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Recruitment of TBP or TFIIB to a Promoter Proximal Position Leads to Stimulation of RNA Polymerase II Transcription without Activator Proteins both *in Vivo* and *in Vitro*

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Eukaryotic transcriptional activators may function, at least in part, to facilitate the assembly of the RNA polymerase II (pol II) preinitiation complex at the core promoter region through their interaction with a subset of components of the basal transcription machinery. Previous studies have shown that artificial tethering of TATA-binding protein (TBP) to the promoter region is sufficient to stimulate pol II transcription in yeast. To test whether this phenomenon is a general one in eukaryotic pol II transcription, the DNA-binding domain of yeast GAL4 was fused to either *Xenopus laevis* TBP or TFIIB in order to enable these factors to be efficiently positioned near the transcription start site in a GAL4-binding site-dependent manner. We found that GAL4-xTBP as well as GAL4-xTFIIB directed an increased level of transcription without involvement of the transcriptional activator, suggesting that incorporation of these basal factors into a preinitiation complex (PIC) is a major rate-limiting step accelerated by activator proteins in metazoans. These results show that transcription activation by artificial recruitment of basal transcription machinery can be observed in general among eukaryotic transcription both *in vivo* and *in vitro*. Furthermore, failure of recovery of transcription by adding GAL4-xTFIIB after depletion of endogenous TBP with TATA oligo competitor suggests that recruitment of TBP cannot be bypassed for Pol II transcription.

Key Words: **transcriptional activation; recruitment assay; TBP; TFIIB; GAL4; *Xenopus*.**

Promoter-specific transcription by RNA polymerase II (pol II) in eukaryotes requires the assembly of a PIC (preinitiation complex) composed of a collection of general transcription factors (GTFs) including TFIID, TFIIB, TFIIE, TFIIF, and TFIIH (1). The rate of transcription of a gene can be greatly enhanced by transcriptional activator proteins, and one of the ways transcriptional activators exert their regulatory effect is by directly interacting with one or more GTFs and thereby facilitating the recruitment of basal factors to the promoter (1). Several GTFs have been shown to serve as targets for activators including TFIID, TFIIB, and TFIIH (2–4). Once TFIID, composed of TATA-binding protein (TBP) and a number of TBP-associated factors (TAFs), is positioned at the promoter, TFIIB can associate with a TATA–TFIID complex.

A series of activator bypass experiments in yeast demonstrated that fusion of a heterologous DNA-binding domain to TBP (5–8), TFIIB (9, 10), and several components of holoenzyme (11–13) resulted in high transcriptional activity at genes bearing the cognate DNA binding site in the absence of any activator. These findings imply that an activator protein stimulates transcription by facilitating recruitment of the transcriptional machinery to the promoter, and that the recruitment of TBP to a certain promoter is a rate-limiting step for transcriptional activation in yeast. However, it has been noted that the mechanism of pol II transcription in yeast manifests considerable dissimilarity from that of higher eukaryotes (14). TAFs, which act as targets for many activators in *Drosophila* and humans (15–18), appear to be less tightly associated with TBP in yeast (19), and are not generally required for transcriptional activation in yeast (20, 21). Certain yeast activator proteins were shown to direct transcriptional activation by interacting with a

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mediator, a multiprotein complex, which associates with RNA pol II (22–24). Because of these differences in the mechanism of transcriptional activation between yeast and metazoans, it was important to determine whether transcriptional activation observed in yeast artificial recruitment assays could be directly applied to the mechanism of pol II transcription in higher eukaryotes.

In this study, the *Xenopus* transcription system was employed to test whether artificial recruitment of TBP or TFIIB to the promoter would be sufficient for transcriptional stimulation. Our results show that the tethering TBP or TFIIB to the promoter-proximal position leads to stimulation of pol II transcription both *in vivo* and *in vitro*. The data of this study also suggest that binding of TBP to a promoter is a major rate-limiting step accelerated by activator proteins for pol II-dependent transcription in metazoans.

