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GAL4-VP16 Stimulates Two RNA Polymerase II Promoters Primarily at the Preinitiation Complex Assembly Step

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We previously demonstrated that RNA polymerase II promoters may be limited in strength not only at the step of transcription complex assembly, but also at initiation or promoter clearance. Here we report on experiments designed to test the possibility that steps following transcription complex assembly might be stimulated by transcriptional activators. Using an in vitro system in which we can independently measure the efficiency of assembly, initiation, and promoter clearance, we have investigated the mechanism by which the model activator GAL4-VP16 increases transcription from two promoters: a weak variant of Ad 2 ML with an altered TATA box, which is inefficient in transcription initiation, and the mouse β-globin promoter, which is inefficient in promoter clearance. We found that whereas GAL4-VP16 is effective in stimulating both promoters, this increase resulted only from greater transcription complex assembly; the initiation and clearance steps were not affected. Because recent studies have suggested that the core transcription factors TFIIE and TFIIH might be important in promoter clearance, we also attempted to increase the initiation and clearance efficiencies of the Ad ML-TATA mutant and globin promoters by direct addition of excess TFIIE and TFIIH to partially purified preinitiation complexes assembled at each of these promoters. These factors had no effect on transcription by either of the preinitiation complexes.

RNA polymerase II promoters Complex assembly Transcriptional activators Initiation Promoter clearance

THE initiation of synthesis of pre-mRNAs by RNA polymerase II is an exceptionally complex process. Assembly of the transcription machinery begins with promoter binding by factors TFIID and TFIIB (in some cases in conjunction with TFIIA). RNA polymerase II, accompanied by TFIIF, then joins the complex, followed by TFIIE and TFIIH [reviewed in (7,28)]. Nonregulated, basal transcription can occur with only a subset of these factors (26,27,31). Once the preinitiation complex is assembled, it must convert to an open complex, which requires ATP [see (17), and references therein]. Transcription initiation then occurs, which may result in either abortive production of short (less than 10 nt) transcripts or in clearance of RNA polymerase from the promoter and entry into productive elongation (14,22).

This multistep pathway provides many opportunities for regulation of transcription. To date, mechanistic studies of the regulation of eukaryotic transcription initiation have focused on preinitiation complex assembly (5,13,21,30) and the transition into open complex (16,32,33). However, it seems likely that control of initiation and clearance might also be exerted in higher organisms. In prokaryotes, examples of control at these steps

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have been described (6,8,18,24,25). A recent report has shown that activation domains can bind specifically to TFIIH (36), which is thought to be involved in promoter clearance (12,23). Also, elongation competence has been shown to be influenced by activators (37). We reasoned that promoters that are inefficient at initiation or clearance are the most likely to be regulated at these steps. We had demonstrated previously that the mouse β-globin promoter is as effective as the strong adenovirus 2 major late (Ad 2 ML) promoter in directing transcription complex assembly but is considerably less effective in supporting promoter clearance (15). We also showed that pML5C, a weak variant of Ad 2 ML that has a base change in the TATA element, is not only much less effective at directing transcription complex assembly but also fails to support efficient transcription initiation (15). In this article we report the results of stimulating in vitro transcription of the mouse β-globin and pML5C promoters with the strong acidic activator GAL4-VP16. We found that although transcription from each promoter was increased by GAL4-VP16, clearance at the globin promoter and initiation at pML5C remained inefficient. Increased promoter effectiveness could be attributed to more efficient preinitiation complex assembly in each case.

MATERIALS AND METHODS

DNA Templates

The pML5C plasmid was described in detail previously (14). It contains an adenovirus 2 major late (Ad 2 ML) promoter sequence, from −174 to +35 relative to transcription start, inserted into pUC18. Several base pair substitutions were made near the transcription start site to facilitate cloning or the generation of paused transcription complexes. The pML5C derivative that contains five GAL4 binding sites cloned 49 bp upstream of the transcription start site, called $pG_5ML5C\Delta$, was derived from pML5C by cleavage with EcoR I and BspE I, which removed all Ad 2 sequences upstream of −49. The plasmid DNA ends were filled in and a blunt-end 126-bp DNA fragment bearing five near-consensus GAL4 binding sites (34) was inserted. This fragment was obtained from a Hind III-Xba I digestion of the plasmid pG_5BCAT (a gift of Dr. Steve Triezenberg). The pMβ5T plasmid, also described in detail previously (15), was generated by subcloning the mouse β-globin promoter from −43 to +61 into pUC18; again several substitutions were made to facilitate cloning and to allow for the generation of paused transcription complexes. $pG_5M\beta5T$ was generated by subcloning the same blunt-end 126-bp GAL4 binding site-containing fragment into the BamH I site of pMβ5T, which is 48 bp upstream of transcription start. To exclude vector transcription from the study of $pG_5M\beta5T$, an EcoR I-Hind III fragment of $pG_5M\beta5T$ was used as template. This 281-bp fragment includes sequence from −198 upstream to +84 downstream of transcription start. The fragment includes the five tandem GAL4 DNA binding sites upstream of TATA. The fragment was isolated on an low melt agarose gel and purified with a Schleicher and Schuell Elutip-d. All plasmids were prepared by alkaline lysis, cesium chloride gradient banding, and gel filtration.

