Identification of Limiting Steps for Efficient Trans-activation of HIV-1 Promoter by Tat in *Saccharomyces cerevisiae* *

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Cellular context is an important determinant for the activity of Tat, the trans-activator of human immunodeficiency virus (HIV). We have investigated HIV-1 promoter expression and trans-activation in *Saccharomyces cerevisiae* to provide clues about the limiting steps for Tat activity in this organism. A minimal 43-nucleotide HIV promoter (HIV43) has the activity of a weak yeast promoter in the presence or absence of various enhancer binding sites (bs), whereas the entire long terminal repeat is not expressed. None of these constructs could be trans-activated by Tat. Fusion proteins Gal4 binding domain (BD)-Tat48 and Gal4BD-Tat72 are active with different efficiencies on various yeast promoters that have Gal4 bs. They have 70 and 50% of Gal4 wild type activity on hybrid HIV promoters fused to Gal4 bs only in the presence of AP1 bs. This study shows that trans-activation of the HIV-1 promoter by Tat occurs in yeast when Tat is targeted to the promoter and a functional enhancer activity is present. Sp1 function and Tat trans-activate from the RNA to the promoter and to the major elements for in vivo trans-activation of HIV-1 that are defective in *S. cerevisiae* but can be replaced by functional equivalents.

Transcriptional activation is a conserved mechanism in eukaryotes from yeast to mammalian cells. Common features include the requirement for general transcription factors (GTFs),1 RNA polymerase, transcriptional activators, and co-activators (1–3). In each organism, these proteins support specific activity due to their unique structure and to their interactions with other factors (4–6). Among the GTFs, the TATA box-binding protein (TBP), first identified as a component of transcription factor TFIID, plays a central role in the assembly of the transcription complex by binding to the TATA sequence (7). This complex is then modulated by its direct and indirect interactions with activators and repressors. Typical activators have separate DNA binding and activation domains. Binding specificity is directed by the DNA sequence that positions the activation domain in the proximity of the transcription complex. Activators exert their activity by chromatin structure disruption, direct interaction with GTFs, or contacts with TBP-associated factors (TAFs) (3, 8, 9). Activation domains can be divided into classes with respect to their amino acid sequence and their functional conservation from yeast to human. Acidic activators including the AP1 family, the *Saccharomyces cerevisiae* Gal4, and the herpes simplex virus VP16 have a high content of aspartate and glutamate residues and function in either lower or higher eukaryotes (10–12). The proline-rich activators such as AP-2 and CTF/NF1 can stimulate transcription in yeast only from a TATA-proximal position (11, 12). Sp1 and octamer activators contain glutamine-rich domains. They stimulate transcription from a proximal position in the yeast *Schizosaccharomyces pombe* and higher eukaryotes, but they do not support activation in *S. cerevisiae* even if the yeast TBP is replaced by human TBP, indicating a non-conserved mechanism between species for this activator family (11–13).

The HIV-1 Tat protein is essential for trans-activation of the HIV-1 LTR promoter (14–17). Unlike conventional activators of transcription, Tat interacts with a cis-acting RNA sequence (TAR) located from +19 to +42 downstream of the transcription start site (18, 19). The TAR RNA can fold into a phylogenetically conserved, stem-bulge-loop structure, which is essential for a correct Tat-TAR interaction and for an optimal activated expression of the LTR (19–22). TAR may primarily act as a tether to bring Tat to the transcriptional apparatus as the Tat protein activates the LTR even when it is directed to the promoter in a TAR-independent manner (23–28). Tat increases dramatically the level of mRNA transcribed from the HIV-1 LTR by promoting the formation of an efficient transcriptional initiation complex (29, 30) at a step occurring after the binding of TBP to the TAR box (31). Tat recruits a kinase that will phosphorylate the carboxyl-terminal domain of the RNA polymerase II. The transcription complex formed with Tat and the phosphorylated polymerase becomes more stable, starts efficient elongation, and promotes promoter clearance followed by rapid reinitiation (32–37). The linkage between mRNA transcription and processing, as well as the characterization of Tat as a component of the preinitiation and the elongation complexes, support this mechanism (38–40).

