Promoter Activation via a Cyclic AMP Response Element in Vitro*

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RNA polymerase II associates with promoters via multiprotein complexes and is subsequently released from these complexes to carry out transcript elongation. The multistep pathway involves both general transcription factors (GTFs) and promoter-specific activator proteins. The roles of many of the GTFs have been studied (reviewed in Refs. 1 and 2). These function primarily either as assembly factors for polymerase or to modify the properties of the polymerase after it has assembled at the promoter (3–7). Activators intervene to promote and facilitate various steps. Many studies suggest that this can occur by diverse mechanisms, including recruiting polymerase and GTFs to the promoter (reviewed in Refs. 8 and 9) in a way that triggers open complex formation (10), releasing polymerase from pause sites (6, 11), or stimulating elongation rates (12).

There is strong evidence supporting the recruitment model (see Refs. 8 and 9). There is less, but accumulating, evidence for postinitiation mechanisms. Postinitiation can be further subdivided into the steps of promoter clearance, elongation, and re-initiation. Several activators, including heat shock factor, VP16, E1a, and E2F (6, 11–13), have been proposed to work at one or more of these steps. For other activators there is uncertainty concerning which steps are affected.

One very important class of activators fits into this uncertain category, the activating transcription factor cyclic AMP response element binding (ATF/CREB) protein family. These proteins work via attachment to promoters containing upstream cAMP response elements (CREs) and mediate cAMP-dependent transcription responses (14–16). There are many members of the protein family and their diversity is increased further by their ability to form heterodimers and to be phosphorylated (14, 15, 17).

Members of the ATF/CREB family of activators have been suggested to work at both pre- and postinitiation steps (18–21). Several members of the family have been shown to bind TFIID (19, 22–24) and in some cases TFIIB (24). These interactions can occur directly or indirectly via coactivators (25, 26). The contact between activator and TFIID apparently occurs whether or not the protein is phosphorylated (18, 19, 23, 24, 27). On this basis the ATF/CREB members have been suggested to be involved in recruitment of the GTFs and the polymerase. However, even individual proteins may contain multiple activation domains, which may function differently (23, 27).

Other studies, conducted in HeLa extracts containing a wide range of factors, have suggested that activation in such extracts occurs instead at the step of promoter clearance (20, 21). In these studies ATF/CREB was not required to form preinitiation complexes in which the DNA start site was melted. Instead the activator was proposed to allow the polymerase, prebound in an open complex, to clear the promoter in a way that led to the observed 3-fold activation of transcription. The protein isoform that bound the CRE and accomplished the activation in nuclear extract was reported to be unphosphorylated CREB-1 (28).

Recently, we found that the effect of a different activator, Sp1, on recruitment can to some extent be bypassed if high amounts (500–1000 ng) of template DNA are used (29). It is possible that some of the uncertainty concerning the step at which ATF/CREB functioned was due to variations in experimental conditions. An additional uncertainty arises from recent work with core promoter sequences analogous to those used in ATF/CREB studies. These suggested that promoter opening is more complex than had been appreciated previously in that initiating nucleotides are involved in addition to ATP (5).

Because of these new uncertainties and the importance of activation via CRE sites, we re-evaluated the activation mechanisms. In contrast to earlier studies, we see little or no effect of ATF/CREB on the rate of promoter clearance. At high concentrations of DNA a strong effect of ATF/CREB on preinitiation complex formation is bypassed. When low amounts of DNA are used the stimulation by ATF/CREB is very great. Its main effect under these conditions is to nucleate formation of a preinitiation complex in which the DNA can be opened. Complete opening requires activator, ATP, and initiating nucleotides. In addition to this primary recruitment effect, there is a
**Promoter Constructs.** Relevant promoter elements (see Ref. 20) are indicated by boxes. Restriction sites used in cloning are indicated. All sequences from the TATA box to the BamHI restriction site are identical to the adenovirus major late (M5) promoter. Primer pBW1 drives activated transcription, while primer pBW2 supports only basal transcription.