Purification of Fusion Protein GAL4-VP16

GAL4-VP16 is a fusion protein that contains residues 1–147 of the amino-terminus of GAL4, which provides a DNA binding domain, joined to the 78 carboxy-terminal residues of herpes simplex VP16 protein using a seven-residue linker (29). Expression of the fusion protein is under control of the *tac* promoter in plasmid pJL2, in *E. coli* strain XA90 (a gift from Dr. Steve Triezenberg). Purification of this protein was adapted from a protocol by Chasman et al. (4). Following induction with isopropylthiogalactoside, cells were harvested, and lysed by sonication. The supernatant was cleared by centrifugation and subjected to polyethyleneimine precipitation to remove nucleic acids. The protein was ammonium sulfate precipitated, followed by sequential chromatographic purifications on DE52 (Whatman) and heparin-Sepharose-4B (Pharmacia). The final product was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and was determined to be greater than 95% pure. Final protein concentration as judged by Bradford assays with BSA as a standard was 1.7 mg/ml. Concentrations that resulted in optimal transcription activation were determined empirically for each plasmid (a molar ratio of 11.7:1 GAL4-VP16 to promoter for $pG_5ML5C\Delta$, and 20:1 for $pG_5M\beta5T$).

Preparation of Preinitiation Complexes

Preinitiation complexes were prepared as described (15) with the following changes. For $pG_5ML5C\Delta$, 6.3 μg/ml intact plasmid was incubated with GAL4-VP16 at 0.98 μg/ml (molar ratio of

11.7:1, protein to DNA) or with an equivalent amount of buffer for 15 min at 30°C at final KCl, NaCl, and $MgCl_2$ concentrations of 73, 7, and 8.4 mM, respectively, in a volume of 200 μl. Control reactions (minus GAL4-VP16) were identical. HeLa cell nuclear extract (300 μl; 7 mg/ml) and additional salts were added to a final volume of 572 μl and salt concentrations of 78 mM KCl, 2.5 mM NaCl, and 8.4 mM $MgCl_2$. Reactions were incubated for 20 min at 30°C. Some preinitiation complexes were assembled in the presence of 0.05% Sarkosyl to provide a control in which assembly was inhibited. Because there is a significant contribution to the transcripts generated from the vector DNA, preinitiation complexes were also assembled on pUC18 vector alone, at 6 μg/ml, without GAL4-VP16. These values were subtracted from the signal generated from $pG_5ML5C\Delta$ to correct for this background transcription, in the tabulation of transcript synthesis. Preinitiation complexes with $pG_5M\beta5T$ were assembled in the same manner but with the following changes. To exclude vector transcription from the study of $pG_5M\beta5T$, a 281-bp EcoR I-Hind III fragment of $pG_5M\beta5T$ was used as template. Preinitiation complexes were assembled as described for $pG_5ML5C\Delta$ with the following changes. The template DNA fragment, at 3.46 μg/ml, was incubated in a volume of 52 μl with 64 mM KCl, 16 mM NaCl, and 8.4 mM $MgCl_2$, and either 0, 1.4, or 2.45 μg of GAL4-VP16 (protein:template equal to 20:1 or 35:1, respectively). HeLa cell nuclear extract (80 μl) and salts were added to a final volume of 150 μl at 74 mM KCl, 6 mM NaCl, and 8.4 mM $MgCl_2$ and incubated for 20 min at 30°C. For experiments involving addition of TFIIE and TFIIH, preinitiation complexes were assembled on pML5C and $pM\beta5T$ (mouse globin promoter) templates and partially purified by gel filtration on Bio-Gel A-1.5m (Bio-Rad) exactly as described previously (15).

In Vitro RNA Synthesis

For the data generated for Tables 1 and 2, transcription reactions were run as described (15), using 1 mM CpA dinucleotide primers, 10 μM dATP, 10 μM UTP, 0.5 μM CTP, and 0.5 μM [α-^{32}P]CTP (800 Ci/mmol, DuPont NEN) as substrates. The longest RNAs that can be made under these conditions are 16 nt for pML5C-based templates and 13 nt for the globin promoter-based templates, because of the template sequence. Transcriptions were done in triplicate with the values in the tables representing the average. For the experiments in Figs. 4 and 5, 18 μl of preinitiation complex assembled on either pML5C or $pM\beta5T$ was mixed with the designated volume of recombinant TFIIE and/or TFIIH (gifts from Dr. Danny Reinberg) and incubated for 5 min at 30°C prior to the addition of nucleotides. Transcription reactions were run as described above. TFIIH was purified from HeLa cell nuclear extract as described (10) with the following sequential fractionation steps: phosphocellulose, DEAE-Sephacel, DEAE-5PW, Mono-S, phenyl-Superose, and Mono-Q. The TFIIE and TFIIH were determined to be transcription competent in reconstituted transcription reactions using recombinant or purified core transcription factors and RNA polymerase II.