MATERIALS AND METHODS

Plasmid constructs. To construct pAS2-xTBP, a *Nde*I digested DNA fragment encoding *Xenopus* TBP (designated xTBP) gene derived from pET-xTBP (25) was subcloned into pAS2 (Clontech) that contains the DNA-binding domain of GAL4 (GAL4: amino acids 1 to 147). To construct pAS2-xTFIIB, a PCR-amplified *Nde*I/*Bam*HI fragment containing *Xenopus* TFIIB (designated xTFIIB) gene derived from pXIIB (26) was subcloned into a pAS2 plasmid. Oligonucleotides used for PCR amplification were XBNE (5′-GGA TCC CAT ATG GCG TCG ACG AGT CGC-3′) and T7 primer. *Escherichia coli* expression plasmids producing GAL4 derivatives were constructed as follows: A PCR amplified *Bam*HI fragment from pAS2-xTBP was subcloned into a pRSET-A (Invitrogen) plasmid to construct pRSET-A-GAL4-xTBP. pRSET-A-GAL4 was constructed by self-ligation after *Eco*RI digestion of pRSET-A-GAL4-xTBP. GN (5′-TTC CCG GAT CCC ATA TGA AGC TAC TGT CTT-3′) and XCOREC (5′-TTC CCG GAT CCG AAT TCT TAC GTT GTT TTT CTG-3′) were used as primers for PCR amplification in the above construction. To construct pRSET-GAL4-xTFIIB, an *Eco*RI fragment containing full amino acid sequences of *Xenopus* TFIIB obtained from pAS2-xTFIIB was inserted into the GAL4 downstream *Eco*RI site of pRSET-GAL4.

Protein purification. GAL4, GAL4-xTBP, and GAL4-xTFIIB recombinant proteins were expressed in *E. coli* BL21(DE3), and purified on a Ni^{2+}-NTA–agarose column (Qiagen) as described previously (27). Purified proteins were dialyzed against HEMG (0.1K) buffer (25 mM Hepes–KOH, pH 7.6, 12.5 mM $MgCl_2$, 0.1 mM EDTA, 10% glycerol, 100 mM KCl).

Yeast one-hybrid assay. pAS2, pAS2-xTBP, pAS2-xTFIIB, and pAS2-ySNF1 were transformed into *Saccharomyces cerevisiae* Y190 (MATa gal4 gal80 his3 trp1-901 ade2-101 ura3-52 leu2-3,-112 URA3::GAL → lacZ LYS2::GAL → HIS3 cyh^r) by lithium acetate method. pAS2-ySNF1 (28) encodes the gene for yeast SNF1 downstream of GAL4. Transformants were spotted onto a media lacking leucine and histidine, but containing different amounts (see Fig. 2B) of 3-amino-1,2,4-triazole (AT). β-Galactosidase assay was carried out according to the manufacturer's protocols (Clontech).

Oocyte injection and chloramphenicol acetyltransferase (CAT) assay. *Xenopus laevis* females were purchased from Nasco (Fort Atkinson, WI). *Xenopus* oocytes were prepared and microinjected with 5 ng of reporter plasmid and 5 ng of each recombinant protein per oocyte as previously described (29). p5GHIV2CAT (30) and pG5E1bCAT (31) were used as reporter plasmids. After overnight incubation at 18°C, five healthy oocytes were selected and homogenized in 300 μl of an extraction buffer (0.25 M Tris–Cl [pH 7.4], 0.1% Triton X-100). The homogenate was clarified by centrifugation and the resulting supernatant was used for CAT activity assay (32). CAT activities were measured using a phosphoimager (Fuji).