Small secondary effect in which ATF/CREB stimulates the rate of transcription re-initiation events.

**Materials and Methods**

Nucleic Acids and Cloning—Fig. 1 summarizes the constructs used. The pBW1 promoter construct is identical to the pSH15 promoter (20). To create this promoter, upstream from sequences used previously to facilitate transcription detection, two partially complementary oligonucleotides were synthesized. B1 has the sequence CCCGAATTCT-GACGTCACAACAGGTATAAAGGGGTTGGGGGCTATCCTGGTCTC. B2 has the sequence CCCGGATCCGACGTCAGGGGCTATCCTGGTGACTGGGAGAGAGAGTGAGGACGAGGCATGCCCCCA. The underlines represent the complementary sequences. Equimolar amounts were hybridized and then mixed with the Klenow fragment of DNA polymerase by mixing the 30-mer extension primer BPM1 (CGCGTGACGTCACAACAG), which hybridizes to the 5′-CTC. B2 has the sequence CGCGGATCCCCCAGCTCCGGCGCGGCCGGGAAGAGAGTGAGGACGAGGCATGCCCCCA. The underlines represent the complementary sequences. Equimolar amounts were hybridized and then mixed with the Klenow fragment of DNA polymerase by mixing the 30-mer annealing reaction with 7.5 mM dithiothreitol, 33 μM each deoxyribonucleoside triphosphate (dNTP), and 3–5 units of Klenow enzyme (Life Technologies, Inc.) in a total volume of 60 μl and incubating for 1 h at room temperature. DNA was purified away from unincorporated nucleotides and buffer over a Qiagen polymerase chain reaction column (Qiagen).

The Klenow extension creates an EcoRI restriction site at the upstream end of the double-stranded oligonucleotide and a BamHI site at the downstream end (see Fig. 1). The product was cleaved with EcoRI and BamHI and was ligated into the EcoRI and BamHI sites of plasmid pSP72 (Promega). This construct was transformed into DH5α-competent cells (Life Technologies, Inc.), and EcoRI-sensitive clones were identified. Candidate clones were verified by direct sequencing.

Promoter construct pBW2 is identical to promoter pAH1 (20) and was created in the same manner as pBW1 except that oligonucleotide B3 (CCCGAATTCCGCTACACAGGTATAAAGGGGTTGGGGGCTATCCTGGTCTC) replaced B1. Double-stranded competitor DNA was made by annealing two perfectly complementary 24-nucleotide CRE sequences (top strand: GGACCAGCTTGACGTCACAACAG, consensus CRE underlined). All primer extensions used the Inr primer (CTTATGTATCATACACATGGATCACGTACATAGTTTACG), which hybridizes to the pSP72 vector up to position +93 relative to the transcription start site (indicated by an arrow in Fig. 1) except bottom strand probing in KmoO4 assays, which used primer BPM1 (CCCGGATCCGAGCTACACAGGTATAAAGGGGTTGGGGGCTATCCTGGTCTC), which hybridizes to the region from −52 to −35 in promoter pBW1. Oligonucleotides were synthesized on a Gene Assembler Plus (Pharmacia Biotech Inc.) and purified on 20% urea-PAGE (19:1 acrylamide:bisacrylamide; 8 μm urea). Plasmids were purified using Qiagen Maxiprep kits.

**In Vitro Transcription—**This assay has been described (29, 30). HeLa nuclear extract was prepared as previously (20–31). The indicated amounts of supercoiled whole plasmid template DNA were mixed with 20 μl of HeLa nuclear extract (6 mg of protein/ml), 8 mM MgCl2, 500 ng of pGEM as carrier DNA and 500 ng each nucleoside triphosphate (NTP) in a total volume of 40 μl. Samples were then incubated at 30 °C for 30 min. 100 μl of stop buffer (10 mM EDTA, 0.3 mM sodium acetate, pH 5.5, 0.2% SDS, 50 μg/ml yeast RNA, 20 μg of proteinase K) was added, followed by incubation at room temperature for 30 min. RNA was isolated and copied using reverse transcriptase (Promega) extension of 5′-32P-labeled Inr primer (29). These labeled cDNA products were separated by % urea-PAGE (19:1 acrylamide:bisacrylamide, 8 μm urea) and visualized and quantified using a PhosphorImager (Molecular Dynamics).

