Abortive Initiation and First Bond Formation at an Activated Adenovirus E4 Promoter*

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Abortive initiation at the adenovirus E4 promoter was studied by following the production of RNA formed from the initiating nucleotides UPA and CTP. Formation of a specific short RNA via a reaction with appropriate α-amanitin sensitivity required promoter, activator, and ATP. In the absence of any of these, an α-amanitin-resistant reaction led to lower levels of a product of unknown origin. The α-amanitin-sensitive reaction required open promoter complexes, as assayed directly by permanganate probing. This reaction was not blocked by the inhibition of polymerase C-terminal domain kinase activity or by the lack of DNA supercoiling. Thus, formation of the initial bond of the mRNA appears to require activator and ATP to open the DNA but not phosphorylation of the polymerase C-terminal domain. In addition, the abortive initiation reaction was strongly suppressed when all elongation substrates were present, suggesting that cycling to produce high amounts of abortive product is strongly disfavored during productive initiation at this promoter.

The mechanism by which RNA polymerase II makes the initial bonds of the mRNA and clears the promoter is not well understood. Recent studies have suggested that a number of factors may influence this process. These include the general factors TFIIH, TFIIE, and TFIIF, which may have components that act primarily at these steps (Parvin et al., 1992; Parvin and Sharp, 1993; Chang et al., 1993; Goodrich and Tjian, 1994). In addition, the overall process requires ATP, independent of the use of ATP as an elongation substrate (Bunik et al., 1982; Sawadogo and Roeder, 1984; Conaway and Conaway, 1988; Jiang et al., 1993). The ATP involvement could be at any of several substeps, including opening the DNA template (Wang et al., 1992a; Jiang et al., 1993), phosphorylating the C-terminal domain (CTD)1 of the polymerase (reviewed by Corden (1990) and Young (1991)), and supporting the helicase activity of TFIIH (Schaeffer et al., 1993; Serizawa et al., 1993b). Each of these activities relies on the hydrolysis of the β-γ bond of ATP, and therefore dATP can act as a substitute. These issues have assumed greater relevance with the emergence of studies, suggesting that some activators may work by facilitating promoter clearance or assisting the resumption of transcription by stalled polymerases (Parada et al., 1995; Narayan et al., 1994; Krumm et al., 1995; Lee and Gilman, 1994; Rasmussen and Lis, 1993; Yankulov et al., 1994).

A critical step in this overall process is the formation of the first bond of the mRNA. This step has been studied using abortive initiation assays (Luse and Jacob, 1987; Jacob et al., 1991, 1994; Goodrich and Tjian, 1994) based on an analogous reaction used for prokaryotic transcription studies (McCleire et al., 1978; Carpousis and Gralla, 1980). In these assays the formation of the first bond is monitored via condensation of a dinucleotide primer and the next encoded nucleoside triphosphate of the transcript. Such reactions appear to properly mimic initiation in the sense that maximal accumulation of the trinucleotide product requires an intact promoter, the dinucleotide primer, nucleoside triphosphate, and an in vitro transcription system in which all components are active. Because the trinucleotide product can in principle be made in very large excess over the amount of template present, the assay allows high detectability even from very inefficient systems.

The specificity of this abortive initiation assay has been established, but the requirements for forming the abortive initiation product are yet to be established. Luse and Jacob (1987) suggested that the reaction at the adenovirus major late promoter used ATP β-γ bond hydrolysis, based on the ability of dATP to stimulate production of trinucleotide. This early work pre-dates the studies showing roles for ATP hydrolysis in promoter opening (Wang et al., 1992a), in phosphorylating the polymerase CTD (Casden and Dahmus, 1987; reviewed by Corden (1990) and Young (1991)), and in generic promoter cleavage (Goodrich and Tjian, 1994), and thus no mechanistic role was proposed. A later study using purified components confirmed the specificity of the abortive initiation reaction at this promoter (Goodrich and Tjian, 1994) but suggested that ATP was not involved in the steps leading up to and including formation of the first bond of the mRNA. Instead it was suggested that ATP hydrolysis was required for the subsequent steps of promoter cleavage, perhaps via an activity associated with factor TFIIH.