Several studies have shown that cellular factors are necessary components of the trans-activation process (17). In *vivo* studies have shown that Tat functions only in mammalian cells and has a weak activity in rodent cells. These cells lack a cellular cofactor that allows a correct Tat-TAR interaction in *vivo* (41, 42), and a newly identified protein, cyclin T, has the properties of this missing co-factor (43).
HIV-1 LTR by Tat has been detected in insect and yeast cells, but the defective steps have not been determined in these organisms (44, 45). Promoter-enhancer elements constitute critical sites required to obtain the Tat effect. In mammalian cells, mutational analyses of the HIV-1 LTR demonstrate that Tat requires the presence of DNA motifs recognized by the cellular transcription factor Sp1 (24, 26–48). Overexpression of TBP (49) and transcription factors such as API, CTF, USF, and ATF can replace Sp1 in Tat responsiveness whereas VP16, E1a, Oct1, or NF-κB confer only a weak response (26, 47, 50, 51). These functional requirements correlate with physical interactions of Tat with Sp1, TBP, and RNA polymerase II (49, 52–54).

The HIV-1 LTR promoter has a weak basal activity in the fission yeast S. pombe. However, unlike in mammalian cells, the expression of Tat in this system did not lead to an increase of HIV-1 promoter activity (45). Protein fusion experiments performed in S. cerevisiae have shown that the activation region of Tat (amino acids 1–48) activates transcription from the yeast GAL1 promoter when bound to DNA upstream of the transcription start site. However, a Tat protein with its RNA binding domain does not activate GAL1 promoter (55).

Because of the similarities and differences between yeast and mammalian cells at the transcriptional level, we anticipated that the well defined yeast genetic system may be useful to dissect the mechanism of Tat-mediated trans-activation. In this study, we have investigated the HIV-1 promoter expression and trans-activation in S. cerevisiae. We made several promoter changes to analyze the potential roles of upstream elements in basal and Tat-mediated trans-activation. We found that a minimal HIV promoter (HIV43) in the presence or absence of various enhancer binding motifs has a basal activity comparable to the weak yeast promoter CYC1. In contrast, the complete HIV-1 LTR is silent and none of these constructs could be trans-activated by Tat. By using Gal4BD-Tat fusion proteins, we demonstrate here that they specifically trans-activate heterologous promoters GAL1 and CYC1. We show that GalBD-Tat also trans-activates the HIV-1 promoter if it combines Gal4 and API DNA-binding sites indicating that two limiting steps for trans-activation in yeast are enhancer elements and Tat transfer from TAR to the promoter.

MATERIALS AND METHODS

Plasmids and Yeast—The yeast indicator strains HF7c, SFY528 and OL1 have been described previously (56–58), W303-1B (Mata, ura3-3, leu2-3, 112, his3-11, 15, ade2-101, trp1-1, can1) was obtained from M. Minet (59).

The yeast shuttle vector pLEV1-Tat (Fig. 1A) is derived from pUT332 (60) in which the phleo gene was replaced by Tat from pCMV1-Tat (61). The integrative, Tat-encoding plasmid pLEV2-Tat was generated by cloning a Xhol-HindIII restriction fragment (containing the TEF1 promoter, Tat coding sequence, and the CYC1 terminator) from pLEV1-Tat into pFL3S (62).

p415-CAT is derived from pRS 415 (63) in which the CAT gene and the SV40 poly(A) from pLTRCAT (47) were subcloned between the unique HindIII and BamHI sites (Fig. 1A). p415-TEF1-CAT and p415-CYC1-CAT were obtained by inserting the TEF1 and CYC1 promoters (from pUT332 and pUT330) respectively, between the HindIII and Xhol sites of p415-CAT (Fig. 1). p415-LTRCAT was constructed by cloning the wild type HIV-1 LTR between the HindIII and Clal sites of p415-CAT. In a similar manner, a deleted version of the LTR containing sequences from position +43 to position +78 (47) was inserted into p415-CAT to generate p415-HIV43-CAT (Fig. 1). For the addition of the heterologous enhancers, synthetic oligonucleotides (Fig. 2A) were ligated into the Sall site of p415-HIV43-CAT. For the addition of the six consensus Gal4 bs (5′-CGAGGACTGTCCCTCGG-3′), oligonucleotides were cloned between the XhoI and ApaI sites of p415-HIV43-CAT, p415-HIV43(AP1)4-CAT, and p415-HIV43(SP1)6-CAT.

