements for upstream promoter elements regardless of the basal activity of the promoter or the levels of Tat transactivation. These results suggest that the mode or pathways of transactivation by Tat and acidic transactivators as typified by VP16 are probably distinct.

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