Thus the requirements for formation of the first mRNA bond and for transcription initiation are unsettled and need further clarification. This is especially true with regard to the various critical steps that use ATP because of its proposed role in three activities: the opening of the DNA and two activities associated with TFIIH. The latter activities are phosphorylation of the polymerase CTD (factor TFIIK) (Feaver et al., 1994; Svejstrup et al., 1995) and DNA helicase activity (Schaeffer et al., 1993; Serizawa et al., 1993b). Each of these three activities has an unknown relationship to forming the first bond of the transcript; thus the role of ATP in first bond formation is especially unclear. An additional unsettled issue relates to the amount of abortive initiation that occurs as a byproduct of productive initiation. Abortive initiation products have been detected un-
under conditions where they could in principle be elongated via nucleotide addition. This is most easily detected at certain promoters greatly weakened by mutation (Jacob et al., 1991, 1994). The extent to which abortive initiation is suppressed during normal, productive initiation at wild type promoters is uncertain.

We address these issues by studying abortive initiation at an activated adenovirus E4 promoter. Recently we proposed that initiation at this promoter involves two successive steps that hydrolyze ATP. In the first step ATP is used to open the DNA, and in a later step it is used to phosphorylate the polymerase CTD, stimulating the ability of the polymerase to move downstream during initiation. In this scheme the formation of the first bond of the DNA would require the first of these ATP-dependent steps but perhaps would not require the second step. The results of these experiments will define the requirements for first bond formation at this promoter and will help to integrate abortive initiation and promoter clearance studies, allowing more general models to be proposed and tested.

MATERIALS AND METHODS

High pressure liquid chromatography-purified ribonucleoside triphosphates and desoxyribonucleoside triphosphates were from Pharmacia Biotech Inc. Sepharose CL-4B and a-amanitin were from Sigma. The CTD kinase inhibitor H8 (N-(2-(methylamino)ethyl)-5-isouquinoline sulfonamide dihydrochloride) was from Seikagaku American Inc. Nuclear extract was prepared as described previously (Dignam et al., 1983). The DNA template contains nine GAL4 binding sites upstream of the adenovirus E4 promoter (Carey et al., 1990). The promoter contains a TATA box as a basal element and has upstream elements allowing first bond formation at this promoter and will help to define the requirements for first bond formation at this promoter and will help to integrate abortive initiation and promoter clearance studies, allowing more general models to be proposed and tested.

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only one that is not diminished by treatment with phosphatase, which changes the mobility of RNA bands with triphosphate ends (not shown); this confirms that the product does not contain CTP at its 5' terminus. If UpA is omitted, the short RNA is the only radioactive band to disappear (lane 2). This band thus has the characteristics expected of the abortive initiation product UpApC. Fig. 3 shows that production of this presumptive abortive initiation product UpApC is strongly inhibited by low concentrations of α-amanitin (lane 3 versus lane 4). The low amount of α-amanitin used is known to selectively block initiation by RNA polymerase II. This is the only band that is inhibited by α-amanitin. These various results support the specificity of the abortive initiation reaction.

These observations apply to reactions involving open complexes, that is to reactions performed in the presence of low amounts of dATP (Fig. 2, lane 4), as established by the permanganate probing of Fig. 1. When dATP is omitted, corresponding to a reaction associated with closed complex conditions, the same band appears but in lesser amount (Fig. 2, lane 3 versus the lane 4 signal from open complexes). However, the product appearing under these closed complex conditions is still made in the presence of α-amanitin (Fig. 3, lane 1 versus lane 2). This is in contrast to the reaction from open complexes formed in the presence of ATP. Thus the products produced in the absence of ATP (closed complex conditions) lack the hallmark associated with RNA polymerase II preinitiation complexes in that their formation is not blocked by α-amanitin.

Previously, we showed that in order to detect functional open complexes at this promoter, a transcription activator must be present (Wang et al., 1992a). Fig. 3B presents the results of abortive initiation assays in which activator was absent. The result is similar to that observed when ATP is absent; although some product is made (Fig. 3B, lane 8), its formation is not reproducibly inhibited by α-amanitin (compare lane 9 versus lane 8 with the loss of signal in the presence of activator in lane 3 versus lane 4). Thus the absence of either ATP or activator leads to a loss of ability to produce the α-amanitin-sensitive abortive initiation product. In addition, inactivation of the promoter by deleting the TATA box also leads to an inability to produce an α-amanitin-sensitive abortive product (Fig. 3C, no loss of signal in lane 14 versus lane 13), an observation that further applies to a promoterless DNA vector (not shown). Thus production of an α-amanitin-resistant product requires the promoter TATA box, an activator, and ATP. These are identical to the requirements for efficient long RNA formation.