The integrative plasmid p26-Phleo (Fig. 1A) was constructed by cloning a restriction fragment containing the Tds ble/phleo gene (which confers resistance to bleomycin and phleomycin) and the CYC1 terminator from pUT332 into the unique HindIII site of pFL26 (62). The TEF1 and CYC1 promoters (from pUT332 and pUT330) were then inserted between the XbaI and HindIII sites of p26-Phleo to generate p26-TEF1-Phleo and p26-CYC1-Phleo, respectively. p26-HIV43-Phleo, p26-HIV43(AP1)4-Phleo, and p26-HIV43(SP1)6-Phleo were constructed by subcloning the relevant promoters from their p415 homologues (described above) between the XbaI and HindIII sites of p26-Phleo.

To generate the plasmids expressing the Gal4-Tat derivatives (pGB9TL-Tat48 and pGB9TL-Tat72), a long version of the ADH promoter was first reconstituted in pGB9 (64) by ligating a Spal restriction fragment of the long ADH promoter of pGB9 (CLONTECH) into the Spal site at the 5′-end of the short ADH promoter of pGB9TL. The resulting plasmid was named pGB9TL. The coding sequences of Tat48 and Tat72 (encoding amino acids 1–48 and 1–72, respectively), were generated by PCR from pCMV1-Tat (61), and cloned into the EcoRI and BamHI sites of pGB9TL to obtain pGB9TL-Tat48 and pGB9TL-Tat72. Plasmids pGB9TL-Gal4 and pGB9TL-Gal4, expressing the native Gal4 trans-activator, were constructed by ligating a Xhol-SmaI restriction fragment encoding the entire Gal4 cDNA from plasmid pCL1 (65) to the XhoI-SmaI-digested vectors pGB9TL and pGB9T, respectively. In each assay pGB9TL-Gal4 and pGB9TL-Gal4 gave similar results, indicating that the short version of ADH promoter allows a maximum activity for Gal4. As data with pGB9TL-Gal4 were inconsistent probably due to the frequent loss of the plasmid, pGB9TL-Gal4 was chosen in each case.

The p414/GAL1-TAR-CAT vector was obtained by inserting the PCR-generated GAL1 promoter from pYEU3 (CLONTECH) in BglII–XhoI digested p15/HIV43-CAT and subcloning the resulting GAL1-TAR-CAT expression cassette into pKPY1-BamHI sites of pRS414 (Stratagene). To generate p414/GAL1-RE-CAT, the TAR element of p415/HIV43-CAT was replaced by a partial Rev-responsive element (SL1B; Ref. 66) by ligating hybridized oligonucleotides (sense: 5′-GATCTATGGGGCCGCGGTCATTACGTACGAGGTTG-3′; anti-sense: 5′-AGGCTTATAACCGGCTTAAAGCAGGCGACCTGAGC-3′) into the BglII and SacI sites in TAR. The expression cassette was then transferred to pRS414 as described previously. The plasmids pAD-Gal4/Tat66 and pAD-Gal4/Rev expressing the Gal4 activation domain (AD) fused to Tat66 and Rev were generated by subcloning PCR-amplified Tat66 (61) and Rev (67) coding sequences respectively, between the EcoRI and XhoI sites of pAD-Gal4 (Stratagene). To construct the control plasmids expressing Tat66 and Rev without fused activation domain, we inserted the PCR-generated Tat66 and Rev coding sequences into the HindIII-digested Gal4AD. Every new plasmid was verified by DNA sequencing.

Yeast Transformation—Yeast strains were transformed either by electroporation (68) or by the modified LiAc method (69). Electroporation was performed with a Jouan apparatus as described (68) using 10 ng of plasmid. The electric field was applied at 3.3 kV/cm for 10 ms using 2-mm spaced electrodes. In these conditions we obtained an average of 104 recombinants in each transformation. Integrative plasmids pLEV1-Tat and p26-Phleo were first linearized by subcloning their respective LEU2 (in BarII restriction site) and URA3 (in BarX) genes before sequential transformations. Plasmid integration was verified by PCR using 1 µg of genomic DNA extracted from three independent clones. For double transformations, 2 or 20 ng of each vector (in the LiAc and the electroporation methods, respectively) were simultaneously added to the transformation mixture. The transformants were then plated on the appropriate yeast drop-out media and grown for 3–4 days at 30 °C.