For promoter clearance and re-initiation assays the above protocol was modified as follows: NTPs were omitted in the first step and preinitiation complexes were formed on the DNA template for 1 h at 30 °C. NTPs were then added to a final concentration of 500 μM each. Reactions were incubated at 30 °C for various times and the RNA quantified as described. In all transcription assays each data point was performed in duplicate except in the competitor titration assay.

**Potassium Permanganate Assay—**This assay has been described (31–34). Briefly, preinitiation complexes were assembled as follows: the indicated amount of supercoiled template DNA was incubated with 20 μl of HeLa nuclear extract (6 mg of protein/ml), 8 mM MgCl2, and 200 ng of pBR322 (Promega) for 30 min at 30 °C. When present, deoxycytidine triphosphate (dATP) was added to a final concentration of 500 μM, and NTPs were added to final concentrations of 100 μM each. Samples were then incubated at 30 °C for 2 min, after which potassium permanganate (KMnO4) was added to a final concentration of 6 μM. Two minutes later the KMnO4 was quenched with 3 μl of β-mercaptoethanol. DNA was purified by extraction with phenol:chloroform: isoamyl alcohol (25:24:1) followed by extraction with chloroform: isoamyl alcohol (24:1) and ethanol precipitation. Pellets were dissolved in water and passed over 1-ml Sephadex G-50 (Sigma) columns. Eluate volumes were equalized and permanganate attack patterns analyzed by extension of the Inr or BPM1 primers in a polymerase chain reaction thermocycler (MJ Research). In the case where 10 ng of template DNA was used, the entire sample was analyzed. When 1 μg of DNA was used, only a 20-ng sample was analyzed. After polymerase chain reaction, samples were recovered and run on 6% urea-PAGE at 36 watts for 1.5 h. Gels were dried and exposed to PhosphorImager screens overnight.

**Aborting Initiation—**This assay was performed as described using dinucleotide primer and radioactive CTP (35) except that Cpa was the dinucleotide primer and a much higher concentration of DNA (1 μg/40 μl) was used. Labeled dinucleotide products were resolved on a 20% (19:1 acrylamide:bisacrylamide)/8 μm urea gel and exposed to PhosphorImager screens (Molecular Dynamics) for 1–5 h.

**Results**

ATF/CREB Stimulation of Transcription in Vitro—Prior experiments regarding activation via ATF/CREB sites in vitro have primarily used plasmid DNA amounts in the range of 500–1000 ng (18–21). In some cases the apparent mechanism of activation is known to depend on the amount of DNA with these amounts representing the highest used (29 and see below). We begin by exploring how these sites activate transcription at high concentrations of DNA.

Fig. 2a, lanes 1 and 8, compare in vitro transcription with and without a single consensus CRE site. Removal of the site (lane 8) leads to a 3-fold loss of transcription, consistent with prior studies done with the same promoter under the same conditions (20, 21). To confirm that this effect is mediated by binding to these sites, and to facilitate clearing ATF/CREB from the extract for experiments described below, a competition experiment was done. A double-stranded oligonucleotide encompassing a consensus CRE (see “Materials and Methods”) was added to in vitro transcription assays prior to addition of template DNA. Its purpose is to specifically sequester CRE-binding proteins (36, 37). As more of this competitor is added the signal gradually decreases (lanes 3–7). At the highest concentration of competitor shown, the amount of transcription has been reduced to basal levels, that is, an amount equal to that formed from a template lacking a CRE site (compare lanes 7 and 8). The competitor has no effect on transcription from the basal template (data not shown). These data confirm that CRE binding proteins are responsible for the activation.