Preparation of transcription extracts and in vitro transcription assays. Oocyte extracts for *in vitro* transcription were prepared using ovaries from mature females as described previously (33). Reactions contained 25 μg of protein in 25 μl of 60 mM KCl, 15 mM Hepes (*N*-2-hydroxy-ethylpiperazine-N′-2-ethanesulfonic acid)–KOH, pH 7.6, 7.5 mM $MgCl_2$, 2 mM dithiothreitol, 6% glycerol, 1 mM nucleoside triphosphate mix, 20U RNasin (Promega), and 250 ng pG5HIV2CAT with or without 50 ng pXL10XP (33). Reactions were incubated at 25°C for 60 min and assayed by primer extension as previously described (34). A 30-mer CAT primer (5′-GGT GGT ATA TCC AGT GAT TTT TTT CTC CAT-3′), which is complementary to the first 30 coding nucleotides of the CAT gene, was used to analyze the transcripts of promoter-CAT fusions after 5′-end labeling with [γ-^{32}P]ATP (Amersham). For the oligonucleotide competition experiments in Fig. 4C, 10 μl of oocyte extract and 12 pmol of double-stranded synthetic TATA competitor (80-fold molar excess over template) were preincubated for 20 min at 25°C (in a final volume of 16 μl) before starting transcription by adding 9 μl of a mix containing all other components including templates and nucleosides (see above). The synthetic TATA competitor has been described previously (35). When GAL4 recombinant proteins were added to the transcription reaction, appropriate amounts of proteins were preincubated with templates for 20 min at 25°C prior to starting transcription.

RESULTS

Xenopus TBP and TFIIB fused to GAL4 binding domain activate transcription from the GAL1 promoter in yeast. To test whether the recruitment of *Xenopus* TBP or TFIIB to the promoter was sufficient for transcriptional activation in yeast, we constructed hybrid proteins containing the heterologous DNA binding domain of GAL4 fused to *Xenopus* derived TBP or TFIIB (Fig. 1). These constructs were introduced into *S. cerevisiae* Y190 strain that carried the *HIS3* and *LacZ* gene under control of the *GAL1* promoter, which bears the GAL4 binding site upstream of the TATA element (Fig. 2A). Activation of these reporter genes by hybrid proteins can be detected by resistance of host cells to AT and by the β-galactosidase assay. When yeast cells harboring the constructs were grown in the presence of AT, strains containing GAL4-xTBP or GAL4-xTFIIB could grow on media containing 30 mM AT, whereas strains containing GAL4 or GAL4-ySNF1 grew poorly even at 10 mM AT (Fig. 2B). The ability of these constructs to activate transcription was also tested by using the *lacZ* reporter. In the X-gal filter assay, cells harboring GAL4-xTBP or GAL4-xTFIIB were stained in dark blue, showing high level expression of the reporter gene *in vivo* (Fig. 2C). The other constructs did not result in a notable color change when the assay was performed. In the liquid culture assay for β-galactosidase, GAL4-xTBP and GAL4-xTFIIB exhibited higher levels of activation of *lacZ* expression which increased by 9.19- and 6.77-fold, respectively, when compared to that of GAL4 (Fig. 2C).

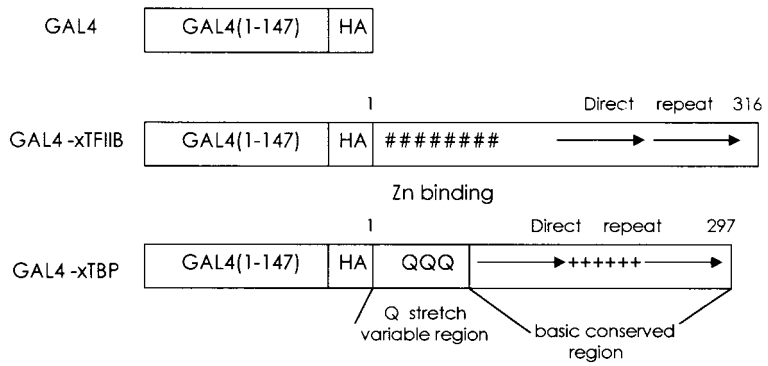

FIG. 1. A schematic representation of the plasmid constructs used in this study. cDNA encoding *Xenopus* TFIIB and TBP were inserted separately into the GAL4 binding domain (1–147) fusion vector and designated GAL4-xTFIIB and GAL4-xTBP, respectively.