Immunoblotting—OL1 cells transformed with pLEV1-Tat or pLEV1 (control) were grown in liquid selective media lacking uracil to an A∞500 of 0.5–1.0 mg/ml cells were then washed twice in phosphate-buffered saline, resuspended in 200 µl of TE buffer, and lysed by four cycles of freeze-thawing with a vortexing step in the presence of glass-beads between each cycle. Cell lysate proteins were resolved by gel electrophoresis (15% SDS-polyacrylamide gel electrophoresis). Two identical gels were run; one was stained with Coomassie Blue, and the second one was transferred to a polyvinylidene difluoride membrane and incubated with a rat polyclonal anti-Tat serum. Western blots were developed with goat anti-rat antibodies conjugated to alkaline phosphatase and a chromogenic substrate as indicated by the manufacturer (Tropics).

Chloramphenicol Acetyltransferase Assay (CAT)—CAT assays were performed with transformed yeast cells grown in liquid selective media to an A∞500 of 0.5 ± 0.01. The cells recovered from 5 ml of culture were washed once in phosphate-buffered saline, resuspended in 200 µl of 0.25 M Tris, pH 7.5, and then lysed by four cycles of freeze-thawing with
a vortexing step in the presence of acid-washed glass beads between each cycle. The cell extracts were cleared by centrifugation and incubated at 65 °C for 5 min (70). Protein concentrations of the extracts were measured using the Bio-Rad protein assay kit and verified to be less than 5% different. The assays were then performed as described previously (61) using 5 ml of cellular extract in a 30-min incubation at 37 °C. CAT activities were quantified in the linear range of acetylation using densitometric computer analysis in a series 400 PhosphorImager by Molecular Dynamics SA.

**RESULTS**

**Replicative and Integrated Forms of the HIV-1 LTR Support Basal Expression in S. cerevisiae**—We initially analyzed the transcriptional activity of the HIV-1 LTR promoter in S. cerevisiae. We first transformed OL1 cells with p415-LTR-CAT. The results in Fig. 2B indicate that no significant CAT activity was detected when compared with a promoter-less construct. We next analyzed a deleted version of the LTR encompassing the sequence from -43 to +80 (HIV43) that contains the HIV-1 LTR sequences from position -43 to position +78 so as to contain the TATA box and the TAR element, which immediately precedes the CAT or Phleor bacterial genes. Oligonucleotides comprising DNA-binding sites for a series of transcription factors (termed enhancers) were subcloned immediately upstream of the TATA box in the HIV43. In a similar manner to the HIV-1 promoter constructs, the CYC1 and TEF1 yeast promoters were linked to the CAT and Phleor reporter genes.
**Type of plasmids** (i.e. integrative versus replicative) and the reporter gene were modified. The HIV43, HIV43(AP1)4, HIV43(SP1)6, CYC1, and TEF1 promoters were linked to the integrative p26-Phleo plasmid carrying the phleo gene and transformed into W303-1B strain. We monitored genomic integration of the various p26-Phleo derivatives by PCR (Fig. 3), and we measured promoter activities by determining the minimal inhibitory concentrations of each stable transformants (Table I). The results were in agreement with those obtained previously. Transformants containing the HIV43 and HIV43(AP1)4 showed the same level of phleomycin resistance (2.5 \( \mu \text{g/ml} \)), whereas HIV43(SP1)6 conferred a weaker resistance (1 \( \mu \text{g/ml} \)). The p26-CYC1-Phleo transformants were found to be slightly more resistant to phleomycin (5 \( \mu \text{g/ml} \)) than those carrying the HIV43 derivatives, whereas the strong TEF-1 promoter conferred the highest resistance (500 \( \mu \text{g/ml} \)). These results indicate that a 43-nucleotide HIV-1 promoter is an active weak promoter in yeast and suggest that it could be trans-activated to a higher level.

