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# Transcription reinitiation rate: a potential role for TATA box stabilization of the TFIID:TFIIA:DNA complex

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## ABSTRACT

**Potential pathways that could account for observed rapid rates of transcription reinitiation were explored. A nuclear extract system was established in which reinitiation rates were observed to be kinetically facilitated and in which the rate was sensitive to TATA box mutation. Kinetic facilitation of functional complex formation could be mimicked by pre-assembling activator and certain general transcription factors on the promoter and then adding nuclear extract. The minimal activated complex with this characteristic contained general factors TFIID and TFIIA. The ability of the TFIID:TFIIA complex to complete assembly rapidly was reduced by the same TATA box mutation that reduced reinitiation rate. Band shift experiments also showed that this same mutation lowered the stability of the TBP:TFIIA complex on the DNA. The results suggest that TATA-dependent variations in retention of the TFIID:TFIIA complex after release of the polymerase could be a primary determinant of reinitiation rate, allowing diversity in promoter strength to be related to diversity in TATA element sequences.**

## INTRODUCTION

Promoters need to specify the conditions under which a gene may be induced as well as the amount of RNA transcribed from the gene. Induction is mediated primarily by upstream regulatory elements, which typically act in conjunction with basal elements near the transcription start site. The proteins that bind to these two sets of elements often cooperate to facilitate production of a functional pre-initiation complex. In many cases this appears to be sufficient for induction to occur.

The elements and factors that specify the strength of the induced promoter are much less understood. For example, yeast promoters can produce transcripts as rapidly as every 6 s or at least as slowly as every 140 s (1). These frequencies represent events that require promoters to be induced but occur after induction, i.e. they reflect the phase of continuous transcription in which the amount of RNA produced is set by the rate of reinitiation. The causes of the differences in rate of RNA production during this phase are not known. If promoter strength is taken as proportional to the rate of RNA synthesis, it is this reinitiation pathway that should make the greatest contribution.

In general, there is little information regarding the relationship between the initial induction and the rate of continuous transcription. For the rates of these two processes to differ, pre-initiation complexes and reinitiation complexes would need to use separate pathways to produce transcript. Several reports have considered the potential separation of these pathways (2–8). These reports consider the possibility that continuous transcription rates are set by reinitiation events which need not have the same controls as induction events. One way of accomplishing this uncoupling would be for factors to remain associated with the DNA template after polymerase escape and promoter clearance. These bound factors might have the potential to complete the remaining reinitiation assembly steps rapidly and could potentially do so through multiple rounds of transcription.

A number of reports have suggested that factors do remain associated with templates after polymerase escape (2–4) consistent with potential implications of early studies (5–7). These factors include TBP, TAFs, TFIIA and some activators. A requirement for activator in reinitiation was suggested by some experiments (8,9). TFIIB and subsequent assembly factors have been shown to be released in the few studies available thus far. These experiments raise the possibility that certain factors have the potential to remain on the DNA and direct continuous transcription reinitiation. If these factors can direct rapid completion of the subsequent assembly steps then high levels of transcription will be obtained after initial induction.

Rapid reinitiation *in vitro* is not universal and thus has the potential to be promoter specific. In early reports reinitiation was inferred to be rapid in one experimental system but slow in another (5,10). Subsequent reports have observed rapid reinitiation in several systems (2,11,12). Recently, we demonstrated that optimal reinitiation rates in one system depended on the sequence of the TATA box associated with the promoter (12); TATA box mutations were shown to slow the reinitiation rate. Those promoters that have been shown to direct rapid reinitiation all have near consensus TATA sequences. However, there have not been enough studies to generalize the contributions of TATA and other elements in specifying the rate of reinitiation.

In this paper we explore potential pathways for reinitiation. The approach includes the assembly and study of selected partial pre-initiation complexes on DNA. There are two aims concerning

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study of these complexes. First, we wish to use a system known to reinitiate rapidly. A goal is to identify a minimal partial complex that can complete the rest of the assembly steps rapidly and thus has the potential to account for rapid reinitiation in that system. Prior reports are consistent with 'rate-limiting' partial complexes containing combinations of TFIID, TFIIA and TFIIB (4,13–17). However, these reports did not study initiation and reinitiation rates directly. Second, we wish to observe the effect of TATA box mutations on such potential reinitiation complexes. The hope here is to identify how TATA sequences might contribute to facilitating rapid reinitiation when it occurs. The results shown below support a model in which TATA holds together a reinitiation complex containing TFIID and TFIIA, thereby allowing reinitiation to bypass the slow process of assembling this partial complex. The model provides insight into how promoter elements can separately specify promoter strength and induction sensitivity.

## MATERIALS AND METHODS

### Plasmids and DNAs

The parental template, G9-TATA-Inr, contains nine Gal4 sites upstream of a consensus TATA box and an Inr element (18). This was made by replacing the GC boxes in GC-TATA-Inr (19) with nine Gal4 sites from a G9E4 plasmid (20) using standard restriction cleavage–ligation procedures. The derivative plasmid that contains a mutated TATA sequence (TAAATAA) in the context of G9-TATA-Inr was made in the same way by replacing GC-TATA-Inr with GC-TAAATAA-Inr (12). DNA fragments (~300 bp containing nine Gal4 sites upstream of the TATA box and Inr element) used in the gel mobility shift assay were generated by PCR. The primers used for PCR were: upstream primer, 5′-CACATACGATTTAGGTGACAC-3′; downstream primer, 5′-GTCGACTCTAGAGGATCC-3′.