GAL4-xTBP and GAL4-xTFIIB hybrid proteins activate transcription from the pol II-dependent promoters in Xenopus oocytes. To examine the ability of the GAL4-xTBP and GAL4-xTFIIB fusion proteins to activate pol II transcription in *Xenopus*, we expressed GAL4, GAL4-xTBP, and GAL4-xTFIIB in *E. coli* and then purified them as described under Materials and Methods. Their binding activity to the DNA fragments containing 5× GAL4 binding sites were confirmed by a gel mobility shift assay (data not shown). The reporter CAT genes (Fig. 3A) bearing GAL4 binding sites were co-injected into *Xenopus* oocytes together with the indicated recombinant GAL4 fusion proteins. GAL4-xTBP and GAL4-xTFIIB resulted in activation of the CAT gene from a HIV2 viral promoter. On the other hand, adenovirus E1b promoter showed high basal transcription activity in *Xenopus* oocytes (Fig. 3B, lane 5). GAL4-xTBP resulted in further increase in the level of E1b transcription by 5.5-fold, whereas GAL4-xTFIIB did not affect the transcription to any notable degree (lanes 6 and 7). The consistent findings in this experiment were that the artificial recruitment of TBP could direct an increased level of transcription for the two promoters used in higher eukaryotes as observed in yeast. GAL4-xTFIIB also showed transcriptional activation without the involvement of activator proteins; however, it seemed to behave in a promoter dependent manner.

GAL4-xTBP and GAL4-xTFIIB hybrid proteins stimulate transcription from the HIV2 promoter in vitro. Since TBP has been shown to possess a fortuitous activation domain (36), it is important to exclude the possibility that GAL4-xTBP functioned simply as a conventional activator working in recruiting endogenous TBP to the TATA element, instead of providing a TBP-inherent function directly at the promoter site (i.e., binding to TATA and sequentially recruiting the pol II holoenzyme). To address this point and to dissect transcription activation by artificial recruitment in detail, we performed several *in vitro* experiments. *Xenopus* oocytes, which have been used extensively *in vivo* transcription assays, were employed to prepare a cell free system to analyze in *in vitro* transcription. *Xenopus* oocyte extracts were found to be self-sufficient in producing pol II dependent transcripts without exogenous protein factors (data not shown).

We first examined whether the transcriptional activity of GAL4 derivative proteins would require the presence of GAL4 binding sites in the promoter. As shown in Fig. 4B, GAL4-xTBP resulted in 1.55-fold increase in the level of transcription from pG5HIV2CAT containing GAL4 sites, but resulted only in 0.46-fold increase from pXL10XP lacking GAL4 sites (lanes 9 and 12). However, the level of transcription stimulated by xTBP increased almost by 1.9 fold from p5GHIV2CAT and by 2.6-fold from pXL10XP (see lanes 5 and 7). High concentrations of xTBP partially inhibited transcription from pXL10XP (lane 8), while GAL4-xTBP did not show such an overdose inhibition of transcription in a similar concentration range (lane 12). These results show that GAL4-xTBP stimulates pol II transcription in a GAL4 site-dependent manner and does not increase the level of transcription in a general manner as xTBP does. A lower concentration of GAL4-xTFIIB stimulated transcription (compare lanes 13 and 14), but the level of transcription dramatically decreased as the concentration of GAL4-xTFIIB increased (lanes 14 to 16 and data not shown). This might be due to the sequestration effect of GAL4-xTFIIB in the transcription extracts (see Discussion). GAL4 did not have any effect on transcription.

Next, we blocked TBP function in transcription extracts by adding TATA-oligonucleotides to mask the function of endogenous TBP. The addition of xTBP to the oligonucleotide-treated extracts overcame the inhibitory effect of the TATA competitors (compare lane 2 with lanes 3 and 4 in Fig. 4C), showing that only the function of TBP was blocked in the extracts. In the event GAL4-xTBP simply acts as an activator that targets the PIC containing an endogenous TBP molecule, inhibition or depletion of TBP activity will block activation caused by GAL4-xTBP. On the other hand, if