**Wild Type Tat Does Not Trans-activate HIV-1 Promoter in S. cerevisiae**—The HIV-1 Tat protein binds the TAR RNA element and activates transcription from the HIV-1 LTR. Tat-mediated trans-activation occurs by increasing the rate of transcriptional initiation, facilitating promoter clearance, and subsequently by facilitating polymerase processivity. We have tested the possibility that Tat may trans-activate the HIV-1 promoter in S. cerevisiae. We first verified Tat expression from pLEV1-Tat and pLEV2-Tat by Western blots. Fig. 4A shows that a polyclonal anti-Tat serum was able to recognize Tat protein in OL1 cells transformed with the replicative plasmid pLEV1-Tat but not in those transformed with pLEV1 alone. Similar results were obtained with strain W303-1B transformed with the integrative plasmid pLEV2-Tat (data not shown).

The yeast strain OL1 was then cotransformed with pLEV1-Tat and the various HIV-1 constructs described above (p415-CAT derivatives). We observed no significant difference in CAT activities between the Tat expressing and the non-expressing strains (Fig. 4B). In a similar manner, W303-1B cells were cotransformed with pLEV2-Tat and the p26-Phleo derivatives. In good agreement with the previous results, Tat expression does not cause any increase in phleomycin resistance (Table I). These data indicate that these enhancer-HIV43 constructs might bring a necessary element to the Tat mechanism but are not sufficient to restore trans-activation.

**Tat48 and, to a Lesser Extent, Tat72 Trans-activate GAL1 and CYC1 Promoters through Gal4 Binding Sites**—On the basis of the above results, the absence of Tat-mediated trans-activation could be related either to the inability of the activation domain of Tat to function in S. cerevisiae or to its incorrect tethering to the TAR RNA. A previous report indicates that the Tat activating region (amino acids 1–48) fused to the DNA binding domain of the yeast transcription factor Gal4 can significantly activate the GAL1 promoter of S. cerevisiae. However, longer versions of Tat hybrids (amino acids 1–58 or 1–67), although comprising an intact activation domain, failed to trans-activate the GAL1 promoter (55). To further extend these results, the yeast strain SFY526 containing the E. coli lacZ reporter gene fused to the GAL1 promoter was transformed with pGBT9L (Gal4BD), pGBT9-Gal4 (Gal4 Wt), pGBT9L-Tat48 (Gal4BD-Tat48), or pGBT9L-Tat72 (Gal4BD-Tat72).
Extracted from three independent clones was amplified by PCR using insert-specific primers. Lane 1: p26-Phleo (promoterless construct); lanes 2 to 7: p26-CYC1-Phleo; lanes 8–10, p26-TEF1-Phleo; lanes 11–13, p26-HIV43(AP1)4-Phleo; lanes 14–16, p26-HIV43(SP1)6-Phleo.

![Fig. 3. Effective genomic integration of the p26-Phleo constructs following transformation of W303-1B cells.](image)

| Promoters | MIC (µg/ml) |
|-----------|-------------|
| 0         | 0.25        |
| CYC1      | 5           |
| TEF1      | >500        |
| HIV43     | 2.5         |
| HIV43 + Tat | 2.5     |
| HIV43(API1)4 | 2.5     |
| HIV43(API1)4 + Tat | 2.5     |
| HIV43(SPI)6 | 1        |
| HIV43(SPI)6 + Tat | 1        |

To determine if the Gal4-Tat hybrids may trans-activate other yeast promoters, the above described HF7c transformants were used in a β-galactosidase assay. Indeed, the HF7c strain also carries a lacZ reporter gene under the control of a yeast CYC1 promoter fused to three Gal4 DNA bs (58). The results, reported in Fig. 5B, demonstrate that the native Gal4 and the Gal4BD-Tat48 constructs efficiently trans-activate the synthetic CYC1 promoter as already observed for the GAL1 promoter. In this third assay, the activity of Gal4BD-Tat72 activity is below the detection level. The overall results in Fig. 5 show that Gal4BD-Tat72 has a low transcriptional activation potential on the GAL1 and CYC1 promoters in S. cerevisiae, whereas Gal4BD-Tat48 can trans-activate 150 times better than its counterpart with an RNA binding domain, and the native Gal4 has maximum activity. The combination of these three assays allows the detection and the quantitation of a low activity of Gal4BD-Tat72 that was not observed in previous studies (55).