### Preparation of proteins

The protocols for preparing protein factors were: his-tagged human TBP (21); holo-TFIID (22); HMK-tagged human TFIIB (23); reconstituted recombinant human TFIIAαβ and HMK-tagged TFIIAγ (24); GalVP16 (25). GalAH was a generous gift of Mike Carey (UCLA) and radiolabeled TFIIA was made by attaching [$\gamma$-$^{32}$P]ATP to the HMK-tagged TFIIAγ subunit within the holo-TFIIA (26).

### Transcription and primer extension

Aliquots of 10–20 ng of parent template, G9-TATA-Inr, and derivative template, G9-TAAATAA-Inr, were used in *in vitro* transcription as described (12). For studies using pre-assembled factors, the indicated protein components were incubated with 10 ng linear template for 30 min at 30°C in 9 µl of 12 mM HEPES–NaOH (pH 7.9), 12% glycerol, 60 mM KCl and 0.12 mM EDTA. A sample of 20 µl of HeLa nuclear extract was then added as the source of remaining factors under the same conditions except that $MgCl_2$ was added to a final concentration of 8.25 mM. At different times 500 µM nucleoside triphosphates (NTPs) were added for 2 min to give one round of transcription. The amounts of protein factors used were: 1–3 ng TBP (or 4–6 µl TFIID made according to Zhou *et al.*; 22), 1 ng labeled TFIIA and 30 ng TFIIB.

All templates were constructed so that RNA could be detected by reverse transcription using CCTTATGTATCATACACATAC-GATTTAGG, leading to a 79 nt cDNA. This cDNA was separated by electrophoresis and quantified using a phosphorimager.

### Gel mobility shift assays

The indicated protein components (1–3 ng TBP or 4–6 µl TFIID preparation, 1 ng labeled TFIIA and 30 ng TFIIB) were incubated with ~5 fmol of PCR fragment for 60–80 min at 30°C in a total volume of 15 µl in 12 mM HEPES–NaOH (pH 7.9), 12% glycerol, 60 mM KCl and 0.12 mM EDTA, 6 mM $MgCl_2$, 10 mM β-mercaptoethanol, 40 µg/ml poly[d(G–C)] and 1 mg/ml BSA. To study the rate of TFIIA dissociation, complexes containing labeled TFIIA were assembled at intervals. A 100-fold excess of cold TFIIA was added to all samples ~1 h later. The timing was adjusted so that all samples could be collected and loaded on the gel simultaneously. In a background control sample, excess cold TFIIA was included during the pre-initiation. The products were resolved on a 6% polyacrylamide gel (59:1 mono:bis ratio, 5% glycerol) in TGEM (27) buffer containing 10% glycerol. Large gels (1.5 × 17 × 20 cm) were pre-run for 1–2 h at 30 mA and run for 1–1.5 h at 400 V at room temperature. For mini-gels (Bio-Rad), the pre-run was at 15 mA for 30 min followed by 35 min of resolving time at 400 V in an ice bath. The temperature of the electrophoresis buffer in the inner tank of the small gel apparatus was monitored and replaced with ice-cold buffer upon reaching 35°C. The bands were visualized and quantified with a PhosphorImager (Molecular Dynamics Inc.).

## RESULTS

The aim was to identify and understand events that have the potential to contribute to rapid reinitiation of transcription. The experimental system was designed to meet two requirements. First, it must exhibit rapid reinitiation. Second, it must be amenable to construction of partial pre-initiation complexes that might mimic those that could contribute to rapid reinitiation. We used a template containing basal elements known to specify facilitated reinitiation in the context of a HeLa nuclear extract (12). It contains a consensus TATA box and a TdT initiator element (19). In order to minimize potential complications due to endogenous activators and to be able to compare with many existing mechanistic studies, upstream Gal4 sites replaced the GC-boxes studied previously (12). The initial experiment tested whether reinitiation in this system was rapid, using the protocol developed previously (12). The amount of template used was very small (10–20 ng) so as to ensure that factors were in great excess. A short 79 nt cDNA was assayed to avoid potential complications associated with the elongation of long transcripts.

### Measurement of reinitiation rate

In the reinitiation experiment one determines the number of rounds of transcription occurring during a time course of continuous transcription. This requires knowing the amount of RNA that corresponds to a single round. This can be estimated by pre-assembling pre-initiation complexes (PICs) in the presence of the activator GalAH and allowing them to transcribe only once by using a very brief pulse of NTPs. This RNA amount can also be measured using the detergent sarkosyl to limit transcription reinitiation (6); as discussed previously, the use of sarkosyl gives

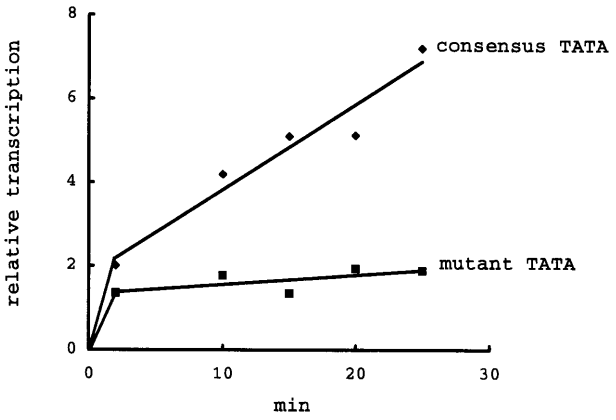


**Figure 1.** Standard reinitiation analysis of *in vitro* transcription of two templates. The activator GalAH, DNA template (G9-TATA-Inr) with either a consensus TATA (◆) or mutant TATA (TAAATAA) (■) box and HeLa nuclear extract were incubated for 60 min. NTPs were then added and duplicate reactions were stopped at 2, 10, 15, 20 and 25 min. cDNA products were analyzed and the relative transcript level is plotted.