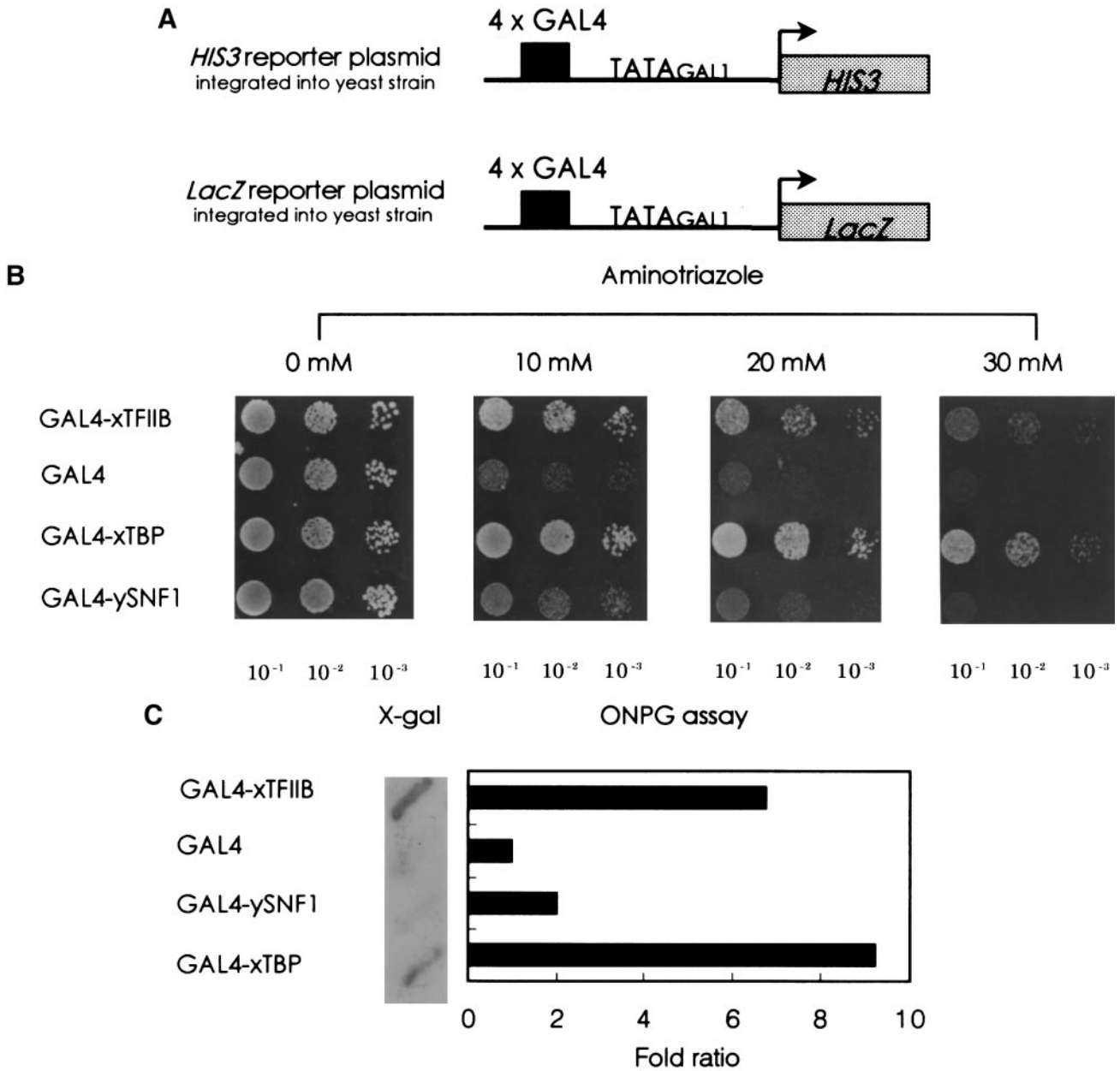

FIG. 2. Yeast one hybrid system. In A, hybrid protein fused to GAL4 DNA binding domain can confer GAL4-dependent transcription of the HIS3 or LacZ reporter sequences that are integrated into the Y190 yeast genome. In B, strains containing the indicated GAL4 derivatives encoding plasmids with a promoter containing 4x GAL4 binding sites upstream of the GAL1 TATA element and HIS3 structural gene were tested for growth in aminotriazole. The degree of aminotriazole resistance is directly related to the level of HIS3 transcription. In C, the full length *Xenopus* TBP (xTBP) and TFIIB (xTFIIB) connected to the GAL4 DNA-binding domain autonomously activate expression of GAL1-LacZ reporters in yeast. The activity of β-galactosidase (average of 20 independent transformants) was measured by an o-nitrophenyl-β-D-galactopyranoside (ONPG) assay in a quantitative manner, and was normalized to the OD_{600} of cells at the time of collection.

