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Variations in Template Protection by the RNA Polymerase II Transcription Complex during the Initiation Process

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Preinitiation complexes (complex 0) or complexes which either made 2 or an average of 10 phosphodiester bonds (complexes 2 and 10, respectively) were assembled in vitro on the adenovirus 2 major late promoter. Each of the complexes was digested extensively with DNase I; the protected DNAs were purified and hybridized to a series of end-labeled oligonucleotides homologous to sequences on the coding or noncoding strands near the initiation site. The hybrids were then extended with reverse transcriptase to map the extent of template protection conferred by proteins in the complex. The downstream protection edge revealed by this approach was approximately +30, +25, and +35 for complexes 0, 2, and 10, respectively. We subsequently found that the apparent inward movement of the downstream protection boundary on initiation could be produced by satisfying the energy requirement for transcription initiation (i.e., by treating with ATP or dATP). The downstream boundary change occurred as rapidly as we could perform the test (<60 s) and was not blocked by α-amanitin. DNAs from trimmed complexes 0, 2, or 10 all supported extension to a single upstream edge at about position −42. Upstream protection was stable in the preinitiation complex, but when postinitiation complexes were incubated for extended periods, protection of the entire upstream region was lost. This decay of upstream protection, like the movement of the downstream boundary, was found to result from exposure to ATP or dATP. Unlike the downstream boundary movement, however, the upstream change was relatively slow; about 15 min was required to lose one-half of the protection.

Transcription initiation is a complex, multistep process which converts the preinitiation, promoter-recognizing form of the transcription complex into an elongating form that is committed to RNA synthesis. At *Escherichia coli* promoters such as *lacUV5* as many as 10 phosphodiester bonds must be made before commitment to chain elongation is achieved (4). During this initial phase of transcription the pattern of template protection changes very little from that observed with the preinitiation complex (4, 16, 17). The process of promoter clearance (conversion from the initiating to the elongating form) has not been extensively studied for the eucaryotic enzyme RNA polymerase II. It is well established that at least one step in the RNA polymerase II initiation process requires energy in the form of ATP or dATP (2, 14); however, the molecular basis for this requirement is unknown. In our laboratory we have recently begun to analyze initiation by RNA polymerase II at the adenovirus 2 major late (Ad2 ML) promoter. We have shown that after the synthesis of two phosphodiester bonds, a relatively stable, nonaborting ternary complex is formed at the Ad2 ML promoter; however, the fully stable elongation state is not reached until 10 bonds have been made (3, 10). As part of these studies, we investigated the extent of downstream template protection during the initiation process by extensively digesting complexes which had made 0, 2, or an average of 10 bonds with DNase I and measuring the length of the RNA synthesized by the trimmed complexes. We were surprised to find (3) that the two-bond complex protected substantially less DNA downstream than did the preinitiation complex. This is consistent with the possibility that a massive conformational change, the loss of a subunit, or both accompanies the acquisition of many of the characteristics of an elongation complex when two bonds are made. In this study we greatly extended the approach of following template protection at the Ad2 ML promoter as a function of the initiation process. We found that the most pronounced changes in the complex, which resulted in the loss of template protection both upstream and downstream of the initiation site, occurred when the energy requirement for initiation was fulfilled.

