Transcription activation by GC-boxes: evaluation of kinetic and equilibrium contributions

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ABSTRACT

Basal and GC-box activated transcription were compared by various assays in order to learn the basis for an 8-fold difference observed under standard conditions. The time required for forming pre-initiation complexes and initiating and elongating RNA synthesis, and the extent of transcription reinitiation were found to be quite similar for basal and activated transcription, with complex formation being the slow step in both cases. The extent of activation was found to vary widely with the amount of template DNA used. Activated pre-initiation complexes were found to have a higher stability than basal complexes. The data are interpreted to indicate that GC-box elements do not stimulate the rate constants for critical steps in this system but rather increase the equilibrium constant for pre-initiation complex formation, probably by 10–30-fold.

INTRODUCTION

High level transcription of RNA polymerase II genes requires activator proteins (reviewed in 1–3). In the absence of activators, transcription can occur but these basal levels are quite low. Activated and basal transcription use a common subset of general transcription factors with additional factors being required to mediate the effect of activators (reviewed in 4). Numerous studies have addressed how different activators enter into the multi-step, initiation pathway to increase transcription levels but a consensus model has not yet emerged. Many of these studies have focused on detecting the interactions of activator proteins with the general transcription factors (reviewed in 2,4).

The first cellular activator protein to be isolated was HeLa Sp1 which was identified initially as a factor that activated transcription by binding to the SV40 GC-box elements (5). Sp1 can function both *in vivo* and *in vitro*. Its activation mechanism has been proposed to involve at least the following general transcription factors: TBP (6), IID (7), IIB (8) and IIA (9) as well as TAF proteins (10,11). The manner in which these interactions lead to enhanced transcription initiation has not been settled (see 12 for a discussion).

Activators can enhance transcription by promoting one or more of a series of broadly defined sequential steps that have been identified in simpler transcription cycles (reviewed in 13). In thermodynamic models basal transcription complexes form poorly and activator increases their number by stabilizing them. In other models the basal transcription complexes are sufficiently stable to form. In such models the activator can increase the rate constant for assembling pre-initiation complexes (14). As a third alternative, activator could cause a pre-initiation complex that initiates RNA slowly to proceed rapidly (15). In this report we study RNA polymerase II transcription activated by Sp1 at GC-boxes and find that the data strongly favor a thermodynamic model.

The approach involves applying a set of assays to compare basal and activated transcription. This uses a HeLa cell nuclear extract that contains all factors necessary for both basal and GC-box dependent activated transcription. Activated transcription is assayed using a template containing a consensus TATA element, an Initiator (Inr element; 16) and the six SV40 GC-boxes located upstream to bind Sp1. This is compared with transcription from a basal template that lacks all GC-boxes but has the same basal elements. Simple assays are established to measure the half-time for forming pre-initiation complexes, for following the time required for initiation, and for following how many complexes form and how stable they are. The results lead to a model that is compatible with prior studies and provides a means of evaluating the effects of transcription activators that complements existing methods.

MATERIALS AND METHODS

The activated template contains six SV40 GC boxes upstream of two optimally placed basal elements: a consensus TATA element and the terminal deoxynucleotidyl transferase (TdT) gene initiator element (plasmid VII of 17). Basal templates were identical in the promoter region except that they lacked upstream GC-boxes (plasmid IV of 17). The HeLa cell nuclear extract was made as described (18).

The *in vitro* transcription assays contain 25 µl HeLa cell nuclear extract in D buffer (18), 8.25 mM Mg^{2+}, 5–1000 ng template (as indicated) and 1000 ng of pGEM as carrier DNA in a final volume of 40 µl. Standard reaction conditions include 0.5 mM nucleoside triphosphates (NTPs) and incubation at 30°C for 30 min. Variations are noted in the text and legends. In single round transcription assays, a 30 min pre-incubation was followed by a 2 min pulse with NTPs. RNA products synthesized in each

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reaction were detected by extension of a labeled primer with reverse transcriptase. The 79 nt labeled cDNA product was then separated in a 6% urea–polyacrylamide gel and visualized by autoradiography. To quantify the RNA product the radioactive bands corresponding to the correct product were scanned with a gel scanner or cut from the gel and counted.