The background activity, producing trinucleotide in the presence of α-amanitin, is similar to that reported previously in studies of a different abortive initiation product at the adenovirus major late promoter (Luse and Jacob, 1987). The above data
show that this activity is suppressed when ATP, promoter, and activator cooperate to form an open complex (Fig. 3, lane 3). We have not further identified this activity that produces amanitin-resistant RNA. Nonspecific reactions of RNA polymerase II and activities of other polymerases are known to catalyze formation of trinucleotide products in an α-amanitin-resistant reaction (de Mercoyrol et al., 1989). We emphasize that the following experiments use the criterion of appearance of the α-amanitin-sensitive RNA product as an indicator of an appropriate abortive initiation reaction.

The Abortive Initiation Reaction Does Not Require DNA Supercoiling or Polymerase CTD Phosphorylation—A number of factors and processes have been suggested to be involved in the transition from preinitiation complex to elongation complex (see the introduction). This overall process includes the critical step of first bond formation. Thus the influences of these factors and processes can be evaluated in the context of the abortive initiation assay. We now test roles for DNA supercoiling and for phosphorylation of the polymerase CTD.

DNA supercoiling has been shown to play an important role in influencing transcription initiation in systems using isolated components (Goodrich and Tjian, 1994; Parvin and Sharp, 1993; Timmers, 1994). Certain components are needed for the transition from preinitiation to elongation complex on linear DNA. When the DNA is supercoiled, the transition occurs in the absence of certain of these factors. It is not known if there is a requirement for supercoiling for formation of an α-amanitin-sensitive abortive initiation product at the promoter studied here. Therefore we repeated the abortive initiation assay but substituted a 350-base polymerase chain reaction fragment (from approximately −240 to +110) for the form I plasmid used in the above experiments. Fig. 4 shows that the α-amanitin-sensitive abortive initiation product is still produced in this protocol (lane 4 versus lane 5). Thus supercoiling is not required for this reaction (as also observed at the adenovirus E4 promoter by Jacob et al. (1991)). We cannot determine if supercoiling influences the extent of either the reaction or its dependence on ATP; this requires two difficult quantitative assays: monitoring any loss of supercoiling in the plasmid and monitoring the amount of active template that survives the column isolation for supercoiled and linear templates.

During transcription initiation the C-terminal domain of RNA polymerase II becomes phosphorylated. Several studies indicate that this reaction occurs during the transition from preinitiation complex to elongation complex (Laybourn and Dahmus, 1989; Payne et al., 1989; Lu et al., 1991). There is considerable uncertainty about the role of this reaction, but it is generally proposed to assist in the transition, especially at activated promoters (reviewed by Drapkin and Reinberg (1994)). Therefore we will test whether inhibiting this activity inhibits the formation of the first bond as monitored by the abortive initiation assay.

The CTD kinase activity is associated with the TFIK component of transcription factor TFIIH (Feaver et al., 1994; Svejstrup et al., 1995). Studies on different promoters have shown that the kinase activity is inhibited by compound H8 (Serizawa et al., 1993a). This compound has been shown to inhibit CTD phosphorylation and transcription levels in the same activated adenovirus E4 transcription system used here (jiang and Gralla, 1994). However, the data of Fig. 4 (lane 6 versus lane 7) show that H8 does not strongly inhibit abortive initiation from open complexes. In addition, the product produced in the presence of H8 is still sensitive to α-amanitin (Fig. 4, lane 10 versus lane 11). We showed previously that H8 does not inhibit the formation of open complexes at this same promoter (jiang and Gralla, 1994). Thus the results suggest that H8 inhibition of transcription, and thus the requirement for CTD kinase activity, occurs after formation of the first bond.