comparable results (12). The experiment to determine the amount of RNA made in a single round is shown in Figure 1 (top curve). The amount corresponds to the 2 min point, which is at the completion of the burst of transcript produced when the pre-assembled pre-initiation complexes synthesize RNA synchronously (figure 3 in ref. 28).

Following this burst, transcription enters a roughly linear phase of continuous RNA synthesis. The rate of reinitiation is calculated from this phase in which protein factors are not limiting (10 ng of plasmid is used; 12). Using the 2 min level as the amount from one round of transcription the slope of the upper line indicates that a round of transcript is made every ~9–10 min during the continuous RNA synthesis phase. This half-time of ~5 min is comparable with results obtained previously using GC-boxes to stimulate a closely related promoter (table 2 in ref. 12).

This time may be compared with the time required to complete the first round of transcription, which includes the slow process of assembling the pre-initiation complex followed by more rapid production of the 79 nt RNA. Such experiments have been done on many promoters, yielding half-times for the first transcription round which are typically 10–20 min (2,5,6,10,29). In this case the time is closer to the lower limit (data not shown). This is somewhat faster than we observed previously (12,28), possibly due to the current use of more concentrated preparations of HeLa nuclear extract. The results indicate that the reinitiation rate on this template is twice as fast as the rate of producing the first round of transcript.

Figure 1 also shows the same experiment repeated on a template with a mutant TATA box. The template differs only in that it contains a TAAATAA sequence in place of the consensus, TATAAAA. The mutation causes a modest reduction in the level of initial pre-initiation complexes that form. The same mutation causes a greater reduction in reinitiation during the continuous phase of transcription (as reported previously on a different template; 12). The consequences of this preferential effect on reinitiation can be seen by comparing transcription from the two templates at 2 and at 25 min after addition of initiating nucleotides. Just after nucleotide addition the amount of transcript made from the consensus TATA template is 130% higher than that from the mutated TATA template, reflecting the modest difference in the number of pre-initiation complexes formed. By 25 min the ratio has increased to 350%, reflecting the dominant effect of differing reinitiation rates in determining the relative strength of these promoters.

## Rates observed when partial complexes are pre-assembled

As discussed in the Introduction, the most likely source of rapid reinitiation on the consensus TATA template is that after polymerase II initiates RNA synthesis, some factors remain associated with the promoter. If this occurs then reinitiation will not require that these factors be re-bound. Thus the time required for their binding will be saved during each round of reinitiation, allowing the rate of the process to be facilitated. Prior studies of fractionated systems have identified various partial complexes whose formation has been suggested to be rate-limiting (Introduction). In this section we assess whether forming these partial complexes allows transcription to go forward rapidly. This characteristic should be a prerequisite for a potential role of such partial complexes in directing rapid reinitiation. The aim is to identify a range of partial complexes that are candidates for directing reinitiation at a facilitated rate.

The protocol differs from most prior studies in using an experimental system that has been shown directly to involve rapid reinitiation (Fig. 1). Various candidate complexes involving general transcription factors are pre-assembled on the DNA. Then the same nuclear extract used in the above reinitiation studies is added to allow completion of the assembly pathway. The goal is to learn which, if any, of the candidate complexes reduce the time required for pre-initiation complex assembly as a consequence of being pre-formed on the DNA. At various times after addition of nuclear extract, samples are removed and the functional complexes are assayed using a 2 min pulse of NTPs. The time required for full assembly is compared with a control where the candidate general factors are not pre-assembled on the DNA, but instead are added in free form along with the nuclear extract. The times chosen for this assay are within the first few minutes after extract addition. This is so that any rate differences that exist will be apparent, in contrast to using long times at which the sample and control reach a common end saturation point. In the initial experiments the activated consensus TATA template is used. In all cases GalAH is employed as the activator.

The minimal complex should contain TFIID, which is required for binding of other factors. Figure 2a shows that TFIID pre-bound on this activated template does not direct faster assembly of functional complexes compared with adding TFIID along with the other free factors (compare bottom two curves). Apparently the addition of TFIID to this activated template is not sufficient to save time during the assembly of functional initiation complexes.