Experiments in which the amount of DNA was varied and transcription was assayed were modeled by considering a simple bimolecular reaction. The reactants are considered to be a nucleoprotein complex containing DNA and a factor that react to form a complex that inevitably leads to transcription. Plots of RNA levels versus amount of DNA were constructed. Best fit binding curves were derived for both activated and basal DNA templates. The relative binding constants associated with these curves were found to differ by 30-fold, assuming that basal and activated transcription reach the same level at infinitely high concentrations of DNA. Curve fitting the data without this assumption reduces the 30-fold difference to 10-fold.

The relative stability of pre-initiation complexes is evaluated with the following protocol. First, pre-initiation complexes are allowed to form on both basal and activated templates during a 1 h incubation. Next, a 10-fold excess of challenge template is added to the reactions and incubation is continued for an additional 45 or 90 min, as indicated. NTPs are then added to allow the remaining stable transcription complexes to produce RNA. This is quantified using primer extension as described above and a phosphoimager. The challenge template was G5TILuc (19) which contains five Gal4 binding sites upstream to the identical consensus TATA and initiator elements used above. It was pre-bound with GAL-AH (20) prior to addition. Transcription from this challenge template is not included in the signal as its RNA does not hybridize to the primer used to detect RNA from the original templates.

RESULTS

Establishment of standard activation conditions

The standard transcription assay uses HeLa nuclear extract, nucleoside triphosphates (NTPs) and a mixture of promoter and non-promoter (carrier) DNA. Each reaction contains 100 ng of the appropriate supercoiled DNA template and 1000 ng of carrier DNA. The inclusion of very large amounts of carrier DNA is a long-established condition to reduce occlusion of the template with non-specific DNA-binding proteins (18). Such conditions minimize activation by anti-repression mechanisms because the activator need not clear the template to make it available for general transcription factors (21). Thirty minute reactions in the presence of all the necessary components including NTPs are done in parallel using the activated and basal templates. This protocol compares continuous transcription from promoters that do or do not contain GC-boxes. The RNAs from both samples are detected by primer extension.

The autoradiograph (Fig. 1) shows that both activated (lane 1) and basal (lane 2) transcription can be detected. The bands were excised from the gel to determine the radioactivity associated with each RNA sample. Repeated experiments showed that the presence of GC-boxes on the template leads to an ~8-fold activation of transcription. This activation is within the lower portion of the range of activation by Sp1 seen in prior experiments which varies quite considerably and can reach several hundred fold for very weak basal promoters. The basal levels in this

Figure 1. Standard assay conditions, yielding an 8-fold activation. *In vitro* transcription reactions contained 100 ng activated template (lane 1) or 100 ng basal template (lane 2) both in the presence of 1000 ng pGEM carrier DNA. The reaction was stopped after 30 min continuous transcription. The autoradiograph shows the 79 nt cDNA of the transcript, denoted with an arrow. The bands were cut out and counted; repeated experiments averaged an 8-fold difference in RNA levels.

experiment are relatively high because the template contains both TATA and Inr elements (22). These experimental conditions, associated with 8-fold activation, will be used in each of the following assays. These assays are designed to separate sub-steps in the transcription pathway to explore whether activator enhances any step in a way that accounts for the 8-fold activation.

The half-time for pre-initiation complex formation

First, we established an assay for the half-time of pre-initiation complex formation by adapting prior protocols (23). There are two purposes for this experiment. One is to learn how fast pre-initiation complexes assemble in this system. The second more important purpose is to learn whether the 8-fold difference in basal and activated transcription levels has its source in an 8-fold acceleration of the rate constant caused by the activator. In this protocol the template is incubated with nuclear extract for various times. At each of these times samples are removed to assay for the number of functional pre-initiation complexes that have formed. This assay is accomplished by adding NTPs and allowing each functional complex to produce RNA. In order to minimize potential complications due to production of more than one RNA from a pre-initiation complex the synthesis is restricted to a 2 min pulse with nucleotides (23 and also see below).

The experiments were done in parallel using the basal and activated template systems. The stronger signal strength of the activated system allows for a more accurate determination. The data show that the formation of a full complement of activated pre-initiation complexes is nearly complete within 1 h (Fig. 2b). The half-time for the reaction is 20–25 min, in agreement with prior studies of assembly of various pre-initiation complexes involving RNA polymerase II (23–25).