**Tat48 and Tat72 Trans-activate HIV-1 Promoter When They Are Targeted to the DNA and in the Presence of AP1 Binding Sites**—The trans-activation observed in Fig. 5 occurs on heterologous promoters. To investigate whether we can obtain similar results from the HIV promoter, we analyzed the ability of the different Gal4BD-Tat constructs to trans-activate the HIV-1 promoter when artificially targeted to the DNA. Similar constructs have been used to study promoter requirements for trans-activation in human cells and reflect the overall Tat activity (24, 26, 27). We fused six consensus Gal4 binding sites to HIV43, HIV43(AP1)4, and HIV43(SPI)6 (Fig. 6A) and co-transformed these CAT reporter plasmids with the Gal4BD-Tat hybrids in SFY526 cells. As shown in Fig. 6B, we observed that Gal4BD-Tat48 and Gal4BD-Tat72 have 70 and 50% of Gal4 wild type activity only in the presence of AP1 bs and not in the presence of SPI bs or a minimal HIV43 promoter. In contrast, the native Gal4 protein is active on all the promoters tested, including the minimal HIV43, with a reduced activity on G6-HIV43(SPI1)6, proportional to the decrease of the basal level. To further demonstrate the requirement of functional AP1 binding sites, we introduced inactivating point mutations in the AP1 sequence (see AP1m in Fig. 2A). As shown in Fig. 6B and C, these mutations completely eliminated Gal4BD-Tat responsiveness, indicating a strict requirement for these sites in trans-activation.
brane (S. cerevisiae). Extracts were then assayed for CAT activity. The values shown are promoter constructs together with pLEV1-Tat or pLEV1 (control). OL1 absence of Tat. OL1 cells were transformed with different HIV-1 LTR conjugated to alkaline phosphatase and a chemiluminescent substrate. Serum. Western blots were developed with goat anti-rat antibodies stained (sates were resolved by gel electrophoresis and then Coomassie Blue- pLEV1-Tat (OL1) or pLEV1 (OL1) were grown in liquid selective media and lysed by three freeze-thawing cycles. The cell lysates were resolved by gel electrophoresis and then Coomassie Blue-stained (left panel) or transferred to a polyvinylidene difluoride membrane (right panel) that was incubated with a rat polyclonal anti-Tat serum. Western blots were developed with goat anti-rat antibodies conjugated to alkaline phosphatase and a chemiluminescent substrate. B, transcriptional activity of the HIV-1 promoters in the presence or absence of Tat. OL1 cells were transformed with different HIV-1 LTR promoter constructs together with pLEV1-Tat or pLEV1 (control). OL1 extracts were then assayed for CAT activity. The values shown are percent conversion of [14C]chloramphenicol product normalized to a 50% activity for the yeast CYC1 promoter (mean values ± S.E.; n = 3).

The Yeast S. cerevisiae Lacks Factors Mediating an Activator Transfer from an RNA Target to the Preinitiation Complex— The above results (Figs. 4–6) showed that AL1 and HIV-1 promoters can only be activated by Tat when the activator is targeted to a DNA sequence and not to an RNA sequence. To determine whether the limiting step was the Tat-TAR interaction or the transfer from the RNA to the promoter, we set up a functional assay based on a known RNA-protein interaction. Rev-RRE interaction is fully functional in yeast (73) and can mediate trans-activation in mammalian cells when fused to VP16 activator (66). As Gal4 is a powerful activator in yeast, we fused its activation domain to Rev or Tat and assayed the activity of the proteins from their respective targets (Fig. 7A). As shown in Fig. 7B, neither Gal4AD-Rev nor Gal4AD-Tat was able to activate GAL1 promoter whereas wild type Gal4 did. We obtained similar results with the G6-HIV43 promoter (data not shown), indicating that one missing step in S. cerevisiae is the ability to transfer an activator from an RNA target to the preinitiation complex.