Numerous earlier studies have suggested that the rate-limiting step in the step-wise assembly pathway may be passed after TFIIB is added (4,14,15,30). Thus we repeated the basic experiment but now pre-assembling factors up to and including TFIIB. When this TFIID:A:B complex is pre-assembled on the activated template a different result is obtained in that this combination of pre-bound factors stimulates the rate at which functional pre-initiation complexes assemble (Fig. 2a, upper curve). The data show that this is true at every time point assayed

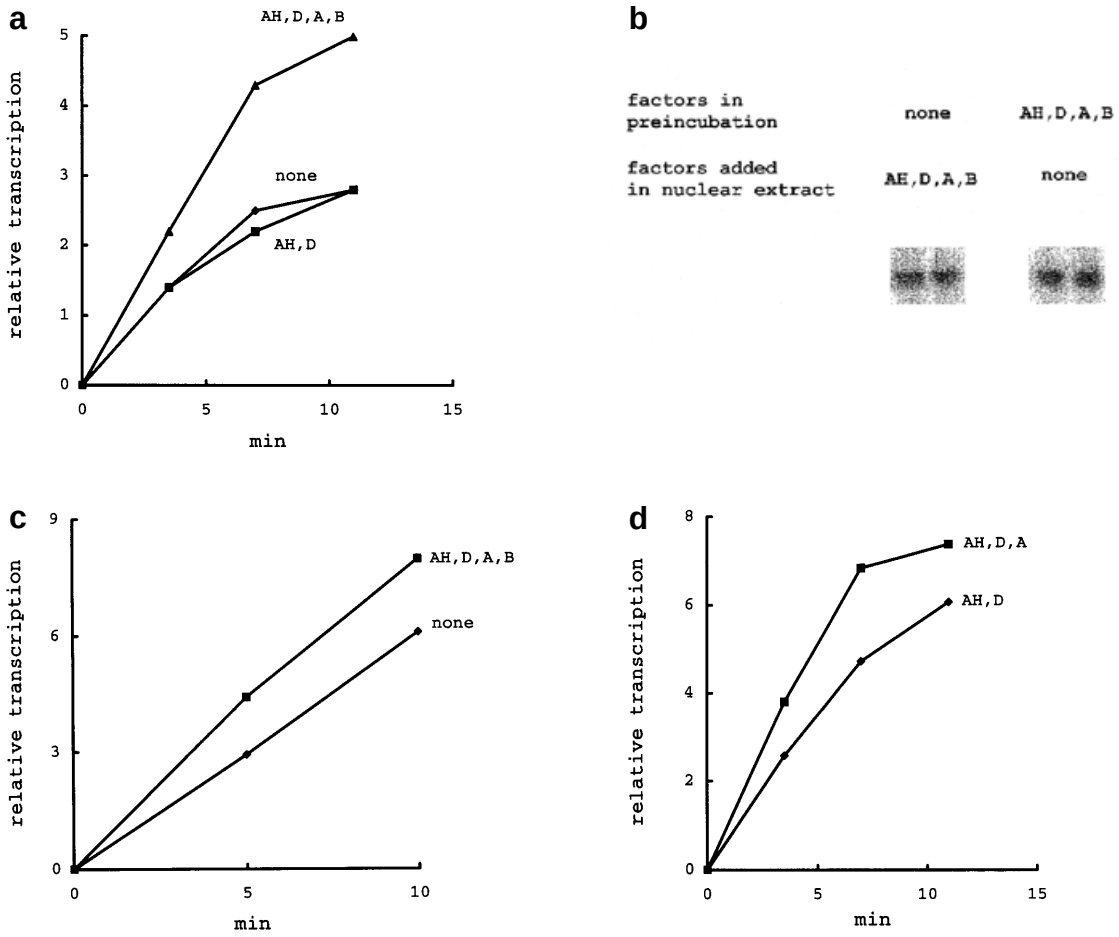


**Figure 2.** The rate of one round activated transcription can be stimulated by pre-assembling GalAH:TFIID:TFIIA:TFIIB on DNA. The indicated transcription factors were mixed with DNA during a 30 min pre-incubation. HeLa nuclear extract as the source of remaining factors was then added for additional times as indicated. One round RNA synthesis was then initiated by supplying NTPs for 2 min. AH, GalAH; D, TFIID; A, TFIIA; B, TFIIB. (**a**) When pre-assembling with G9-TATA-Inr with a consensus TATA box, AH:D:A:B (▲) can make transcription go forward faster. Pre-incubating only AH and TFIID (■) has no effect compared with the control (◆). (**b**) Pre-assembling AH-D-A-B does not increase the signal after a 60 min incubation followed by a 2 min pulse. (**c**) Pre-binding AH,D,A and B (■) on the TAAATAA template gives a modest stimulating effect compared with control without pre-bound factors (◆). (**d**) Dropping out TFIIA from AH:D:A (■) to form AH:D (◆) on a consensus TATA template reduces the rate at which functional pre-initiation complexes form.

with the rate enhancement being ~2-fold. Other data show that this stimulation is of rate rather than an effect of changing the number of complexes that form, i.e. Figure 2b shows the ultimate level of transcript when a long time is used to allow both pre-assembled and free templates to become saturated with pre-initiation complexes. At saturation, pre-binding of TFIID:A:B does not give a greater signal than adding the same free factors to supplement the nuclear extract. The comparison shows that pre-binding stimulates the rate without increasing the number of complexes formed.

This experiment was repeated using the template containing a mutated TATA box, where reinitiation was not strongly facilitated (Fig. 1, lower curve). Figure 2c shows that pre-assembly of the TFIID:A:B complex does lead to a detectable rate enhancement but the effect is less than on the consensus TATA template. To derive a quantitative comparison of the effect on the two templates we performed repeated comparisons at a single time point (7 min). In each experiment the effect of pre-assembling TFIID:A:B was compared with samples in which the same factors were added in free form. Table 1 shows that at 7 min, there is an ~60% increase in signal caused by preincubation with the consensus TATA template and an ~25% increase with the non-consensus TATA template. We infer that the TATA sequence plays a role in directing the rate at which the TFIID:A:B complex can complete assembly of functional pre-initiation complexes.