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MATERIALS AND METHODS

Templates, nuclear extracts, and transcription complexes. Transcription complexes were assembled on the pSmaF-1 plasmid, which contains the Ad2 ML promoter (3), by using nuclear extracts made from HeLa cells (6). Plasmid DNA was cleaved with *Hin*dIII before assembly. Preinitiation complex (complex 0) was obtained by incubating pSmaF-1 DNA in nuclear extract for 1 h, as described previously (3); complex 0 was purified by chromatography on Bio-Gel A1.5m (Bio-Rad Laboratories, Richmond, Calif.) (3). Complex 0 was converted to complex 2 (2 bonds) or complex 10 (5 to 12 bonds) by incubating complex 0 with the appropriate nucleoside triphosphates (NTPs), as described previously (3). Briefly, the coding strand near the initiating A residue (underlined) of the Ad2 ML promoter reads as follows: . . .TCACTCTCTTCCGCAT. . . (19). Thus, incubation of complex 0 for 5 min with the primer dinucleotide UpC (2 mM) plus ATP (20 μM) and CTP (0.5 μM) leads to the synthesis of two bonds; the nascent RNA (UCAC) is retained in a ternary complex (10). When complex 0 is incubated for 10 min with ATP and CTP (each at 50 μM) and very limited UTP (0.13 μM), it elongates to a collection of paused ternary complexes with nascent RNAs of (predominantly) 6, 7, 10, and 13 bases; we refer to this collection of complexes as complex 10. RNA polymerase was run off by incubating complex 10 for 10 min with 0.5 mM A, C, G, and UTP. Mock complex 0 was obtained by incubating nuclear extract and

template DNA under the same conditions used for complex 0, except that 0.025% Sarkosyl (Sigma Chemical Co., St. Louis, Mo.) was also present in the incubation.

DNase digestion and determination of template protection boundary. The Bio-Gel column buffer used in purification of complex 0 consisted of 100 mM KCl, 7.5 mM $MgCl_2$, 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; pH 7.9 at 25°C), 0.2 mM EDTA, and 20% (vol/vol) glycerol (10); digestions of complex 0 were performed by adding DNase I (Sigma Chemical Co., St. Louis, Mo.) to the complex and incubating at 25°C. Complexes 2 and 10 were generated by incubation of complex 0 with the appropriate NTPs at 25°C (see above); DNase was then added directly, and the incubations were continued. Unless noted otherwise, all DNase digestions were done for 2 min with 80 μg of enzyme per ml. Reactions typically involved 20 to 50 μl of complex 0 (see figure legends), containing about 2 ng of pSmaF-1 DNA per μl. The surviving DNA was purified by extraction with 1 volume of phenol-chloroform (1:1), ethanol precipitated with 8 μg of tRNA carrier, and suspended in 10 μl of 50 mM NaCl–6 mM $MgCl_2$–34 mM Tris hydrochloride (pH 8.3 at 42°C). The resuspension buffer also contained one of three 18- or 20-base synthetic oligonucleotides that are homologous to different DNA sequences near the initiation site of the Ad2 ML promoter. The oligonucleotides were gel purified and 5′ end labeled with T4 polynucleotide kinase before use; the molar excess of oligonucleotide to pSmaF-1 DNA was five- to eightfold for reactions involving complex 0 (see figure legends). The reactions were overlaid with mineral oil, heated to 100°C for 5 min, and incubated at 42°C for 25 min. Reverse transcriptase mix (1 μl) was then added, and the 42°C incubation was continued for an additional 30 min. Reverse transcriptase mix was assembled with 1 volume of avian myeloblastosis virus reverse transcriptase (17 U/μl; Life Sciences, Inc., St. Petersburg, Fla.) and 2 volumes each of 20 mM dATP, dCTP, dGTP, and TTP. For the extension shown in Fig. 3, *E. coli* DNA polymerase Klenow fragment (Pharmacia Fine Chemicals, Piscataway, N.J.) was used instead of reverse transcriptase. In this case the purified DNAs and appropriate amounts of primer (see above) were suspended in 8 μl of 113 mM NaCl–19 mM Tris hydrochloride (pH 8)–12 mM $MgCl_2$ and heated to 95°C for 7 min; the mixtures were then incubated at 42°C for 15 min and 37°C for 5 min. A total of 2 μl per reaction of Klenow mix (1 U/μl of DNA polymerase Klenow fragment and 1 mM of each of the deoxynucleoside triphosphates) was added, and incubation at 37°C was continued for 1 h. All extension reactions (reverse transcriptase or Klenow) were terminated by the addition of 5 μl of 10 mM EDTA–90% formamide plus tracking dyes, followed by heating at 100°C for 5 min. Electrophoresis was performed on 20% polyacrylamide gels containing 1% bis and 7 M urea.