DISCUSSION

Promoter activities are modulated by activators and repressors. HIV-1 LTR is a weak promoter that can be activated by upstream enhancers and the trans-activator Tat. Tat and Sp1 act in synergy to enhance the LTR function (74). To reproduce trans-activation of HIV-1 LTR in S. cerevisiae, it is first necessary to start with a basal level similar to a weak yeast promoter. In a previous study in the yeast S. pombe, the HIV-1 LTR showed no activity except for a −117 promoter (45). Similarly, in S. cerevisiae, the entire LTR has no activity, indicating that elements upstream the NF-κB sites have inhibitory effects (Fig. 2B). In contrast, a 65-nucleotide HIV promoter has no detectable activity in S. pombe (45), but an HIV43 promoter has an activity in the same range of order than the CYC1 promoter in S. cerevisiae (Fig. 2B and Table I). This basal level is conserved by the addition of enhancer elements but is reproducibly decreased in the presence of six Sp1 bs (Fig. 2B and Table I). The addition of the human Sp1 transcription factor did not restore or increase the promoter activity (data not shown). These data correlate with the absence of Sp1 function in yeast cells and suggest that a negative factor may recognize the Sp1 bs (12, 13). In each other case, the transcriptional activity of the HIV-1 promoter is between one half and one third of the weak CYC1 promoter and much weaker than the strong TEF1 yeast promoter (Fig. 2 and Table I). These comparisons indicate that the HIV43 is a weak promoter in S. cerevisiae comparable to CYC1 and suggest that it could be trans-activated to a higher level if we can find the appropriate conditions for Tat function. To find these conditions, it was necessary to investigate each possible missing function and to find a counterpart to overcome it.

Tat functions poorly in rodent cells and not at all in insect cells or in the yeast S. pombe; therefore, we expected that it would not function in S. cerevisiae (44, 45, 75, 76). To verify this hypothesis, we expressed wild type Tat on yeast vectors (Fig. 4A) and cotransfected them in S. cerevisiae with the previous HIV43 promoter constructs. Neither the entire LTR nor a minimal promoter could be trans-activated by Tat (Fig. 4B), which correlates with the data in S. pombe. We obtained similar results in different yeast strains, with replicative and integrative expression vectors and with various reporter genes (Fig. 4 and Table I). This absence of activity indicates that S. cerevisiae lacks some factors or some mechanisms that support Tat trans-activation of the HIV-1 promoter. We then elucidated which steps are defective by complementing the function that they support.

Acidic activators function in S. cerevisiae but not the glutamine-rich activators like Sp1 (12, 13). As Sp1 is required for Tat to activate the HIV-1 LTR in mammalian cells (24, 26, 47, 74), it is possible that Tat does not function in yeast because of the absence or the inactivity of Sp1. We cotransfected an HIV43(Sp1) with Tat and Sp1 expression vectors and verified that Sp1 does not induce Tat responsiveness in this system (data not shown). We then investigated if various enhancers would compensate for this lack of Sp1 as shown previously in mammalian cells (26, 47, 51). We did not detect any trans-activation by Tat with the different HIV promoter constructs (Fig. 4 and Table I), indicating that either Sp1 is not the defective step or, more likely, that another factor(s) is missing in addition to Sp1. We anticipated that another defective step could be at the Tat-TAR interaction level.

Despite its acidic NH₂ terminus, Tat has not been classified as an acidic activator and its minimal activation domain re-
quires three functional regions included in amino acids 1–48 (27, 77). Nevertheless, the Tat activation domain is functional in S. cerevisiae on an heterologous promoter when it is targeted to the DNA, indicating a similarity in function with other activators (55). We first reproduced and quantified this assay to determine the conditions for Tat function (Fig. 5B). Whereas the activity of Tat72 was low, Tat48 induced a 150-fold increase in β-galactosidase expression from the GAL1 promoter and the wild type Gal4 protein was 60-fold more effective than Tat48, probably because of optimal cellular settings for this yeast protein. By using the histidine selection (Fig. 5C), we confirmed the activity of Tat72 in this highly sensitive selection system and a Tat48 activity reaching the same level than wild type Gal4. By using a CYC1-derived promoter, we demonstrate that Tat48 is active on two heterologous promoters when targeted to the DNA but here Tat72 activity is below the detection level (Fig. 5D). These data have revealed a trans-activation potential in S. cerevisiae for Tat72 that was not detected in another study, probably because they used a less sensitive and non-quantitative assay (55). The difference in activity between Tat48 and Tat72 is probably due to a competition between the RNA binding domain present in Tat72 and the Gal4 DNA binding domain. Indeed, although the TAR RNA is not present in these systems, it is likely that the RNA binding domain in Gal4BD-Tat72 binds cellular RNA targets and therefore competes for the GAL1 binding. As there are four binding sites in