**Table 1.** Effect on PIC assembly of pre-binding GalAH, TFIID, TFIIA and TFIIB on DNA[a]

| TATA sequence | Increase (%)[b] |
|---|---|
| Wild-type TATAAAA | 61 ± 12 |
| Mutant TAAATAA | 27 ± 17 |

[a]After pre-binding the nuclear extract was added into the reaction and incubated for 7 min before the addition of NTPs. [b]The data represent average values from three or four experiments. The increase is calculated compared with values obtained without pre-binding of factors to the DNA.

Many early studies implicated TFIIB in rate-limiting steps (4,14,15,30). However, these were done using systems typically lacking TFIIA and did not measure rates directly. Subsequent direct rate studies using activated transcription have suggested that TFIIA is a key factor in overcoming potential rate-limiting steps (13,16,17,24,31). Figure 2d uses the above protocol to assess the role of TFIIA in this activated system. The experiment compares the rates of completion of assembly of pre-initiation complexes with and without TFIIA added to a TFIID-containing template.

The comparison of the two curves shows that addition of TFIIA to a TFIID-containing template is sufficient to lead to a rate enhancement. The extent of the enhancement appears to be somewhat less than that observed with the TFIID:A:B complex; however, repeated experiments do not confirm that this small reduction in stimulation is statistically significant. Thus addition of TFIIB appears to have a modest effect. From these experiments we infer that addition of TFIIA to a TFIID:DNA complex is a key step in stimulating the rate of assembly in this system. The result is in agreement with studies that used fully fractionated systems (13,16,17). We attempted experiments that used a pre-assembled TFIID:B:DNA complex to assess the effect of bypassing TFIIA (data not shown). However, in contrast to the effect of pre-forming TFIID:A:DNA complexes, those pre-incubations were problematic in that they led to significant reductions in the ultimate number of pre-initiation complexes formed. This is expected based on the role of TFIIA in activation and anti-repression (reviewed in 32,33). The reduced number of pre-initiation complexes did not appear to assemble more rapidly although the interpretation is complicated by the lowering of the number of complexes formed.

In summary, these experiments have used a common system to demonstrate: (i) the minimal activated complex that can direct subsequent assembly steps at a stimulated rate contains TFIID and TFIIA; (ii) the extent of this rate stimulation depends on the sequence of the TATA element; (iii) in this system reinitiation also occurs at a stimulated rate and also depends on the sequence of the TATA element (Fig. 1). These comparisons suggest that the ability of templates to hold together the TFIID:TFIIA complex through TATA could be a key determinant of reinitiation rate. This view is supported by a recent result demonstrating that a stable TFIID:TFIIA complex is left behind after initiation at an activated promoter that directs rapid reinitiation (2). In the next section we explore whether the TATA sequence can contribute to the stability of TFIIA-containing complexes (34–36).

## TFIIA dissociation and the effect of TATA sequence

The aim of these experiments was to explore what contributes to the stable association of TFIIA with the promoter. We developed a new approach that assays directly the extent to which TFIIA is present in various complexes. HMK-tagged TFIIA (26) was radioactively labeled. Band shift experiments were done with TFIIA as the only source of radioactivity in the system. Autoradiography provided a simplified pattern as compared with using radiolabeled DNA; only those complexes that contain TFIIA will be visible upon autoradiography. The stability of labeled TFIIA in all complexes can be followed directly by addition of unlabeled TFIIA and then following the subsequent loss of label. The potential contributions to the stability by the activator GalAH, TBP and TFIIB and the TATA sequence on the template will be assayed.

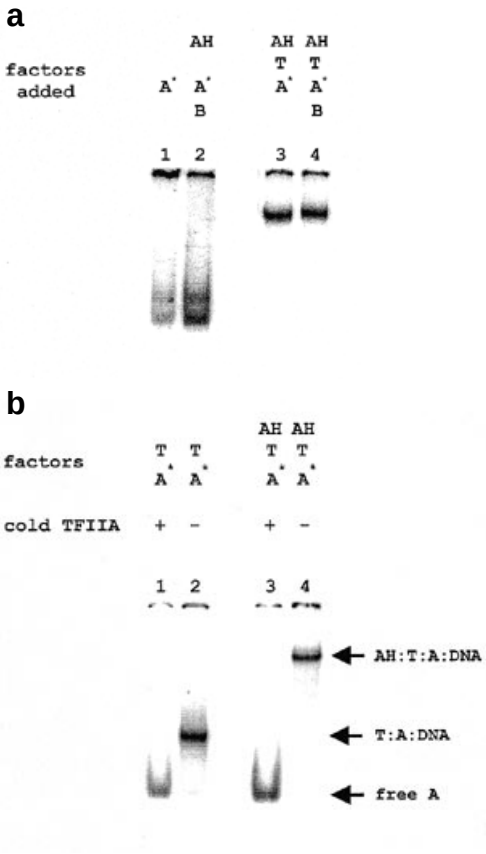


**Figure 3.** Gel mobility shift assay using radiolabeled TFIIA. AH, GalAH; T, TBP; A*, labeled TFIIA; B, TFIIB; these are present as indicated. All lanes include the DNA fragment with TATA initiator and Gal4 sites. (**a**) Formation of complexes containing labeled TFIIA depends on TBP. Lanes 1 and 2, migration of free TFIIA; lane 3, the AH:TBP:TFIIA complex; lane 4, the AH:TBP:TFIIA:TFIIB complex. (**b**) Addition of an excess of unlabeled TFIIA in lanes 1 and 3 abolishes the labeled TFIIA in complexes (see arrows).