Figure 2a shows the same experiment done using the basal template, which lacks GC-boxes. The experiment shows that yet again the reaction is nearly complete within 1 h. The half-time for
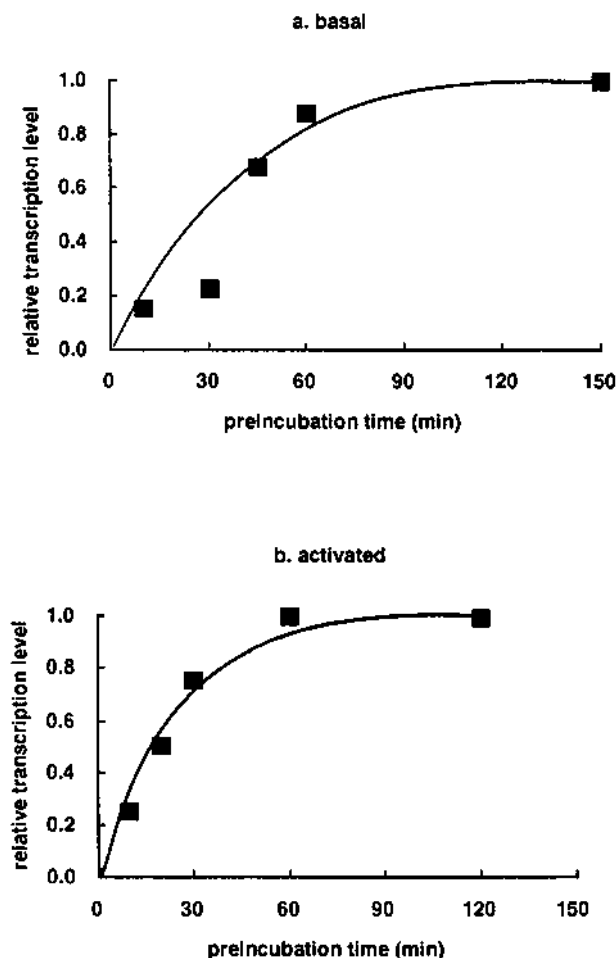
Figure 2. The time required to form a pre-initiation complex. 100 ng of basal (**a**) or activated (**b**) template was mixed with Hela nuclear extract and 1 µg pGEM carrier DNA. The reactions were incubated at 30°C for various times as indicated. NTPs were then added to allow initiation and the reaction was stopped 2 min later. The relative transcription level was plotted versus the reaction time. The primer extension products of the RNA were detected and counted as in Figure 1.

forming the pre-initiation complex is ~30 min. Due to the lower signal strength in the basal system the data are not as accurate and the result cannot be considered to differ significantly from that for activated transcription, although a lag in accumulation of signal cannot be excluded. In any case the data show that the 8-fold difference between the levels of basal and activated transcription does not have its source in a 8-fold reduced half-time for forming activated pre-initiation complexes. The two half-times differ very little, if at all. Instead the activated signal is simply ~8-fold higher than the basal signal at each experimental time point.

The half-time for initiating RNA from pre-initiation complexes

Next we measured how long it took for these pre-initiation complexes to form RNA when presented with NTPs. The experiment begins with a 30 min incubation of DNA and transcription extract. Because this is done in the absence of added nucleotides, pre-initiation complexes will assemble but not initiate, as just described. After this accumulation, NTPs are added to initiate RNA synthesis. Samples are removed at various subsequent times and the RNAs are detected as just described. The assay for RNA is a primer extension assay that requires hybridization to an RNA sequence 79 bases downstream from the start site. Thus the assay detects only those transcription complexes that have initiated and transcribed as far as position +79. The time required for pre-initiation complexes to initiate and reach this downstream position is determined by quantifying the amount of RNA produced at the various times after initiation was begun upon the addition of NTPs.

This experiment was done in parallel for basal and activated transcription and the results are shown in Figure 3. There are two aspects of this experiment worth noting. First, transcription is very rapid once NTPs are added to pre-initiation complexes. Under these conditions the 79 nt RNA is initiated from the pre-formed pre-initiation complex and completed in <1 min. This burst is followed by a slower gradual increase in RNA production from re-initiation events (unpublished data). In contrast, the results of Figure 2 showed that it takes ~100 times longer to complete the formation of pre-initiation complexes. Thus under these conditions the slow step in the pathway is clearly forming pre-initiation complexes; once formed they initiate and elongate the RNA very rapidly.