The competitor titration experiment was repeated several times with varying amounts of template DNA. In all cases complete inhibition of activation occurred with approximately 250 ng of competitor oligonucleotide, as shown in Fig. 2b. Thus we conclude that this amount of competitor is sufficient to sequester the endogenous ATF/CREB proteins in the nuclear context.
Open Complexes Can Form without ATF/CREB at High Concentrations of DNA—The source of the 3-fold activation by ATF/CREB has not been settled and was originally suggested to occur after the opening of the DNA (20). That is, it was reported that ATF/CREB is not required to create a permanganate-sensitive open complex at these promoters. There is also some confusion as to the nature of the opening reaction itself. Opening in general was reported to require ATP or dATP (31, 32, 34). Subsequently, Holstege et al. (5) showed that at the adenovirus major late promoter, the sequence of which is largely preserved in the constructs used here (see Fig. 1), initiating nucleotides were needed in addition to ATP to obtain a substantial permanganate signal. Therefore we investigated the formation of open complexes at these promoters.

We looked first at opening of the upper (nontemplate) strand of the DNA. The data show strong permanganate signals downstream of the start site, and weaker signals at upstream sites, when the initiating nucleotides ATP and CTP are added (Fig. 3a, lane 3 versus the lane 1 control). Addition of only dATP (lane 2) or only ATP (not shown) does not yield a significant permanganate signal. These nucleotide requirements are consistent with prior reports using the adenovirus major late promoter (5), the core sequence of which is retained in the promoter studied here. The open complexes formed in this manner appear to be functional in that the addition of nucleotides that allow elongation causes the disappearance of the signal (compare lanes 2 and 3 of Fig. 3b). This is expected based on studies of open complexes at several promoters in which the melted bubble has been shown to be chased to downstream positions (31, 33).

FIG. 2. In vitro transcription. a, varying amounts of competitor oligonucleotide from 50 to 1000 ng (5–100-fold molar excess over template DNA) were added to in vitro transcription reactions prior to template. The 93-nucleotide primer extension product on 6% urea-PAGE is indicated. The presence (+) or absence (−) of a CRE in the promoter is indicated. b, bands from a were quantified and plotted versus the amount of competitor added.

FIG. 3. Potassium permanganate footprinting at high DNA concentration. a, 1 µg of pBW1 (activated) template was KMnO₄-footprinted in nuclear extract. When present, the indicated nucleoside triphosphates are denoted. Saturating amounts of competitor oligonucleotide (250 ng, cf. Fig. 2b) were added prior to template DNA where indicated. The transcription start site and direction are represented by an arrow. The bar indicates the open region. b, 1 µg of either pBW1 (lanes 1–3) or pBW2 (lanes 4–6) were KMnO₄-footprinted. Nucleoside triphosphates were added as indicated. c, KMnO₄ footprinting of bottom (template) strand of pBW1 (activated). The +1 thymidine is indicated by an arrow. d, the sequence of the open complex region. The transcription start site is indicated by an arrowhead. Reactive thymidines are indicated in bold.

The formation of functional open complexes does not require ATF/CREB under these conditions. When an excess of competitor oligonucleotide is added to HeLa extract prior to the template, the permanganate signal still appears (compare Fig. 3a, lane 6, to the negative control in lane 4). However, the permanganate signal (denoted by a bar) is weaker relative to background reactivity (region below the bar) when basal (Fig. 3a, lane 6) and activated (Fig. 3a, lane 3) conditions are compared. A similar result is seen when basal and activated templates are compared directly (Fig. 3b); the permanganate signal persists on the basal template, but is somewhat weaker (compare lane 5 with lane 2). As expected from Fig. 3a the signal depends on addition of initiating nucleotides (Fig. 3b, lane 5) and disappears when a full complement of elongation substrates is present (lane 6). We also explored the opening of the other DNA strand, since that strand had been reported previously to open in response to dATP alone (20). In this experiment, dATP alone did not cause detectable opening (not shown, but see Fig. 3a and below), but initiating nucleotides did (Fig. 3c).