Quantitation of Preinitiation Complexes

Preinitiation complexes were quantitated using a nuclease protection assay as described (15). Briefly, preinitiation complexes were assembled with or without 0.05% Sarkosyl, which provides a control in which assembly was inhibited. The complexes were subjected to complete digestion by DNase I at 200 U/ml for 3 min at 25°C. The digested DNA was purified, denatured, and hybridized with an end-labeled oligonucleotide that hybridized to the nontemplate strand from +15 to −5. This region has been shown to be protected from digestion with DNase I by the preinitiation complex and therefore represents the template that was in complex (2). The oligonucleotide was extended with avian myeloblastosis virus reverse transcriptase (Super reverse transcriptase, Molecular Genetics Resources). The extended products were electrophoresed on a denaturing 19% acrylamide, 1% bisacrylamide gel (15). The extension products were seen as a set of bands about 60 bases long, corresponding to the DNase I footprint upstream of the TATA box at about −45 (+15 to −45) (2). The intensity of the extension product bands was directly proportional to the number of preinitiation complexes assembled. By also synthesizing extension products from known amounts of pure DNA in otherwise identical reactions, the amount of preinitiation complex was computed by comparing the intensities of the extension product bands obtained with the preinitiation complex DNA and the pure DNA controls (pure DNA controls are not shown in Fig. 2; see Fig. 1). The signal generated by the preinitiation complexes assembled in the presence of Sarkosyl was used as a background control and subtracted

from the signal of the complexes without Sarkosyl.

RESULTS

To compare efficiencies of transcription initiation between promoters it is necessary to quantitate transcription complex assembly without relying on the measurement of RNA. We showed that the ability of the preinitiation complex to protect the promoter from extensive DNase I digestion could be exploited to measure the number of preinitiation complexes (2). When we applied this approach to a set of RNA polymerase II promoters (15), we found that all of the preinitiation complexes detected by nuclease protection gave rise to transcripts in a single-round transcription assay for the relatively strong adenovirus 2 major late (Ad 2 ML) and mouse β-globin promoters (pMβ5T). However, a TATA box mutant of Ad 2 ML called pML5C, which is an order of magnitude weaker in supporting long RNA synthesis, did not support effective initiation. For every mol of pML5C transcription complex detected by nuclease protection, only about 0.35 mol of transcript was obtained in a single-round assay (15). Once transcription initiation has occurred, a fraction of the RNA polymerases may make from one up to about nine bonds and then cease RNA synthesis, releasing the nascent RNA (22). This abortive initiation process can claim a substantial fraction of the newly initiated transcripts. We found that at the Ad 2 ML and pML5C promoters about 15% of the initiations were lost to the abortive pathway whereas at the β-globin promoter a majority (about 70%) of initiations were abortive (15).

In the present study, we examined the effect of the model activator GAL4-VP16 on the pML5C and mouse β-globin promoters. Appropriate templates were created by substituting five tandem GAL4 DNA binding sites upstream of the TATA box in both the pML5C and β-globin constructs, creating new plasmids that we named pG_5ML5CΔ and pG_5Mβ5T. Preinitiation complexes were assembled on pG_5ML5CΔ templates with or without GAL4-VP16 and assayed for abortive and productive RNA synthesis and for transcription complex assembly essentially as described previously (15), except that lower concentrations of template DNA were used to maximize stimulation by GAL4-VP16. Single-round transcription assays employed a CpA dinucleotide primer, UTP, and radiolabeled CTP as substrates as well as dATP to satisfy the energy requirement for transcription initiation (22); this mixture supports the production of transcripts of 16 nt or less (14) from the pG_5ML5CΔ promoter. As shown in Fig. 1, under these conditions RNA synthesis from pG_5ML5CΔ was nearly completely dependent on the activator. Most of the α-amanitin sensitive RNAs were 15 or 16 nt long, as expected. These RNAs were in active ternary complexes since they were all extended when an excess of the four NTPs were added [data not shown here, but see (15)]. Transcripts less than 10 nt long were abortively initiated, because they were not chased with excess NTPs [see (15)]. The amounts of RNA produced in three separate experiments of the type shown in Fig. 1 are given in Table 1.

To determine initiation efficiency (the ratio of single-round transcripts to preinitiation complexes) it was also necessary to quantitate the amount of preinitiation complex assembled. We relied on our earlier observation that the template DNA within the preinitiation complex is resistant to extensive digestion by DNase I (2). The promoter DNA that survived the DNase I digestion was hybridized with an excess of an oligonucleotide complementary to the nontemplate strand near the transcription start site. The hybrids were then extended with reverse transcriptase. Extension proceeded to a relatively sharp edge of protection just upstream of the TATA element. The amount of extended primer obtained with DNase-digested promoters was compared to the level of extension product obtained with known amounts of pure promoter DNA, allowing us to determine the amount of DNA originally protected by transcription complexes [see also (15)]. Figure 2 shows the extension products generated from a typical analysis, done in triplicate. The bracket marks the bands generated by extension to the upstream edge of the nuclease-trimmed preinitiation complex, at −41 to −46. To confirm that these bands were specific to the transcription complex, DNase digestion and primer extension were also performed on reactions assembled in the presence of 0.05% Sarkosyl, which prevents preinitiation complex assembly (compare lanes 4–6 with lanes 10–12). The levels of preinitiation complex for each of the preparations are given in Table 1. From this analysis we found that on average only 26% of the pG_5ML5CΔ preinitiation complexes could support RNA synthesis. Considering the margin of error with these determinations, this is not significantly different from the value of 35% previously obtained for pML5C complexes produced without activation. Because pG_5ML5CΔ transcription in