The second relevant aspect of the data concerns the comparison of basal and activated transcription. The data shows that the two systems take a similar time to complete initial RNA production (compare Fig. 3a and b). The rapid completion of this step, coupled with the lack of difference in the two systems, demonstrates that a difference in the initiation step cannot account for the 8-fold difference in transcription between basal and activated templates. Again the ratio of activated to basal transcription simply remains roughly constant at each experimental time point.

Re-initiation of transcription

These experiments have focused on the formation and properties of pre-initiation complexes derived from the interaction of free DNA and factors in the nuclear extract. However, the RNA produced in a continuous transcription reaction, such as that shown in Figure 1, can include an important contribution from events occurring after these steps. This is because some of the RNA can be produced by the process of re-initiation wherein templates are used a second time. The rate of re-initiation can be quite fast in some circumstances (26) and thus, in principle, it would be possible to activate transcription by causing multiple re-initiation events selectively on the activated template.

We tested this possibility by comparing RNA levels at long times to the RNA levels associated with primarily the first round of transcription. Pre-initiation complexes were assembled during a pre-incubation and then RNA synthesis was begun by the addition of nucleotides. As shown in Figure 3, there is a burst of initial RNA synthesis, in which the first round is completed within 1 min. Samples were removed at 2 min, which measures the amount of RNA produced in this first round and a small fraction of second round RNA (26; unpublished data). Samples were also removed after multiple rounds had occurred at 30 min. The results (Fig. 4) show that roughly three times as much RNA is produced at 30 min compared with 2 min (Fig. 4, lanes 2 versus 1 and 4 versus 3). However, the important point is that this ratio
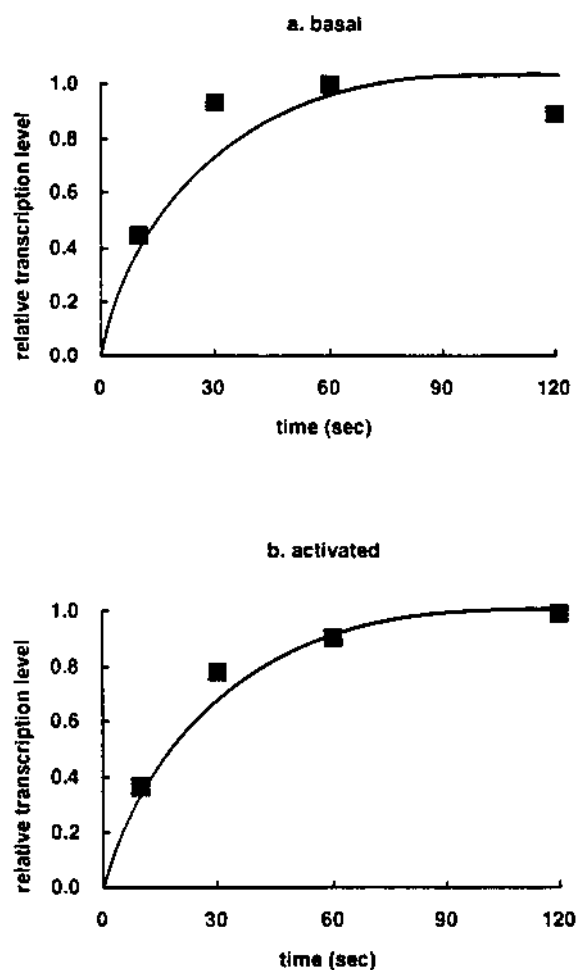
Figure 3. The time required to produce RNA from a pre-initiation complex. Pre-initiation complexes were pre-assembled during a 30 min pre-incubation. Transcription was then initiated by supplying NTPs and stopped after various times, as indicated. RNA was quantified as in Figure 1. The relative transcription level was plotted versus time.

Figure 4. Comparison between one round transcription and 30 min transcription. After a 30 min pre-incubation transcription was initiated with NTPs. Reactions were stopped after either 2 or 30 min. 100 ng basal template transcribed for 2 min (lane 1) or 30 min (lane 2); 100 ng activated template transcribed for 2 min (lane 3) or 30 min (lane 4). Reactions were done parallel for both basal and activated templates. The autoradiograph was exposed longer in order to visualize the basal transcription. Quantitation showed an ~2–3-fold difference for both templates as measured by scintillation counting.

does not differ dramatically for activated (lanes 3 and 4) and basal templates (lanes 1 and 2). Thus the 8-fold higher level of transcription for activated templates is not a consequence of an 8-fold increase in re-initiation events.