GAL4-xTBP can replace the endogenous TBP in the PIC assembly, it could still support pol II transcription without TBP activity in the transcription extract. When GAL4-xTBP was added to the transcription reaction after preincubation with the templates harboring GAL4 binding sites, the inhibited transcriptions were recovered (lanes 9 and 10). Also, relatively small amounts of GAL4-xTBP were required to restore the transcription to a high level compared to that of xTBP (see legend to Fig. 4C). These results excluded the possibility that the TBP domain of the GAL4-xTBP hybrid protein might act as a cryptic activation domain

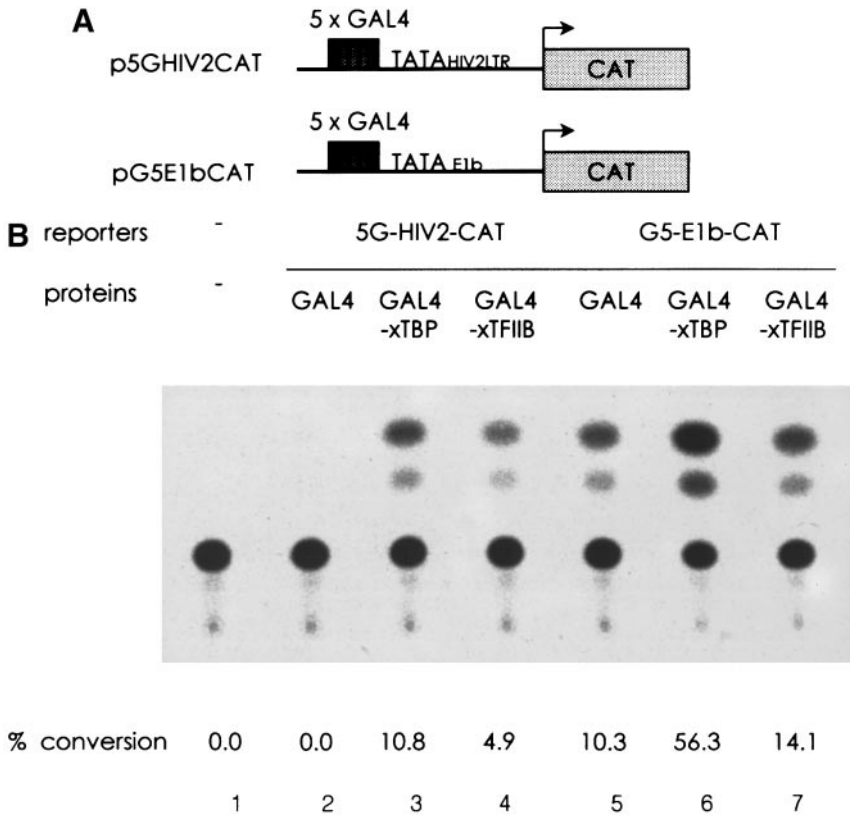

FIG. 3. CAT activity assay. In A, CAT reporter templates carrying 5× GAL4 binding sites upstream of the HIV2 and E1b TATA elements are shown. In B, CAT activity was assayed in *Xenopus* oocytes following microinjection of GAL4 recombinant proteins and a CAT reporter as indicated. The level of CAT activity measured as percentage conversion was determined by the Imagequant program supported by the Phosphoimager (Fuji).

and supported the idea that efficient recruitment of TBP to the promoter by the GAL4 binding protein resulted in transcriptional activation.