Overall, these data are in significant, but not full, agreement with prior studies. The promoter opening behavior is consistent with that observed at the analogous adenovirus ML promoter (5), but not the same as reported elsewhere (20). As reported (20), promoter opening did not require ATF/CREB. The stimulation of transcription under these conditions was 3-fold (Fig. 2), also as reported in prior studies (20, 21). However, the permanganate results on the top strand showed some strengthening of the open complex signal by the activator. It appears that at least some of the 3-fold effect is due to the stimulation of open complex formation.

ATF/CREB and Promoter Clearance—In a prior study the 3-fold stimulation was attributed not to formation of open complexes but to postinitiation stimulation of promoter clearance (20). To clarify this issue we re-evaluated the rate of promoter clearance. Preinitiation complexes were formed by a 1-h incubation of template with transcription extract in the absence of nucleoside triphosphates. This drives the DNA into
closed transcription complexes that lack the nucleotides needed to complete the opening and elongation reactions. All four NTPs are then added to begin these reactions synchronously. At various subsequent times the appearance of the 93-nucleotide-long RNA is quantified. In the very short time course of this experiment each template that forms an open complex can only produce a single RNA (31). Thus, this is a one-round transcription assay, and the amount of RNA is a direct measure of the number of preinitiation complexes that complete opening and clearance at the indicated times (31).

Fig. 4 shows the time required for the preinitiation complexes to complete these steps. The activated (pBW1) and basal (pBW2) templates are compared to assess the effect of activator. In both cases the addition of NTPs leads to an initial burst of RNA synthesis as the preinitiation complexes synchronously initiate and elongate the short RNA transcript (as seen in prior studies of other promoters (38, 39)). The activated templates appear to have assembled approximately 2.5 times the number of active preinitiation complexes, as indicated by the 2.5-fold greater amount of RNA produced. However, the results show no effect of ATF/CREB on the rate of promoter clearance. On both templates the reactions are mostly complete within the first minute. The approximate half-time (t_{1/2}) for this reaction is 15 s for both templates under these conditions. Thus, the activated template produces more RNA, but this is due to a greater number of active preinitiation complexes; both templates complete promoter clearance at the same rate (t_{1/2}).

This result agrees with those of the free transcription and permanganate experiments of Figs. 2 and 3. In those experiments the activator caused a 3-fold stimulation of transcription and also caused more open complexes to form. We conclude that some active preinitiation complexes can form without ATF/CREB but that ATF/CREB causes a modest increase in their number, accounting for the approximately 3-fold activation.

**ATF/CREB Functions Prior to Transcription Initiation**—The above results demonstrate that ATF/CREB does not stimulate the rate of promoter clearance (t_{1/2}) but does cause an increase in the number of promoters that clear. The permanganate results (Fig. 3) suggest that at least some of this stimulation occurs at the level of open complex formation. However, the permanganate assay is not quantitative, and the possibility remains that ATF/CREB could increase the activity of open preinitiation complexes in a way that allows more of them to clear the promoter and synthesize the 90-nucleotide-long transcript. In this experiment we measure the quantitative effect of ATF/CREB on preinitiation complex formation using an abortive initiation assay.

We have previously used abortive initiation to measure the number of open preinitiation complexes in both prokaryotic (40) and eukaryotic systems (35). CpA has been used as the dinucleotide primer in abortive initiation assays at the adenovirus major late promoter (see Ref. 5), which has the same initiation sequence used here. The assay was done using the high DNA concentrations (1 μg of plasmid/40 μl) that gave the 3-fold activation described above.

Fig. 5a shows that the activated template pBW1 (lanes 1 and 2) produces significantly more labeled trinucleotide (indicated by an arrow) than does the basal template (lanes 3 and 4). The product has the appropriate characteristics, as shown in Fig. 5b, by its disappearance when dinucleotide is left out (lane 2) or when α-amanitin is added (lane 3). The trinucleotide bands in Fig. 5a were quantified, and the results from three trials were averaged. The data indicate that the activated template (Fig. 5a, lanes 1 and 2) produced 4-fold greater abortive product than the basal template (lanes 3 and 4) under these conditions. This 4-fold stimulation of formation of preinitiation complexes that can initiate transcription agrees well with the 3-fold stimulation of both open complex formation and transcript (above). We conclude that, under these conditions, the primary effect of ATF/CREB is to stimulate the formation of functional open preinitiation complexes and that ATF/CREB has no significant effect on promoter clearance.