A large DNA concentration effect on activation and a thermodynamic model

These experiments have shown that activation does not involve reduction in the time required for pre-initiation complexes to form or to initiate and re-initiate RNA synthesis. An alternative possibility is that the number of templates engaged in forming pre-initiation complexes is increased by the activator. This could occur by promoting any of the succession of bimolecular reactions between DNA and transcription factors that lead to formation of functional pre-initiation complexes. In this way activator drives more components from the free state into the bound state and thus more transcription complexes form. Components of bimolecular reactions can also be driven together by mass action by simply increasing their concentration. Thus, if aspects of thi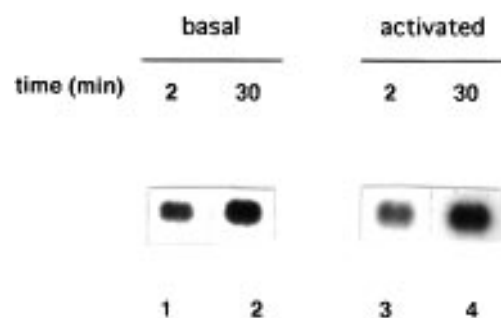s thermodynamic model are applicable then increasing the concentrations of factors or DNA may lessen the need for activator to drive them together. The hypothesis may be tested in this system by varying the concentration of DNA and assaying transcription; technically it is not possible to vary the concentration of factors over a wide range because of lack of control over the unknown factors in the transcription extract.

In preliminary experiments (not shown) we found that increasing the amount of DNA from 100 to 400 ng led to an increase in transcription, but increasing the concentration of DNA further led to slight declines in activated transcription. Declines were not observed over this range for basal transcription but did set in when additional promoter or non-promoter DNA was added (a presumptive artifact also reported in ref. 27). We also found that lowering the amount of DNA led to progressive decreases for both activated and basal transcription, although the extent of the decrease was not comparable in the two cases. We decided to assay transcription systematically over the lower part of this range of concentrations.

At each concentration of DNA, parallel reactions were done using basal and activated templates. The amounts of RNA produced were measured and are plotted in Figure 5A. One simple observation is probably the most important aspect of this experiment. This is emphasized in Figure 5B which plots the ratio of activated to basal transcription as calculated from the two curves of Figure 5A. The analysis shows that the quantitative effect of activator varies dramatically with the concentration of DNA used. Activation is very high at low concentrations of DNA (25-fold at 10 ng) and very low at high concentrations of DNA (3-fold at 400 ng). Because the addition of multi-microgram amounts of DNA causes a general inhibition of transcription, we cannot reliably determine if this ratio will continue to decrease with higher concentrations of DNA. This larger effect of activator at low DNA concentrations is consistent with a variety of observations, although the phenomenon has not been studied directly (25,28).