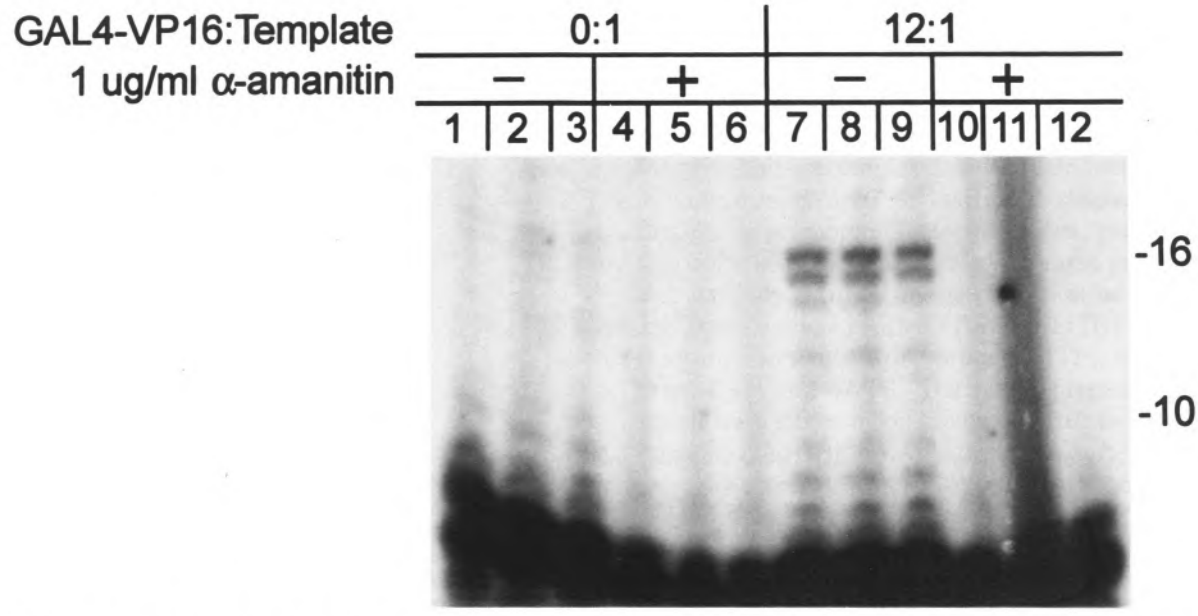

FIG. 1. The effect of GAL4-VP16 on in vitro transcription reactions using limiting nucleotides with $pG_5ML5C\Delta$ template. Preinitiation complexes were assembled on $pG_5ML5C\Delta$ in the absence (lanes 1–6) or presence (lanes 7–12) of GAL4-VP16 at a molar ratio of 12:1 activator:DNA template as described in the Materials and Methods section. Reactions were performed in triplicate. Substrates for the transcriptions were CpA, UTP, [α-^{32}P]CTP, and dATP; RNAs were synthesized, purified, and resolved on 25% polyacrylamide, 3% bisacrylamide gels as described in the Materials and Methods section. The indicated reactions contained 1 μg/ml α-amanitin. The lengths of selected transcripts are indicated in the margin.

the experimental conditions used here was nearly completely dependent on the activator, we may conclude that GAL4-VP16 is stimulating preinitiation complex assembly but is not improving the characteristic low initiation efficiency of the pML5C promoter.

When we studied transcription complex assembly and transcription initiation at the mouse β-globin promoter, we found that assembly was as efficient as that seen with the strong Ad 2 ML promoter (15). Initiation from the β-globin complexes was essentially complete, in that all of the preinitiation complexes gave rise to transcripts. However, the majority of the β-globin transcripts stopped at positions +5, +6, or +7; only about one-fourth of the RNAs could be extended into longer transcripts. We repeated these transcription experiments with the $pG_5M\beta5T$ template containing five GAL4 binding sites upstream of the globin promoter. Low levels of DNA template were employed, and in half of the reactions a large molar excess of GAL4-VP16 was preincubated with the template before transcription complexes were assembled. Transcription was performed with a CpA primer, UTP, labeled CTP and dATP, which should support production of transcripts of 13 nt or less from the globin promoter. As shown in Fig. 3, the expected 13-mer was generated, along with numerous shorter RNAs. We have shown that the 11 and 13 base RNAs chase quantitatively when excess NTPs are added, whereas the prominent shorter transcripts cannot be extended and are therefore abortive [data not shown here, but see (15)]. Amanitin-sensitive transcription from the globin promoter was strongly stimulated by GAL4-VP16. We determined that a 20:1 molar

TABLE 1
QUANTITATION OF TRANSCRIPTION INITIATION EFFICIENCY OF PREINITIATION COMPLEXES ASSEMBLED ON $pG_5ML5C\Delta$ IN THE PRESENCE OF GAL4-VP16

Trial	Productive Transcript (fmol)	PIC (fmol)	Initiation (%)
1	0.110	0.247	44.5
2	0.145	1.090	13.3
3	0.087	0.404	21.5
Average			26.5

Productive RNA synthesis and the extent of promoter occupancy, as assessed by protection against DNase I cleavage, were measured for three independent sets of preinitiation complexes (PIC) assembled on $pG_5ML5C\Delta$ in the presence of GAL4-VP16 as described in the Materials and Methods section. Values are the average of triplicate measurements. Percent initiation is the molar ratio of productive transcript and preinitiation complex.