**DNA Concentration and Transcriptional Activation**—The conclusions stated above apply to conditions involving high amounts of template DNA (1 μg of plasmid DNA). Prior experiments assessed the effect of DNA concentration on GC box-activated transcription (29). Modest effects of activator were seen at high DNA concentrations, similar to the 1000 ng used here and in other studies (see Introduction). At lower amounts of DNA much greater effects were observed, more in accord with expectations based on strong requirements for activator in vivo. Such considerations raise the possibility that the use of high amounts of DNA in vitro bypasses a major effect of transcription activators. Therefore we investigated the role of activator at lower concentrations of template.

In the set of experiments shown in Fig. 6, either the activated or the basal template was simply mixed with transcription extract and all four nucleotides. The RNA was isolated 30 min later. The amount of DNA was varied down from the 1000 ng used in the above studies. At each concentration the amount of basal and activated transcription was compared. An activation ratio, representing the stimulation due to the presence of a CRE site, was calculated for each experiment.

Fig. 6 shows that very high levels of stimulation by ATF/CREB sites are observed at low concentrations of DNA. As the DNA amount is lowered from 1 μg to 20 ng, the activation ratio...
increases from 3-fold to nearly 60-fold. The use of even lower amounts of DNA leads to even greater increases with apparent ratios greater than 100-fold. The absolute value of these high ratios are somewhat uncertain due to the small amounts of RNA made from basal templates. Nonetheless the data show that the effect of activator is small at high DNA concentrations compared with the much greater effect observed at low DNA concentrations. As seen previously with GC box-activated transcription, it is the severe restriction of basal transcription at low amounts of DNA that accounts for the high activation ratios (Ref. 29 and data not shown).

**Open Complex Formation at Low DNA Concentration**—We infer that the use of high amounts of DNA allows the transcription machinery to artificially bypass much of the stimulation by CRE sites. Therefore, we now assess the effect of a CRE site under conditions where activation is very strong. The experiments described below will use amounts of DNA where the activation is typically greater than 50-fold rather than the 3-fold associated with prior experiments.

The permanganate experiment of Fig. 7a is essentially the same as that of Fig. 3a, but conducted under conditions where activation is very high. Recall that at high DNA concentrations (1000 ng/reaction) the activator was not required to open the start site (Fig. 3a, lane 6, and Fig. 3b, lane 5). Under the low DNA conditions of this experiment (10 ng/reaction), a quite different result is seen. Lanes 3 and 7 of Fig. 7a show the appearance of a substantial permanganate signal using the activated template. When activator is titrated out with a CRE competitor oligonucleotide, as described above, this same template fails to open (lane 6). The opening on the activated template requires initiating nucleotides (no signal in lane 1) and is not detectable when dATP is the only source of nucleotide (lane 2). α-Amanitin counteracts the stimulating effect of nucleotides (lack of signal in lane 8), indicating that first bond formation is required for opening to be detected by the permanganate assay (35). Thus to detect opening of the DNA under these conditions, one requires binding to the CRE as well as first bond formation.

Fig. 7b confirms the requirement for activator in opening the start site under strong activating conditions. The activated template displays a strong permanganate signal in response to initiating nucleotides (lane 2 versus control in lane 1), whereas the basal template fails to open in response to the same nucleotides (no signal in lane 5). Recall that the basal template opened efficiently in response to initiating nucleotides when high levels of DNA were used (Fig. 3b, lane 5). The open complexes that form on the activated template are functional as judged by the disappearance of the permanganate signal upon addition of the remaining nucleotides (Fig. 7b, lane 3).

We conclude that under conditions where the effect of activator is maximal, activator is required to reach the stage where a detectable open complex is formed; under these conditions the basal template simply does not open efficiently.