This lesser reliance on activator is not caused by the increasing amounts of DNA acting as a sink for histones or other non-specific DNA binding proteins that might act to repress transcription (29). All the reactions occur in the presence of 1000 ng of non-template carrier DNA. Thus when the activation ratio falls from 25 to 10, by increasing the amount of template from 10 to 50 ng, the total amount of DNA serving as a sink for histones
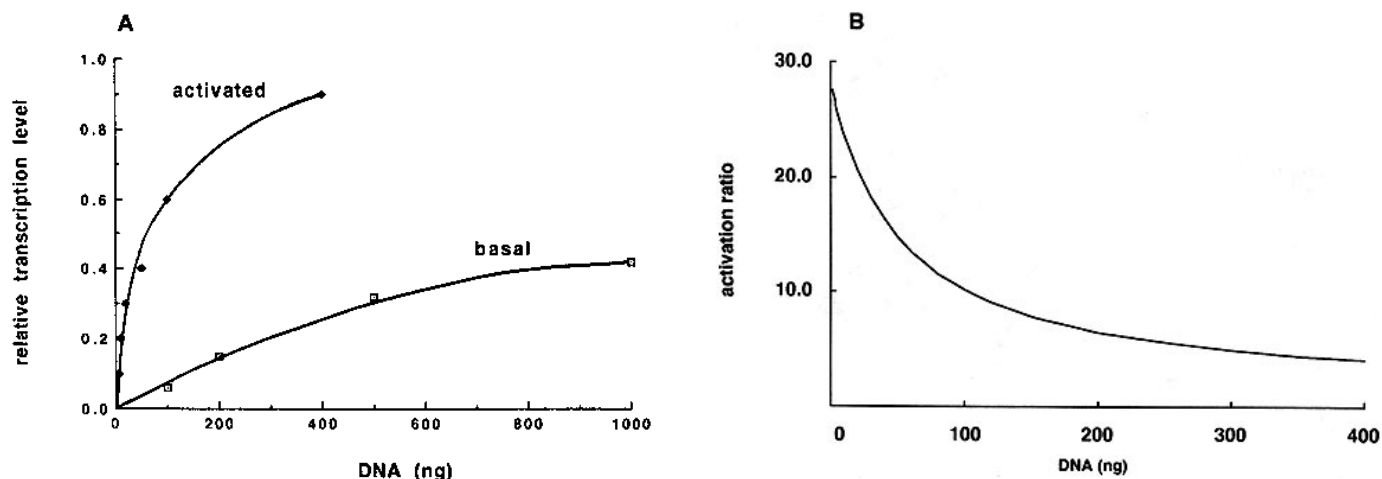
Figure 5. The extent of transcription activation strongly depends on the amount of DNA template used. (**A**) Relative transcription levels using various amounts of basal (□) or activated (◆) templates. The solid lines are best fit model curves assuming a simple binding equilibrium (see Materials and Methods). (**B**) The expected ratio of activated to basal transcription as calculated from the two model curves in (A).

and other non-specific DNA-binding proteins increases only from 1010 to 1050 ng. Thus the presence of a large excess of carrier minimizes the potential contribution of such anti-repression mechanisms.

This reaction can be modeled using a simple bimolecular scheme where two components, one containing DNA and one containing an essential factor, react to form an intermediate complex that inevitably leads to a pre-initiation complex. The solid lines in Figure 5A show the shapes of two best-fit binding curves expected from a simple bimolecular reaction in which DNA is one of the reactants. The experimental data points fit such a simple theoretical model. No doubt the actual reaction pathway is much more complicated but this simple model can be used to account for the drastic changes in activation level seen as different amounts of template are used.

The analysis suggests that a major role of activator in this system is to stabilize the binding of an essential factor, which would enhance the stability and increase the number of functional pre-initiation complexes. This is consistent with a wide variety of models proposed on the basis of studies of fractionated components (8,30–32). In addition the analysis allows a preliminary estimate of the increase in affinity caused by the activator. We assign an arbitrary equilibrium constant of 1 to the upper curve (Fig. 5A) for activated transcription and then attempt to estimate the relative binding constant associated with basal transcription. Curve fitting is difficult for basal transcription due to the low signal strength at low DNA concentrations and uncertainty as to whether the signal will ever reach the level of activated transcription. The result of this calculation indicates an ~10–30-fold increase in equilibrium constant caused by the activator (see Materials and Methods). The 30-fold estimate may be an upper limit because it assumes that all the effect of activator (25-fold at 10 ng of DNA) can be overcome by increasing the DNA concentration; in fact Figure 5B shows that at the highest accessible DNA concentration (400 ng) ~12% of the activator effect remains.

In principle, one can confirm such predicted increases in stability by attempting to detect the increase in lifetime of the critical

transcription complex caused by GC-boxes. Because the effect of GC-boxes is presumably associated with stabilization of an unknown intermediate transcription complex, such an assay cannot be done directly at the present time. However, as there may be a residual increase in stability associated with full pre-initiation complexes we measured their stability indirectly using a challenge protocol. In this experiment pre-initiation complexes are allowed to form on both basal and activated templates during 1 h of incubation. Then the complexes are challenged with a 10-fold excess of competitor template. At two subsequent times NTPs are added to assay for the number of remaining functional basal and activated pre-initiation complexes, that is those that have resisted the effects of this challenge. The competitor template consisted of an activated TATA and initiator promoter that had GAL-AH pre-bound to it; we found this to be the most effective competitor of several tried.