![Diagram A](image1.png)

**Fig. 5.** Gal4BD-Tat48 and, to a lesser extent, Gal4BD-Tat72 trans-activates yeast GAL1 and CYC1 promoters from upstream DNA bs. A, schematic representation of the Gal4 derivatives used in the transfection studies. B, the reporter strain SFY526 expressing the indicated hybrid proteins were replica-plated on filters and then tested for β-galactosidase activity. The blue staining indicates an efficient trans-activation of the GAL1 promoter (each patch represents an independent transformant). The levels of β-galactosidase expression, shown on the left, were quantified from three independent transformants using the CPRG substrate (mean ± S.E.). C, HF7c transformants were analyzed for histidine auxotrophy. Growth in the absence of histidine indicates an efficient trans-activation of the GAL1 promoter. D, the same HF7c transformants were also analyzed for the production of β-galactosidase as this strain also carries a lacZ gene under the control of a minimal CYC1 promoter fused to three consensus Gal4-binding sites.
the natural GAL1 promoter and three in Gal-CYC1, this hypothesis is compatible with our results. We therefore assumed that an increase in the number of Gal4 bs should improve the DNA affinity compared with the RNA affinity as seen in other contexts (27, 78).

In the assays shown in Fig. 5, Tat is active in the absence of Sp1 bs. Similarly, it has been suggested that Tat can act independently from SP1 on heterologous promoters. In each case, an alternative activator whose binding site is present on the promoter could be used to act in concert with Tat (79). In the case of GAL1 and CYC1 promoter, Tat may also cooperate with a yeast DNA binding protein present on the promoter.

Tat-mediated trans-activation of the HIV-1 promoter in yeast has only been studied in S. pombe. In this case, only an HIV117 promoter (with NF-κB and SP1 bs) has a basal level of expression, and no HIV constructs support trans-activation by Tat (45). Based on previous studies and the above results, we investigated which conditions might be required for trans-activation of the HIV-1 promoter in S. cerevisiae. 1) A minimal HIV43 has the activity of a weak promoter but cannot be trans-activated by Tat (Figs. 2 and 4 and Table I). 2) Tat functions in S. cerevisiae when tethered to a DNA binding domain (Ref. 55 and Fig. 5). 3) Sp1 is required for trans-activation in mammalian cells but can be replaced by other enhancer elements, including AP1 (47). 4) Sp1 does not function in yeast, but AP1 binding sites are recognized by GCN4 and YAP1 (80). 5) Despite the replacement of Sp1 by AP1 bs, we did not observe a trans-activation by the wild type Tat protein in the presence of TAR RNA (Fig. 2). To circumvent a possible incorrect or inefficient Tat-TAR targeting, we investigated the activity of Gal4BD-Tat fusion proteins on the HIV-1 promoter in S. cerevisiae.

Fig. 6 shows that the necessary conditions to obtain trans-activation by Tat in S. cerevisiae are both a DNA targeting and the presence of AP1 bs. In these conditions, Gal4BD-Tat48 and Gal4BD-Tat72 have 70 and 50% activity of the strong Gal4 activator, which represents a very good level of activation. Their close activity is probably the result of an increased DNA binding affinity of the Gal4BD fusion proteins. Indeed, we used six Gal4 DNA bs that should bind the Gal4BD more strongly and be less efficiently competed by the RNA binding domain in Tat72.

Why is Sp1 not active in yeast and not active for trans-activation whereas AP1 is active? This lack of Sp1 function is not due to an absence of interaction with TBP as yeast TBP mediates Sp1 activity (81). Recently, some activators have been
shown to directly contact the TAFs that mediate their function. For example, VP16 acidic domain binds TAF140 and Sp1 binds TAF1110 (7, 82, 83). Within the last few years, a large number of TAFs have been cloned from *Drosophila*, human, and yeast. For most of them, there is a species conservation suggesting a functional similarity. One exception is for TAF110, which has two limiting steps that prevent trans-activation of HIV-1 by Tat and that each one is a necessary component to the Tat multi-step mechanism (17): the presence of a functional Sp1 enhancer element and the transfer of Tat from TAR to the preinitiation complex. Whether this last function could be complemented by recently identified cofactors (33, 36, 43) will be a key issue to elucidate this mechanism.

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