Figure 3 establishes the feasibility of using this new assay procedure. Various combinations of TBP, TFIIB, GalAH and radiolabeled TFIIA were mixed to form complexes on a DNA fragment containing nine Gal4 binding sites upstream of the consensus TATA box and an initiator element (Inr) (Materials and Methods). The complexes were resolved on a native polyacrylamide gel in a standard gel mobility shift protocol. The experiment differed from a conventional assay only in that it was a labeled TFIIA-containing (rather than a labeled DNA-containing) band whose shift was observed. Note that the free TFIIA sometimes appeared as a doublet (as in lanes 1 and 2). This may be due to partial dissociation of the labeled γ subunit of holo-TFIIA while migrating through the gel; this should not strongly influence the analysis as the isolated γ subunit cannot bind a TBP–TATA complex (37,38).

Inclusion of all components, TBP, TFIIA, TFIIB, GalAH and DNA, led to a shifted band at the highest position on the gel (lane 4), as expected from the known assembly pathway (39). If TFIIB was omitted (lane 3) the complex had a slightly increased mobility, as expected. If TBP was omitted the TFIIA was not shifted at all (lane 2); this was expected as TBP is required to recruit TFIIA to the DNA. The presence of DNA was required for efficient formation of all these bands (data not shown). We confirmed the identity of these various bands by comparison with

parallel experiments using labeled DNA (data not shown). We conclude that the labeled protein band shift assay is suitable for identifying complexes containing TFIIA.

The additional control experiment of Figure 3b illustrates the advantage of the system for studying the dissociation of TFIIA from such complexes. The point here was to show that simple addition of unlabeled TFIIA can serve as a chase and allow direct measurement of the loss of TFIIA from various complexes. In the presence of 100-fold excess unlabeled cold TFIIA, the labeled hot TFIIA did not detectably mark the TBP:TFIIA:DNA complex (compare lanes 1 and 2) or the same complex with added activator (compare lanes 3 and 4). Thus one can follow release of TFIIA from multiprotein complexes simply by adding excess cold TFIIA and observing the replacement of the hot TFIIA as it dissociates. The protocol is more simple than prior successful ones that, for example, chase the various complexes with pre-assembled TBP:DNA complexes (35) or AH:TBP:DNA complexes.

Having established the assay, the initial objective was to measure the dissociation of TFIIA from various complexes formed on DNA with a consensus TATA box. The TBP:TFIIA:DNA radioactive complex was formed by pre-incubation. Then a 100-fold excess of cold TFIIA was added. Samples were removed at various times and run on a native polyacrylamide gel. After electrophoresis, the radioactive bands (Fig. 4, bottom left) corresponding to the original complex were quantified and normalized to the 0 min challenge time. This monitors the rate of loss of labeled TFIIA from the complex.

The half-time ($t_{½}$) of TFIIA dissociation from the ternary complex was ~30 min (Fig. 4, upper curve; time corresponding to loss of half the signal), which is somewhat faster than reported previously using different conditions (reported as 49 min; 34). This dissociation does not appear to be influenced by a spontaneous decomposition of components during the 1 h chase time; control experiments without excess cold TFIIA showed that the level of labeled TFIIA in complexes did not diminish (data not shown). We infer that the assay can measure the stability of TFIIA in complexes and that the half-time is ~30 min on this DNA template under the conditions of this experiment.

We next determined whether a mutation in the TATA box would alter the stability of TFIIA within the TBP:TFIIA:DNA complex. The experiment follows that just described with the sole change being that the TATA box sequence was mutated. The absolute level of complex formed initially was reduced by ~50% from that using the consensus TATA DNA (data not shown); this is consistent with the lower level of pre-initiation complex formation on the non-consensus TATA promoter (Fig. 1, compare the first data points measured at 2 min of both curves). In order to compare half-times for dissociation the curves were individually normalized to the observed retention of labeled TFIIA at the outset of each experiment.

Figure 4 (bottom curve) shows that TFIIA dissociates more rapidly from complexes in which the TATA box has been mutated away from the consensus. The $t_{½}$ of TFIIA dissociation was ~3-fold reduced from the 30 min observed with the wild-type TATA sequence. We conclude that TFIIA retention in TBP:TFIIA:DNA complexes can be influenced by the sequence of the TATA box.

Finally, we investigated whether other protein factors could influence the lifetime of TFIIA within partial pre-initiation complexes. We used the mutant TATA DNA because its lesser