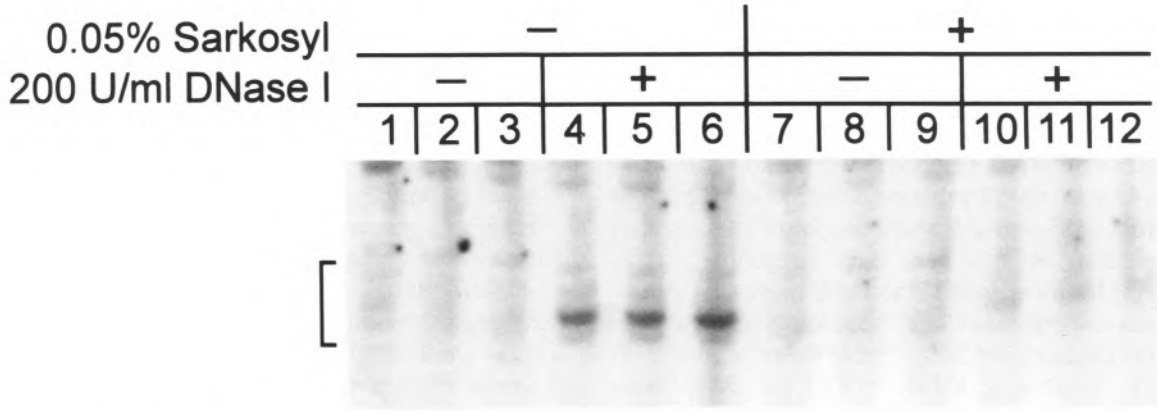

FIG. 2. Protection against 200 U/ml of DNase I digestion conferred by preinitiation complexes assembled on $pG_5ML5C\Delta$ in the presence of GAL4-VP16. Preinitiation complexes were assembled on $pG_5ML5C\Delta$ with GAL4-VP16 at a molar ratio of 12:1 activator:DNA template, in the presence or absence of 0.05% Sarkosyl. Protection against DNase I digestion was quantitated by a primer extension analysis as described in the Materials and Methods section. Triplicate reactions were incubated with 200 U/ml DNase I for 3 min at 25°C (lanes 4–6 and 10–12). Primer extension was also performed on complexes not subjected to DNase I digestion but that were instead linearized with EcoR I (lanes 1–3 and 7–9). The extension products were purified and analyzed on 19% polyacrylamide, 1% bisacrylamide gels as described in the Materials and Methods section. The bracket in the left margin indicates the region of each lane that was used to quantitate promoter occupancy using a Phosphorimager. The signal generated in this region from the Sarkosyl-assembled complexes was subtracted from the corresponding signal generated from complexes assembled without Sarkosyl.