Figure 6A shows that there is a strong decrease in the amount of basal transcription after a 45 min challenge (compare lanes 5 and 6 with lanes 1 and 2). This corresponds to a loss of ~55% of the original signal. There is a modest further decrease after an additional 45 min (lanes 7 and 8 compared with lanes 3 and 4). When a parallel experiment is done using activated transcription the loss of signal after 45 min is much less (~20%; Fig. 6B, compare lanes 5 and 6 with lanes 1 and 2). Thus the activated complexes are significantly more stable against the 45 min challenge than the basal complexes. The difference is much smaller by 90 min. Because the reduction does not exhibit a smooth decrease to a low baseline (not shown) it is not possible to accurately assess the dissociation half-time. Nonetheless, the results support the thermodynamic model for activation in that the GC-boxes have enhanced the stability of pre-initiation complexes, allowing them to strongly resist the 45 min challenge in this protocol.

DISCUSSION

These results allow evaluation of the contributions to transcription activation via GC-boxes in an *in vitro* transcription system. Kinetic experiments showed that forming a pre-initiation com-
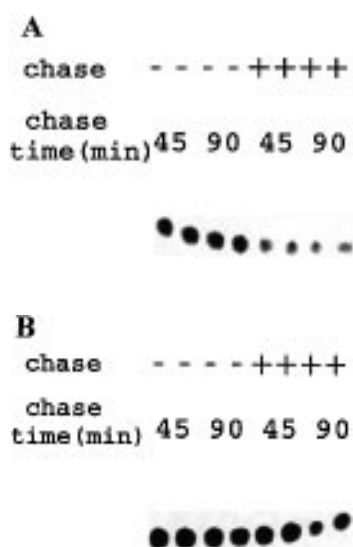
Figure 6. Activated pre-initiation complexes resist a 45 min challenge better than basal complexes. Transcription complexes (75 ng) were formed for 1 h on basal (**A**) or activated (**B**) templates. Challenger template (750 ng G5TILuc, pre-bound with Gal-AH) was then added to the reactions where indicated. After an additional 45 or 90 min, as indicated, 500 μM NTPs were added and the reaction stopped 30 min later. The reverse transcripts are shown. Experiments for basal and activated templates were done at the same time but different exposures are shown due to the lower levels of basal transcription, as described above.

plex is the rate-limiting step in this system for both activated and basal transcription. Although the activator does work at this step to enhance transcription it does not do so by decreasing the half-time for pre-initiation complex formation. Instead it increases the number of such complexes that can form, presumably by increasing their thermodynamic stability; each complex, however, forms and proceeds through the pathway at a rate that is similar to that observed for basal transcription. Thus more transcripts are produced because the activator has caused more templates to become engaged in the pathway, but each step in the pathway proceeds with an unchanged rate constant.

Activation occurs without changing the rate constants of observable steps

In an attempt to identify a step that was facilitated by activator we assayed the time required to complete various steps in the transcription pathway. Using a standard condition that involved an 8-fold activation by GC-boxes we found that the half-times for the following steps were not affected by GC-boxes: (i) forming a pre-initiation complex; (ii) initiation and elongation of RNA by the pre-initiation complex; (iii) re-initiation. The formation of the pre-initiation complex was found to be the slow step with a half-time on the order of 20–30 min. In contrast, when starting with a pre-initiation complex it was possible to initiate and elongate a 79 nt transcript in <1 min. We did not directly measure the rate of re-initiation but simply showed that the activator did not lead to a large increase in the number of rounds of transcription occurring in 30 min. These experiments led us to conclude that the 8-fold stimulation by activator was not due to its enhancing the rate constant for any of these steps.

These conclusions are in accord with a variety of prior studies using HeLa cell extracts and fractionated systems, although the

rates of all of these steps have apparently not previously been compared directly with each other. The half-time of pre-initiation complex formation has been studied in a variety of mammalian systems and estimates are in the range of 10–30 min (23–25,33). Pre-formed mammalian pre-initiation complexes can produce RNA rapidly (23). In a *Drosophila* transcription extract both steps occur more rapidly but formation of the pre-initiation complex is still rate-limiting (34). These studies have used a variety of promoters and activators which suggests that it will be quite common that formation of a pre-initiation complex is rate-limiting. In addition, the data indicate that the rate constant for this step is not strongly affected by activator, although it is too early to assume that this important conclusion will be true generally.