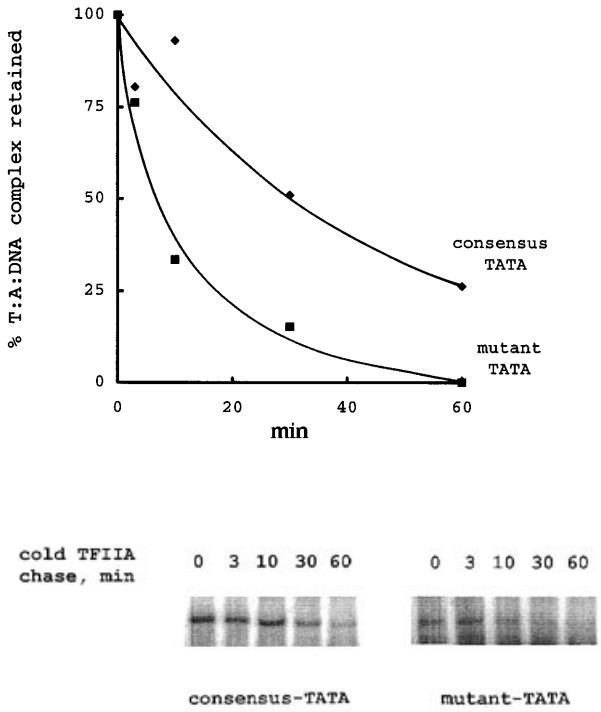


**Figure 4.** Labeled TFIIA (A*) in the ternary complex TBP:TFIIA:DNA is released with different rates depending on the TATA sequence. Complexes containing the indicated combinations of factors (abbreviations as Fig. 3) were pre-assembled on DNA and then a 100-fold excess of cold TFIIA was added for the indicated times (including 0 min) before being resolved in a native gel (radioactive bands in lower panel). Control reactions (not shown) which contained an excess of cold TFIIA during binding reactions effectively abolished shifted bands and served as background. Data from Phosphorimager analysis (upper panel) were normalized to the 0 min chase time.

lifetime of holding TFIIA allows for higher sensitivity in observing potential increases caused by addition of other factors. To increase the sensitivity further we altered the electrophoresis system to reduce running times, thus maximizing the signal. This increased the apparent lifetime of TFIIA retention somewhat (27). The wild-type half-life is now 35 min and the TATA mutation reduces this by a factor of 2 to 15 min (Fig. 5 and data not shown). The experiment will determine whether other factors enhance the retention of TFIIA on the mutant TATA template by assaying the amount remaining at this 15 min point.

In this protocol radioactive TFIIA-containing complexes are assembled with the addition of either the activator or TFIIB. An excess of cold TFIIA is then added. After 15 min the experimental and control samples (cold TFIIA added with no chase time) are compared using the band shift analysis and the amount of hot TFIIA retained is determined.

The results (Table 2) show that activators GalAH and GalVP16 do not lead to higher retention of TFIIA. TFIIB addition leads to a reduction in retention, suggesting that it may somewhat destabilize TFIIA within the complex. The same destabilization was noted when the wild-type TATA sequence was used (Table 2). We also replaced TBP with TFIID but at accessible concentrations the TFIID:TFIIA complex on mutant TATA DNA was too weak to obtain reliable data on the TFIIA lifetime. Using the wild-type TATA the signal improved somewhat, but no significant change

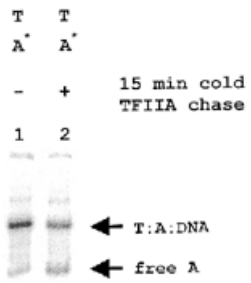


**Figure 5.** Example of mini-gel band shift assay using labeled TFIIA and mutant TATA DNA. Lane 1, TBP:TFIIA:DNA complexes; lane 2, the ~50% retention after a 15 min chase with excess cold TFIIA. Abbreviations are as in Figure 3.

in TFIIA retention was noted in preliminary experiments (data not shown). Overall the data suggest that the dominant factors in holding TFIIA at the promoter are the TATA sequence and of course TBP, with TFIIB potentially playing a destabilizing role.

**Table 2.** Effect of other proteins on retaining TFIIA on DNA

| | TFIIA remaining in complex (%)[a] |
|---|---|
| Mutant TATA (15 min challenge time[b]) | |
| T,A | 56 ± 9 |
| T,A,B | 30 ± 4 |
| T,A,AH | 50 ± 9 |
| T,A,VP16 | 44 ± 13 |
| Wild-type TATA (35 min challenge time[b]) | |
| T,A | 51 ± 9 |
| T,A,B | 28 ± 6 |

[a]The data represent average values from two to four experiments. T, TBP; A, TFIIA; B, TFIIB; AH and VP16, Gal fusion activators.
[b]The lifetime of TFIIA in TBP:TFIIA:DNA is ~15 min if complexed with the mutant TATA box (TAAATAA) and ~35 min when complexed with the wild-type TATA box (TATAAAA) (text). After the indicated complexes were formed with labeled TFIIA, a 100-fold excess of cold TFIIA was added for the times indicated, followed by analysis of TFIIA retention.

## DISCUSSION

In this report we explored potential pathways that could lead to diversity in rates of transcription reinitiation and thus in promoter strength. There are now several reports indicating that reinitiation can occur faster than initiation (2,5,11,12,40). Recently, we suggested that the sequence of the TATA box can be an important determinant of reinitiation rate, which in turn dominates the overall rate of transcription (12). A primary goal of the current data was to suggest potential mechanisms for the role of TATA sequences in this process. This required exploring potential pathways for transcription reinitiation.

It is now well established that the pathways for formation of pre-initiation complexes and reinitiation complexes can be quite different (2,4,5,7,11,12), i.e. certain factors have the potential to remain associated with the promoter after escape of the polymerase into elongation phase ('promoter clearance'). We used this information to explore whether pre-assembled partial complexes containing these factors have properties consistent with the expected properties of promoters in reinitiation. These include: an ability to complete pre-initiation complex assembly more rapidly than when the factors are not pre-assembled; a role of TATA sequence in the stability of the complex; evidence from the work of others that such a complex could be left intact after promoter clearance. In view of the above experiments the TFIID:TFIIA:TATA complex, whose properties have been studied in detail (35,41), best meets these criteria.