The Abortive Initiation Reaction Appears To Be Reiterative

Fig. 3. Specificity of abortive initiation. A, α-amanitin sensitivity in the abortive initiation assay. All samples contained 2 mM UpA and 1 μM labeled CTP. When indicated (+), α-amanitin was present at 1 μg/ml and dATP was present at 10 μM. B, activator requirement. As in A except no activator was present. C, on a template with the TATA box deleted. Activator was present.

Fig. 4. Effects of supercoiling and the H8 CTD kinase inhibitor. A, abortive initiation using a fragment template. All samples contain 1 μM labeled CTP and the standard concentrations of the other indicated compounds. B, effect of 2 mM H8, which was preincubated with enriched preinitiation complexes for 5 min when present (+). All samples contain standard amounts of UpA and CTP.
Fig. 5. Test of reiterative synthesis. Standard abortive initiation was initiated by the addition of nucleotides, and the amount of product was determined at the indicated subsequent times (diamonds). Samples were removed after 5 min and added to either elongation substrates (triangle) or α-amanitin (circle). 5 min later the amount of product was measured in both cases, as shown. The numbers on the y axis are arbitrary units from PhosphorImager analysis.

The presence of elongation substrates. The standard abortive initiation reaction is represented by the combination of compounds shown in lane 1. Suppression of the reaction by the addition of 50 μM elongation substrates (indicated by a bar) is shown in lanes 3, 4, 7, and 8.

The products that had formed could not be chased efficiently. In addition, the addition of elongation substrates appears to have essentially halted the abortive initiation reaction (see also below). The result is similar to the one obtained if α-amanitin is added at the same time in a parallel reaction (circle in Fig. 5). These characteristics are those expected to be associated with a reaction that proceeds reiteratively in the absence of elongation substrates.

Finally the experiment of Fig. 6 tests whether the abortive initiation reaction occurs reiteratively primarily because elongation substrates are absent. That is, it asks if a very significant amount of abortive product can accumulate during normal productive initiation that takes place in the presence of all elongation substrates. The question arises because abortive initiation does occur during normal initiation at certain prokaryotic promoters and has been suggested to occur at certain weak RNA polymerase II promoters (Carpousis and Gralla, 1980; Jacob et al., 1994).

Fig. 6 shows the amount of abortive product obtained in parallel reactions with and without elongation substrates. When 50 μM UTP, GTP, and either ATP (lane 4) or AMPPNP and dATP (lane 3) are added to the standard abortive reaction containing UpA and CTP, there is a strong decrease in the signal (compare with a standard reaction in lane 1). The comparison shows that significantly less product is seen when all four elongation substrates are present. Comparable reductions were not seen when any of the elongation substrates were added separately (not shown). In addition no new bands are seen (see Fig. 6) that might correspond to new abortive products that could accompany normal transcription initiation. Thus at this promoter the presence of elongation substrates suppresses abortive initiation, perhaps by favoring RNA extension over release, thus disfavoring the reiterative reaction.

Inhibition of CTD kinase activity by H8 can slow the escape of the polymerase from this promoter.3 It is not known how this happens, but one possibility is that the polymerase stalls during first bond synthesis and is caught in an abortive initiation mode. Thus it is possible that under these conditions there will be an enhanced amount of abortive initiation even though all elongation substrates are present. However, the results shown in Fig. 6 suggest that this effect is either weak or absent (lanes

3 Y. Jiang and J. D. Gralla, unpublished observations.
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7 and 8 in the presence of H8 and elongation substrates versus lane 5 without elongation substrates). Thus inhibition of CTD kinase activity does not seem to trap polymerase in abortive initiation mode.

DISCUSSION

These experiments have established an abortive initiation reaction at the activated adenovirus E4 promoter and used it to determine requirements for first bond formation. There has been considerable uncertainty about these requirements, especially with regard to the role of ATP (see the introduction). We consider four activities that have previously been proposed to influence the transition from preinitiation complex to elongation complex, which of course includes the critical step of first bond formation. These are polymerase CTD kinase activity, DNA supercoiling, ATP hydrolysis, and activator function. The results of this study suggest that two of these are required for efficient first bond formation at the adenovirus E4 promoter (ATP hydrolysis and activator), whereas the other two activities are not required. We discuss the evidence for these conclusions and attempt to place them in the context of several prior studies.