Increasing the amount of DNA can significantly lessen the dependence on activator

High concentrations of DNA were shown here to largely overcome the need for activation via GC-boxes. Thus GC-boxes stimulate transcription 25-fold in reactions containing 10 ng of DNA but only 3-fold in reactions containing 400 ng of DNA (always buffered by the presence of 1000 ng of carrier DNA to minimize anti-repression). Prior studies of stimulation by GC-boxes have led to estimates ranging from 3- to 500-fold (11,17,35). We suspect that some of this variation is due to variations in the amount of DNA used. It is likely that some is also due to the use of different promoters and anti-repression effects in cases where high amounts of non-promoter DNA was lacking. The result re-emphasizes the need to standardize reaction conditions when evaluating the strength of activators. It also emphasizes the desirability of using very low concentrations of DNA when attempting to assay for transcription activators. Similar phenomena have been observed in other systems although explanations have not been discussed (25,28).

Activation involves an increase in the apparent equilibrium constant for pre-initiation complex formation

Two experiments suggest that activator increases the equilibrium constant of pre-initiation complex formation to form a complex with enhanced stability. First, activated complexes were found to be significantly more resistant to destabilization in a template challenge protocol. Secondly, as just discussed, the effect of activator becomes minimal at high amounts of DNA, which suggests that mass action has partially substituted for activator in driving complex formation. We attempted to put these observations into semi-quantitative terms by modeling the complex reaction in terms of a simple bimolecular scheme. The rationale is that one effect of activator may be to facilitate the binding of a factor to a stable nucleoprotein complex containing DNA template and possibly other factors. The reaction scheme was simplified to that between two components: an unknown factor, whose concentration is fixed, and DNA, the concentration of which may be varied *in vitro*. Because the concentrations of factors cannot be determined, the absolute value of the binding constant cannot be estimated from these data. Modeling indicated that the activator increases the equilibrium constant by 30-fold, although this could be an overestimate by up to a factor of 3, as discussed above (see Materials and Methods). This change presumably reflects an increase in the affinity of some critical factor for the nucleoprotein complex containing the template DNA. The analysis allows this contribution of activator to be

interpreted in this manner, but by no means excludes additional effects of activator in more complex pathways.

Integration of these data with results involving partially purified factors

These conclusions are compatible with a wide variety of studies using partially purified transcription factors. Many studies have led to the suggestion that activators can contribute to transcription by 'recruiting' certain general transcription factors into the assembling pre-initiation complex (reviewed in 2). Evidence points toward activators changing the ability to bind TFIIB although this is not sufficient to account for the full level of activation (8,36). This evidence may be reconciled with the current data by proposing that basal templates are poorly transcribed in large part due to a low affinity for TFIIB and that Sp1 increases the affinity of TFIIB for promoter by up to the factor of 30 calculated from the new binding data.

The current data do not show a change in the half-time for pre-initiation complex assembly (consistent with 24,25,37). This implies that the critical factor is not recruited more rapidly by activator. Instead once the factor is bound it associates with more stability; the longer lifetime of the complex formed gives it a greater opportunity to bind the subsequent factors and complete the pathway to functional pre-initiation complex. This is consistent with proposals that activation can involve promoting a form of the activator–TFIID–TFIIA complex that can bind TFIIB in a stable and functional manner (31,38). One problem with this simple model is that adding a large excess of purified TFIIB causes only small increases in transcription rather than the large increases that might be expected if transcription is limited by its low affinity for the nucleoprotein template (8,25; also see 12 for discussion). However, TFIIB apparently is not recruited as an isolated protein but rather as part of a large multiprotein transcription complex (39,40). Thus adding excess isolated TFIIB may only lead to the modest effects observed.

These data also provide a rationale for the large differences in extents of activation that can be observed *in vitro*. The up to 30-fold increase in equilibrium constant seen here will be predicted to have its greatest influence when low concentrations of DNA are present or when promoters with weak, low affinity, basal elements are used. Additionally, experiments that omit large amounts of carrier DNA may include an additional activation by anti-repression. These considerations should be very useful in comparing different experiments involving activated transcription and in providing a continuing basis for rationalizing the mechanism of activation in terms of traditional enzymology.

ACKNOWLEDGEMENTS

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