The addition of TFIIA to the TFIID:DNA complex occurs early in the ordered assembly of general transcription factors at the promoter (39). The current data show that the complexes that immediately precede or follow TFIIA addition in the pathway have fewer characteristics expected to be associated with the facilitation of rapid reinitiation. First, consider the TFIID:DNA complex. In prior studies of the issue, TFIID has been found to be left behind after promoter clearance (3,4,7). However, the key consideration is that preformation of this TFIID:DNA complex does not save time in the assembly pathway at the promoters studied here and in some prior reports (16,17). Thus if such a complex was left behind after promoter clearance one would expect reinitiation to be no faster than the original assembly that led to initiation.

One caveat is that *in vitro* experiments use relatively low concentrations of TFIID (as opposed to TBP). Thus pre-bound TFIID may not fully occupy the promoter because when it dissociates it may re-bind slowly. In this context the critical role of TFIIA may be indirect in preventing transient dissociation of TFIID, consistent with its well-known role in stabilization of TFIID/TBP binding (13,16,24,34,36). Indeed we have found that at high concentrations pre-binding of TBP can save some time during pre-initiation complex formation even in the absence of other pre-bound factors (unpublished results). This has been observed previously by Hawley and colleagues (34) who also note potentially complex effects of TATA mutation.

Addition of TFIIB to form a TFIID:A:B:DNA complex does not meet certain criteria for directing rapid reinitiation. Prior reports indicated that complexes containing TFIIB have passed the 'rate-limiting' step (4,14,15). We confirm that a TFIID:A:B:DNA complex can proceed rapidly along the assembly pathway using the same promoter in which we show reinitiation to be rapid. However, in several studies TFIIB has been found to be released rapidly following promoter clearance (2–4,7), making the TFIID:A:B:DNA complex an unlikely candidate to direct reinitiation. Our data also raise the possibility that retention of TFIIB could actually somewhat destabilize the critical association of TFIIA with the promoter.

These considerations suggest that the TFIID:TFIIA:TATA complex is the most likely candidate to be left behind as the initiation complex breaks up and to have the capacity to direct rapid reinitiation. The current data make the connection between certain properties of this complex and the rate of reinitiation, i.e. a TATA mutation was shown to have two effects in a defined promoter system; it reduced the rate of reinitiation and it reduced the stability of the TBP:TFIIA:DNA complex. Thus at a TATA mutant promoter one would expect a lower retention of TFIIA. This in turn could lead to the slower rates of reinitiation that were observed. The central role of TFIIA addition in reinitiation adds to its prominent role in initiation as a stabilizer of TFIID/TBP

binding (34–36,42) and a mediator of activation and anti-repression (13,24,37,38,42–44).

Among the questions raised by this potential reinitiation pathway are: how common is it and how does the TATA sequence have this important influence? These two considerations are closely related. It is well established that the primary role of TATA is to recruit TBP and that TATA mutations destabilize the association of TBP (45–47). The existing studies on retention of factors after initiation have in common that TFIIB was released and TFIID was retained (2–4,7); only one of the studies involved TFIIA (2) and it was retained. However, all of these studies used promoters containing TATA elements that bind TBP very strongly. We speculate that when poor TATA elements are used, the retention of the TFIID:TFIIA:DNA complex would be only transient. This would lower the probability of it being present to direct reinitiation and thus lower the reinitiation rate. In this mechanism the TATA box exerts control over the reinitiation rate, as observed in the few experiments thus far conducted to address this issue (5,10,12). This should make a major contribution to the known effects of TATA sequence on transcription levels (45–47).

One important unanswered question relates to the involvement of activators. In certain systems the removal of activator appears to block the reinitiation pathway (2,8,11,40). An activator requirement would have the advantage of providing a physiological mechanism to turn off continuous reinitiation. The current data did not reveal a stabilizing effect of activator on TFIIA retention. We can only speculate that such effects may exist and would be revealed in systems using a more complex mix of factors than the simple purified components used in the current experiments.

In any case, the reinitiation pathway is seen as relying on competition in which the lifetime of TFIIA (or that of the TFIID:TFIIA:DNA complex) is critical. The competition is between the loss of TFIIA and the re-binding of TFIIB and subsequent factors. If the TATA sequence is strong, the TFIID:TFIIA:DNA complex should stay together long enough to bind subsequent factors before dissociating and this would direct rapid reinitiation. TATA mutation is known to reduce TBP retention times (45–47) and also, as shown here, to reduce TFIIA retention times; promoters with non-consensus TATA sequences would thus have a lower probability of binding TFIIB and subsequent factors to direct rapid reinitiation. Observed rates of reinitiation could vary from fully facilitated for consensus TATA promoters to non-facilitated for poor TATA promoters to intermediate levels for other TATA elements. Thus promoters that were simultaneously induced by common signal transduction pathways could differ greatly in promoter strength due to differences in reinitiation rate. Diversity in basal promoter elements (41,48), including the TATA sequence, would contribute to these differences, allowing DNA sequence to specify different RNA levels for genes induced by the same signals. Such diversity in promoter strength within sets of commonly induced genes should make important contributions to the diversity of physiologically appropriate gene transcription.

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