The two factors that are required to produce significant amounts of α-amanitin-sensitive abortive initiation product are a source of β-γ hydrolyzable ATP and a transcription activator. Previous studies have shown that these two factors together are necessary to produce significant amounts of open transcription complexes (Wang et al., 1992a; Jiang et al., 1993). That is, in the absence of either, the preinitiation complexes that accumulate are primarily in the closed form. We conclude that in this system only open complexes produce significant amounts of proper abortive initiation product. The role of ATP hydrolysis in this case is to trigger open complex formation, and it is therefore required indirectly in the process of first bond formation.

This conclusion may be compared with two prior studies of abortive initiation, both at the adenovirus major late promoter. Neither prior report studied open complex formation directly, but both addressed the role of ATP. In one case ATP was reported to stimulate abortive initiation (Luse and Jacob, 1987), in agreement with the current findings at the adeno E4 promoter. In addition, they found that abortive initiation in the presence of ATP was inhibited by α-amanitin, as was also found here. In the other case, no ATP stimulation was detected, and the response to α-amanitin was not tested (Goodrich and Tjian, 1994). Of the three studies, the two that agree in these respects have in common the use of Hela extracts as a source of transcription factors and the use of activated transcription (either added GAL-AH or endogenous USF). In the other study a purified basal system was used. In such systems high concentrations of both DNA template and factors allows the requirement for activator to be bypassed. It is possible that this system has also bypassed the requirement for ATP to open the DNA. Alternatively, the abortive initiation product observed in that system might result from the α-amanitin-resistant process described here at the E4 promoter and previously at the adeno major late promoter. The process that produces this product is not known, but an undefined activity associated with purified RNA polymerase II has been reported to produce such products on poly(dA·dT) templates (de Mercroy et al., 1989).

The data also indicate that neither DNA supercoiling nor the activity that phosphorylates the polymerase C-terminal domain is required for first bond formation. Of these the lack of requirement for polymerase phosphorylation is of greater interest because it has been proposed to affect the transition from preinitiation complex to elongation complex (Laybourn and Dahmus, 1989; Lu et al., 1991; Payne et al., 1989; Usheva et al., 1992). Studies of activated transcription have shown that the CTD phosphorylation inhibitor H8 depresses transcription levels. In the same system studied here, transcription was inhibited by H8, but open complex formation was not inhibited, suggesting that CTD phosphorylation can facilitate a step after open complex formation. The current observation is that H8 also does not inhibit formation of the first bond of the mRNA, and thus we infer that the step that is facilitated by CTD phosphorylation occurs after formation of the first bond of the mRNA. As discussed elsewhere, this may occur by giving assistance to initiated polymerases in moving out of the initial transcribed region.

The data are also relevant to abortive initiation, both as an assay for first bond formation and as a phenomenon that might occur during normal transcription initiation. The experiments show that abortive initiation is strongly suppressed when all nucleoside triphosphates required for elongation are present. This observation suggests that abortive initiation is probably a relatively infrequent event during normal productive transcription initiation at the activated E4 promoter. This is consistent with a prior report that showed that allowing extension of the abortive initiation product by a single nucleotide at the adeno major late promoter leads to a reduction in signal (Luse and Jacob, 1987). In addition, repeated attempts (as in Fig. 6) have failed to detect significant amounts of longer products when nucleotides that allow elongation are present (but our experiments use only 10 ng of DNA/sample). This suggests that such longer RNAs do not accumulate in very high amounts as products of a reiterative abortive initiation reaction at this promoter. It has been reported that mutations that weaken a promoter can lead to increases in the amounts of short RNA products, including ones longer than trinucleotide (J acob et al., 1994). Thus there appear to be cases where abortive initiation may occur during normal productive initiation, but there is not yet an example of this at a strong promoter.

Taken together with other data, a view of the initiation pathway can be developed. Closed preinitiation complexes assemble in response to activators, and then the DNA within them is opened by ATP hydrolysis. The first bond of the mRNA can then form and then several more bonds. Further extension of the transcript is stimulated by the ATP-dependent phosphorylation of the polymerase. At some point the polymerase clears the promoter, allowing entry of a new polymerase for reinitiation. Further challenges include testing this model and determining whether there are important promoter-dependent variations within it.

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