**ATF/CREB and Transcription Reinitiation**—Activators can have additional functions after the DNA is open (see Introduction). Among these functions is the facilitation of continuous reinitiation of transcription. We evaluated this possibility for ATF/CREB by adopting prior assays (31, 38). In this experiment, basal and activated templates are incubated for 1 h in the absence of nucleotides. This allows the full level of preinitiation complexes to form (38). The amount of DNA is adjusted so as to be on the linear phase of a titration curve where DNA is varied and transcription assayed (below 50 ng of plasmid/40-μl reaction in this case. This ensures that the template is limiting in that transcription factors are in functional excess (Refs. 29 and 38 and data not shown). The four nucleotides are then added, allowing each complex to open, initiate, and complete the 93-nucleotide-long RNA, which is then detected by primer extension.

A rapid burst of transcription accompanies NTP addition, as shown in Fig. 4. When 35 ng of DNA are used the burst is complete within 2 min, as shown using high amounts of DNA (Fig. 4 and data not shown). As discussed above, the signal at this time reflects RNA production from the complement of preinitiation complexes formed during the long incubation. Samples are also taken 30 and 60 min after the addition of NTPs. The long time allows for subsequent rounds of RNA synthesis to occur. If the CRE site mediates more rapid reinitiation then the ratio of activated to basal transcription should be higher at 60 min than at 2 min.

The results from several experiments are summarized in Table I. At the 2-min point there is approximately eight times more RNA from the activated template compared with the basal. By 60 min the amount of activated transcription has increased 7.5-fold, whereas basal transcription has increased only 4-fold. Thus the apparent activation ratio is 8 at the earliest time when preinitiation complexes have fired only once, but has increased to 15 at the later time when re-initiation has been occurring for 60 min. If one calculates the apparent number of rounds of reinitiation per time the activated template has a continuous transcription rate double that of the basal template.

The results suggest that under these conditions, activation via ATF/CREB can be partitioned into two effects. The main effect is stimulation of preinitiation complex formation, which is 8-fold under these conditions. The secondary effect is on the continuous transcription rate, which is 2-fold under these con-
cases, 35 ng of template DNA was used. Data represent the averages of two to four trials each.

| Time (min) | Relative transcription | Activation ratio |
|-----------|------------------------|------------------|
|           | Basal                  | Activated        |               |
| 2         | 1                      | 8                | 8             |
| 30        | 3                      | 32               | 11            |
| 60        | 4                      | 60               | 15            |

The main effect of the activator could be bypassed by the use of high DNA concentrations (1000 ng), as seen previously for GC box-activated transcription (29). Under these conditions a residual 3-fold activation occurs, in agreement with prior studies and attributed to promoter clearance effects (20, 21). However, we see no effect on the rate of promoter clearance; activator causes three times the number of functional preinitiation complexes to form and both basal and activated complexes fully clear within a minute of adding NTPs. The entire effect is quite minor in view of the 20–100-fold activation that is observed under optimal conditions using 10 to 50 ng of whole plasmid DNA.

Prior studies in similar nuclear extracts have indicated that the major activator bound to the CRE is CREB-1 and that it is not phosphorylated (28). CREB has been shown to contact TFIIID in nuclear extracts and to activate transcription without serine phosphorylation (23, 24). Thus the mechanism observed here likely applies to nonphosphorylated CREB-1. Phosphorylation has been proposed to promote interactions with the coactivator CBP/p300, which in turn interacts with TFIIIB (25, 26). This interaction could explain the enhancing effect of phosphorylation on preinitiation complex formation (21, 28), even at higher concentrations of DNA. CBP/p300 also interacts with P/CAF, a histone acetylase, which may be involved in chromatin remodeling in vivo (42). Thus one would expect that phosphorylation might increase the ability of CREB to recruit polymerase to a remodeled template via the TFIIID-TFIIIB complex and allow it to reach the fully open complex stage. However, this remains to be tested, as does whether phosphorylation alters the secondary ability of CREB to promote higher rates of re-initiated transcription.

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