DISCUSSION

This study has demonstrated that in yeast and *Xenopus*, recruitment of TBP to the promoter by a heterologous DNA binding protein is sufficient for transcriptional activation (Figs. 2 and 3). Transcriptional activation *in vitro* by GAL4-xTBP depends both on GAL4-binding sites (Fig. 4B) and the TATA element (data not shown). The possibility of existence of cryptic activation domains within GAL4-xTBP polypeptide has been excluded as GAL4-xTBP recovered transcription was suppressed by TATA oligonucleotides (Fig. 4C). Taken together, these results suggest that activation by GAL4-xTBP involves increased interaction of the TBP moiety with the TATA element as a result of its physical connection to a GAL4 binding domain that binds to a nearby site. Consistent with previous *in vivo* studies in yeast (5–7) and in mammalian cells (37), these *in vivo* and *in vitro* data propose and generalize the idea that the recruitment of TBP and its stable binding to the promoter can be a major limiting step for transcriptional activation in eukaryotes.

Our results show that GAL4-xTFIIB stimulated pol II dependent transcription in the absence of transcriptional activators and this strongly supports the idea that certain activators act at steps subsequent to the recruitment of TBP including incorporation of TFIIB to the PIC. GAL4-xTFIIB could not support Pol II transcription in the extracts in which endogenous TBP had been masked by TATA oligonucleotides, indicating that artificial recruitment of TFIIB by the GAL4 binding domain cannot bypass the requirement of TBP and that proper positioning of TFIIB in the PIC should occur by formation of the TATA-TBP-TFIIB ternary complex as revealed by its crystal structure (38). At high concentrations, GAL4-xTFIIB dramatically decreased the level of Pol II transcription *in vitro* (lanes 15 and 16 in Fig. 4B). One possible explanation for this observation is that an excess amount of GAL4-xTFIIB may bind and thereby sequester partial subsets of basal transcription complexes devoid of one or more essential component of PIC. Thus they cannot be involved in transcription initiation (39). As GAL4-xTFIIB successfully activated transcription from