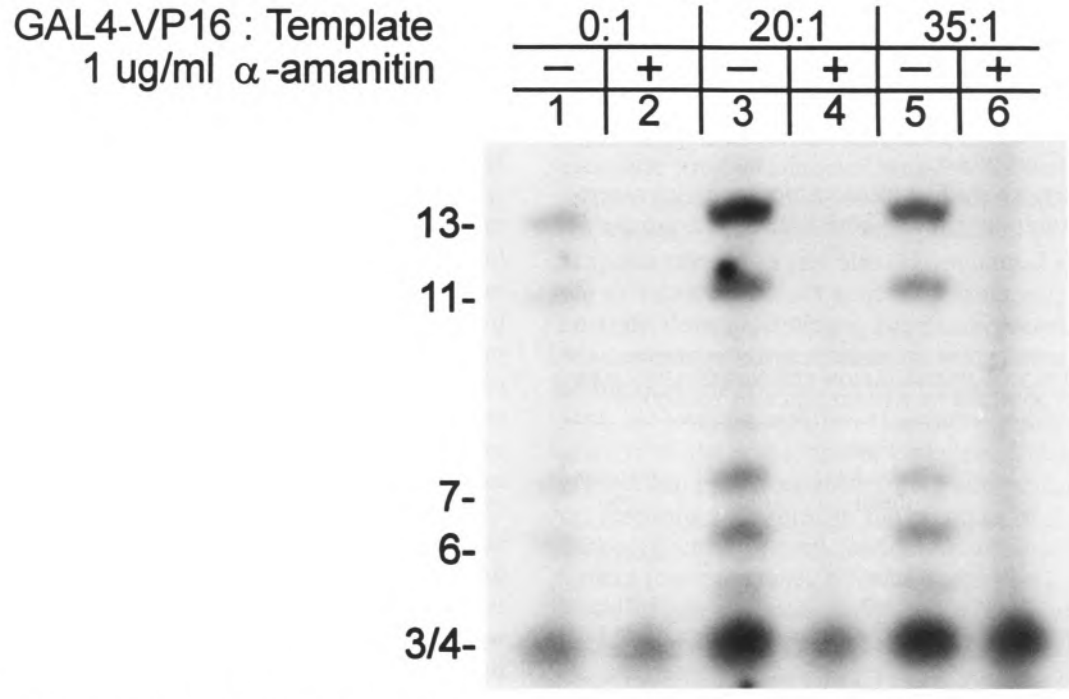

FIG. 3. The effect of GAL4-VP16 on in vitro transcription reactions using limiting nucleotides with $pG_5M\beta5T$ template. Preinitiation complexes were assembled on $pG_5M\beta5T$ in the absence (lanes 1, 2) or presence of GAL4-VP16 at a molar ratio of either 20:1 (lanes 3, 4) or 35:1 (lanes 5, 6) activator:DNA template as described in the Materials and Methods section. Substrates for the transcriptions were CpA, UTP, [α-^{32}P]CTP, and dATP; RNAs were synthesized, purified, and resolved on 25% polyacrylamide, 3% bisacrylamide gels as described in the Materials and Methods section. The indicated reactions contained 1 μg/ml α-amanitin. The lengths of selected transcripts are indicated in the margin.

TABLE 2
QUANTITATION OF TRANSCRIPTION ACTIVATION AND PROMOTER CLEARANCE ON PREINITIATION COMPLEXES ASSEMBLED ON pG$_5$Mβ5T IN THE PRESENCE OF GAL4-VP16

Round	Fold Stimulation	Abortive Transcripts / Productive Transcripts	
1	3.51	unstimulated	4.00
		stimulated	3.55
2	3.50	unstimulated	3.55
		stimulated	3.76
3a	3.56	unstimulated	3.55
		stimulated	3.76
3b	2.41	unstimulated	4.88
		stimulated	3.76
4	2.03	unstimulated	4.00
		stimulated	4.00
5	2.13	unstimulated	3.76
		stimulated	3.55
Average	2.86	unstimulated	3.96
		stimulated	3.73

Abortive and productive RNA synthesis were measured in triplicate for five independent sets of preinitiation complexes assembled in the absence and presence of GAL4-VP16 as described in the Materials and Methods section. Complexes in Experiment 3 were assayed twice (3a and 3b). Fold stimulation is the ratio of productive transcripts with and without the addition of GAL4-VP16 to the preinitiation complex assembly reaction. Promoter clearance is the ratio of productive transcripts to total transcripts.

ratio of GAL4-VP16 to DNA gave us optimal stimulation of transcription (Fig. 3, lane 3 vs. lane 1), whereas additional factor actually decreased transcription (compare lanes 3 and 5). This may represent squelching, as reported previously (11).

The results in Fig. 3 are representative of the five separate pG$_5$Mβ5T preinitiation complex preparations assayed. On average we saw a 2.9-fold increase in productive transcripts (Table 2) with GAL4-VP16. In all cases both stimulated and nonstimulated reactions gave a ratio of productive (11 and 13 nt RNAs) to abortive (less than 8 nt RNAs) of about 1:4. Because transcription increased without any change in the partitioning between abortive and productive transcription, activation by GAL4-VP16 had no significant effect on promoter clearance.

It is important to note that protection of the template against DNase attack should be primarily provided by TFIID and the RNA polymerase. Thus, our nuclease protection assay is probably unable to distinguish preinitiation complexes that have assembled the full complement of factors from complexes without TFIIE and/or TFIIH, which arrive after the RNA polymerase. However, recent results from several groups suggest (12,23) that failure to load these factors could explain defects in initiation or promoter clearance. We therefore thought it was important to explore the possibility that a deficiency in these factors within the preinitiation complex could explain inefficient initiation on pML5C or inefficient clearance on the globin promoter. To test this idea, preinitiation complexes were assembled on these promoters, partially purified by gel filtration as usual, and then incubated with TFIIE and TFIIH before transcription with CpA primer, UTP, radiolabeled CTP and dATP. The factor preparations, which were either recombinant (TFIIE) or highly purified (TFIIH), were the generous gift of Dr. Danny Reinberg. Roughly 1 μl of the IIE and IIH preparations that we used was sufficient to support maximal in vitro transcription in reactions similar to ours but containing purified components (D. Reinberg, personal communication). Figure 4 shows transcription reactions on pML5C (lanes 1–4) or β-globin (lanes 5–8); reactions in lanes 3, 4, 7, and 8 were preincubated with the indicated volumes of transcription factor for 5 min before addition of nucleotides. In Fig. 5, globin promotor preinitiation complexes were preincubated with TFIIE or TFIIH alone before the addition of transcription substrates. In neither case did we detect any effect of the additional factor(s). Transcription initiation did not increase on pML5C, and the partitioning of transcripts between the abortive and productive pathways on the globin promoter was not affected.

DISCUSSION

We report here on the molecular mechanism by which GAL4-VP16 stimulates transcription at the mouse β-globin promoter and at pML5C, which is a mutated version of the Ad 2 ML promoter. Even though both of these promoters are inefficient at a step beyond the stage of assembling the preinitiation complex, we did not find evidence that GAL4-VP16 increased transcription at either promoter by any means other than facilitating transcription complex assembly. These findings represent the first instance in which stimulation of transcription has been studied in a system in which effects on transcription complex assembly may be easily distinguished from effects on initiation or promoter clearance. Interestingly, the addition of either TFIIE or TFIIH to partially purified preinitiation complexes did not affect initiation or promoter clearance at either pML5C or the globin promoter.

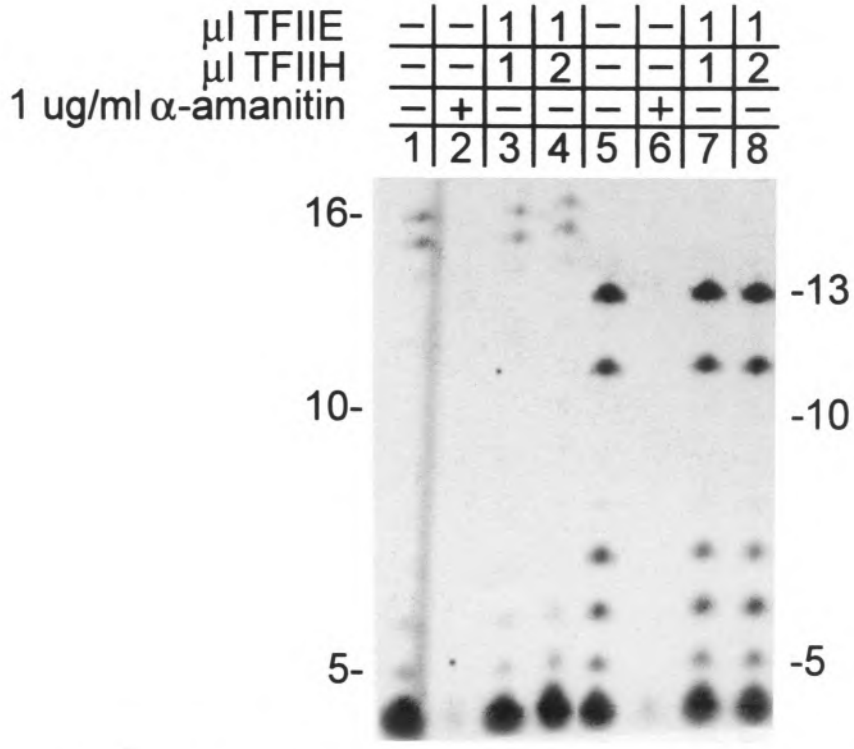

FIG. 4. The effect of adding TFIIE and TFIIH on transcription initiation and promoter clearance by preinitiation complexes assembled on either pML5C or pMβ5T templates. Preinitiation complexes were assembled on pML5C (lanes 1–4) or pMβ5T (lanes 5–8) as described in the Materials and Methods section. Prior to the addition of transcription substrates complexes were incubated with the indicated volumes of TFIIE and TFIIH for 5 min at 30°C. CpA, UTP, [α-^{32}P]CTP, and dATP were added as transcription substrates and RNAs were synthesized, purified, and resolved on 25% polyacrylamide, 3% bisacrylamide gels as described in the Materials and Methods section. The indicated reactions contained 1 μg/ml α-amanitin. The lengths of selected transcripts are indicated in the left margin for transcripts generated on pML5C and in the right margin for transcripts generated on pMβ5T.

Many previous studies have also addressed the question of how GAL4-VP16 activates transcription. The experiments of Lin and Green (20) showed that another acidic activator, GAL4-AH, can increase the number of transcriptionally active complexes through facilitated loading of TFIIB into the preinitiation complex. It was suggested that GAL4-VP16 worked through a similar mechanism. This work was later extended by Choy and Green (5), who demonstrated that in addition to the recruitment of TFIIB, a later step in transcription complex assembly was stimulated by GAL4-VP16. White et al. (35) also concluded that GAL4-VP16 stimulated transcription complex assembly; they determined that the step that was activated occurred after TFIID binding, which is consistent with the findings of Green and colleagues. Chang and Gralla (3) noted that a synergistic response to GAL4-VP16 during in vitro transcription required the use of chromatin templates, which is consistent with a model in which GAL4-VP16 serves to prevent nucleosomal repression by increasing transcription complex assembly. Jiang et al. (15) showed that GAL4-VP16 mutants that fail to activate transcription also fail to support open complex formation. This result could be interpreted to indicate that GAL4-VP16 affects the transition from closed to open complex, but because no independent measure of transcription complex assembly was obtained in this case the observation is also consistent with stimulation at the assembly step.