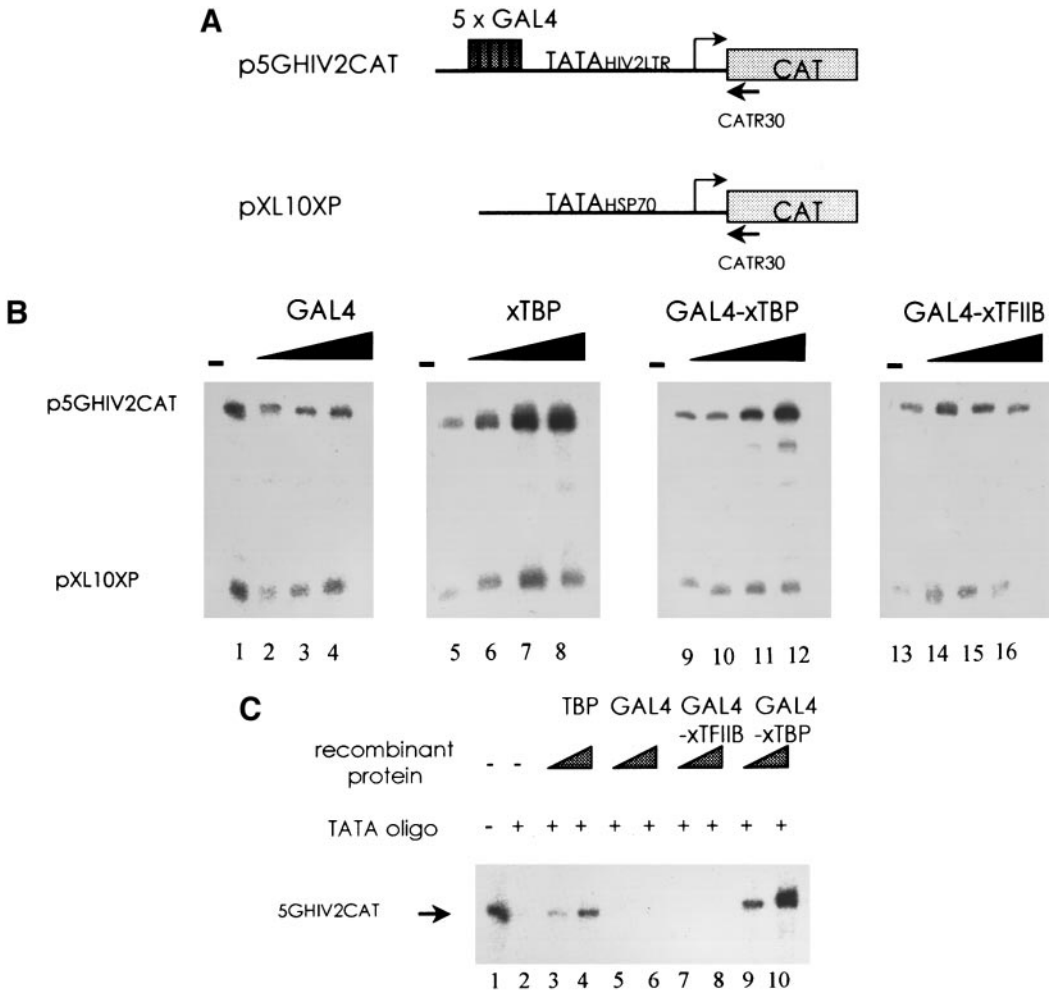

FIG. 4. Effect of recombinant proteins on the *in vitro* transcription. In A, *in vitro* transcription templates are shown. Inserted upstream of HIV2 promoter were five binding sites for GAL4. In B, *in vitro* transcription reactions were performed in 30 μg of whole-cell extracts derived from *Xenopus* oocytes with 250 ng p5GHIV2CAT and 50 ng pXL10XP as templates. The concentration of GAL4 recombinant proteins supplemented in the reaction mixtures was as follows: GAL4 at 0, 12, 20, 36 nM; xTBP at 0, 12, 20, 36 nM; GAL4-xTBP at 0, 12, 20, 36 nM; GAL4-xTFIIB at 0, 0.032, 0.8, 20 nM. RNA products were analyzed by primer extension followed by electrophoresis on a 5 or 8% denaturing polyacrylamide gel. In C, *in vitro* transcription experiments were performed in *Xenopus* oocyte extracts incubated for 20 min at 25°C with 12 pmol oligonucleotides containing a synthetic TATA box prior to the start of transcription. Reactions were carried out in the presence of xTBP (3.5, 7 pmol), GAL4 (0.5, 0.75 pmol), GAL4-xTFIIB (0.5, 0.75 pmol), and GAL4-xTBP (0.5, 0.75 pmol), respectively. The resulting transcripts were analyzed by primer extension.

GAL1 and HIV2 promoter *in vivo* in yeast and in *Xenopus*, respectively (Figs. 2B and 2C, and lane 4 in Fig. 3B), this sequestration effect seems to be alleviated *in vivo* due to the complex regulation mechanism. Although GAL4-xTFIIB stimulated *in vivo* transcription from the *GAL1* and the HIV2 promoters, it showed little effect on transcription from the adenovirus E1b promoter (lane 7 in Fig. 3B). The E1b promoter manifested an elevated level of transcription with the GAL4 DNA binding domain alone (lane 5 in Fig. 3B). This observation likely resulted from stimulation of the transcription by a cryptic activation domain located within the GAL4 DNA binding domain (40). Even in this case, GAL4-xTBP increased transcription level up to 5.5-fold (lane 6 in Fig. 3B) indicating that TBP recruitment did enhance the transcription level from the E1b promoter. These findings may reflect differential requirement of activation steps among Pol II promoters composed of differential combinations of core promoter elements, such as the TATA box, initiator, recently defined downstream promoter element (40) and/or subtle contextual effects.

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