Given that enhancement of preinitiation complex assembly by GAL4-VP16 has been demonstrated in a number of cases, we were not surprised that this factor promoted increased assembly in our system as well. However, it seemed reasonable to expect that stimulation of

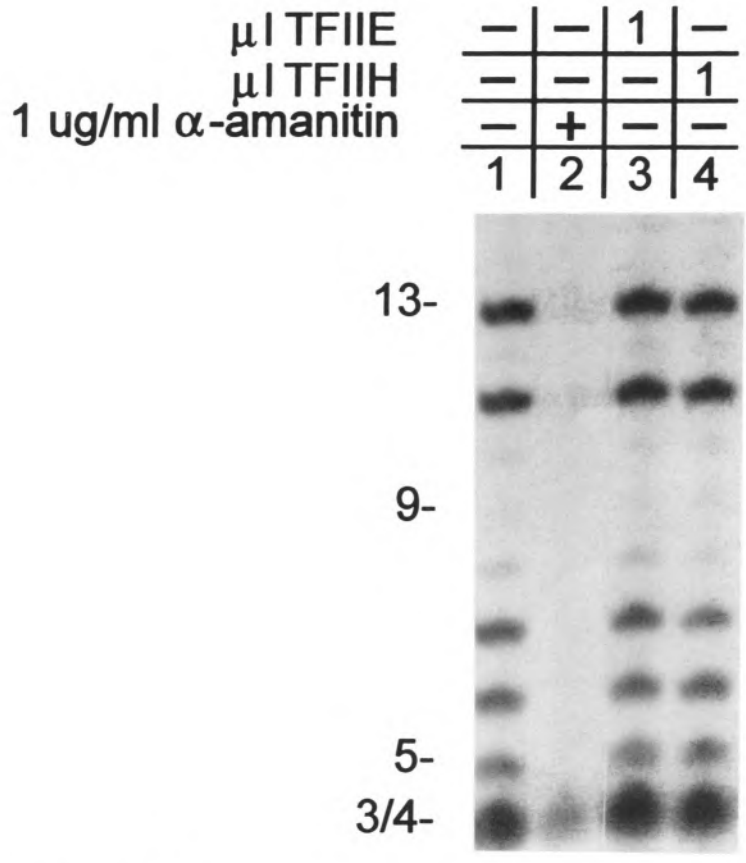

FIG. 5. The effect of exogenously added TFIIE or TFIIH on promoter clearance by preinitiation complexes assembled on pMβ5T templates. Preinitiation complexes were assembled on pMβ5T as described in the Materials and Methods section. Prior to the addition of transcription substrates, complexes were incubated with the indicated volumes of TFIIE or TFIIH for 5 min at 30°C. CpA, UTP, [α-^{32}P]CTP, and dATP were added as transcription substrates and RNAs were synthesized, purified, and resolved on 25% polyacrylamide, 3% bisacrylamide gels as described in the Materials and Methods section. The indicated reactions contained 1 μg/ml α-amanitin. The lengths of selected transcripts are indicated in the left margin.

initiation or promoter clearance might also be observed in our experiments because we deliberately selected promoters for our study that were defective at steps other than transcription complex assembly. Failure to see such stimulation could be attributed to several causes. Initiation and clearance efficiencies might be intrinsic properties that cannot be altered by regulatory factors. In this context, it is relevant to note the very recent report by Lee and Hahn (19) on the ability of TFIIB to bind to the TATA box region in a TBP-TFIIB-DNA ternary complex. TFIIB occupies the opposite DNA face from TBP in this complex, binding within the sharp bend created by TBP's interaction with TATA. If the mutation in pML5C (which changes TATA to TATC) interferes with the bending of the TATA element by TBP, this might create a preinitiation complex in which TFIIB has difficulty in interacting with its entire binding site; this could result in a preinitiation complex that is intrinsically poor in initiating transcription. If GAL4-VP16 increases the extent to which such defective complexes are assembled but cannot alter the inappropriate alignment of TBP and/or TFIIB within them, the activator would be unable to increase the poor efficiency of initiation at the pML5C promoter.

It is also possible GAL4-VP16 in particular, or acidic activators as a group, might be unable to affect steps in transcription beyond preinitiation complex assembly. There are no previous reports on initiation or clearance regulation in eukaryotes

with which a comparison may be made. However, GAL4-VP16 has been shown to interact directly with TFIIH (36), which is thought to be involved in promoter clearance (12,23); GAL4-VP16 has also been shown to stimulate transcriptional processivity in transfection and oocyte microinjection experiments (37). Our extracts might not contain sufficient levels of components necessary for GAL4-VP16 to stimulate initiation or clearance. For example, it now seems likely that adaptor factors, in addition to the TAFs present in TFIID, are required for the action of many transcriptional regulatory factors [recently reviewed in (38)]. Stimulation of steps after transcription complex assembly could require a different set of adaptor molecules from assembly stimulation; the former adaptor set might be absent or inactive in our extracts. It is also worth noting that GreA factor (9) has recently been reported to suppress abortive initiation in favor of productive transcription at an *E. coli* promoter. The GreA protein stimulates transcript cleavage by paused *E. coli* RNA polymerase ternary complexes (1) and in this sense is a functional analog of the SII elongation factor in eukaryotes. Thus, it is possible that an elongation factor(s) is required in addition to GAL4-VP16 (and adaptors) to increase promoter clearance. Finally, the points just raised can also be applied to the failure of added TFIIE and TFIIH to stimulate initiation or promoter clearance. Because we added these factors to gel-filtered transcription complexes, it is possible that additional components necessary for the loading or action of these factors might not have been present. Unpublished work of Kumar and Reinberg [cited in (38)] suggests that it might be necessary for TFIIE and TFIIH to be loaded prior to initiation in order for these factors to stimulate promoter clearance.

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