To determine the extent of survival of upstream protection and of transcriptional activity after the energy requirement was satisfied (see Table 1), aliquots of complex 0 were incubated at 25°C for up to 32 min with 10 μM dATP plus 5 mM creatine phosphate and 100 μg of creatine kinase per ml to maintain dATP levels during the extended incubation. The aliquots were then divided into two portions and either digested with DNase I at 80 μg/ml for 2 min, followed by hybridization of the purified DNA to the 20u primer and extension with reverse transcriptase, or incubated at 25°C for 5 min with 50 μM ATP and CTP and 0.13 μM [α-^{32}P]UTP. The extent of upstream protection was measured by scintillation counting of the extension products in the −40 to −45 range (see Fig. 4B). Transcriptional activity was measured by scintillation counting of the 6- to 13-base RNAs.

RESULTS

We have shown previously (3, 10) that at least three functionally distinct RNA polymerase II transcription complexes can be identified during the process of accurate initiation at the Ad2 ML promoter. These complexes are (i) a preinitiation complex which synthesizes RNA immediately on exposure to NTPs; we refer to this as complex 0 (see also reference 7); (ii) the earliest stable ternary complex, which has made two phosphodiester bonds and which we refer to as complex 2; and (iii) a later elongation complex, which contains 6 to 13 bases in the nascent transcript and which we refer to as complex 10. When substrates are supplied which allow only one phosphodiester bond to be made, no ternary complex is obtained (10). We investigated the extent of downstream template protection conferred by these three complexes by digesting them extensively with DNase I and then allowing them to synthesize RNA from the truncated templates. We found that at comparable levels of digestion, complex 0 protected 10 bases further downstream than did complex 2 (3). We wished to study this effect in detail; we also wished to investigate the possibility that promoter protection caused by transcription factors might persist after the polymerase cleared the promoter. We therefore adopted the strategy of mapping the boundaries of template protection, both downstream and upstream of the initiation site, as a function of transcription initiation. This involved extensive digestion of the various complexes with DNase, followed by hybridization of labeled oligonucleotides to the protected DNA; the hybridized primers were then extended to whatever length the trimmed templates would support. Based on our own results (3) and those of Sawadogo and Roeder (15), we anticipated strong protection from about bases −40 through +30, at least in complex 0. We therefore synthesized three primers which would hybridize in this region: primer 18d, bases −8 to +10 of the coding strand; primer 20d, bases −20 to −1 of the coding strand; and primer 20u, bases +15 to −5 of the anticoding strand. The primer designations consist of the length, in bases, followed by a letter indicating whether that primer is elongated in the upstream or downstream direction.

Before we could employ these primers, it was necessary to establish certain experimental parameters. In our previous study (3), we showed that incubation at 25°C with 80 μg of DNase I per ml for 2 min gives an essentially limit digest of complexes 2 and 10, in that further digestion does not shorten further the runoff RNAs made by these complexes. Results of preliminary experiments with end-labeled pSmaF-1 DNA, which was digested with 80 μg of DNase I per ml for 1 to 3 min, showed that most of the DNA was reduced to a length of 12 to 25 base pairs (data not shown). This raised the possibility that fully digested (non-protein-protected) DNA would support some minimal extension after hybridization to our primers. This point is particularly important, since the majority of DNA that is incubated in crude nuclear extracts is not assembled into active transcription complex (10); the question of the exact fraction of template that is active in transcription in these studies is addressed below. Increasing amounts of purified pSmaF-1 DNA were digested with DNase I (80 μg/ml) for 2 min; end-labeled 18d, 20d, or 20u primer was hybridized to the surviving fragments and extended by reverse transcription (Fig. 1). (Note that in all of the figures the length of each extension product is given as

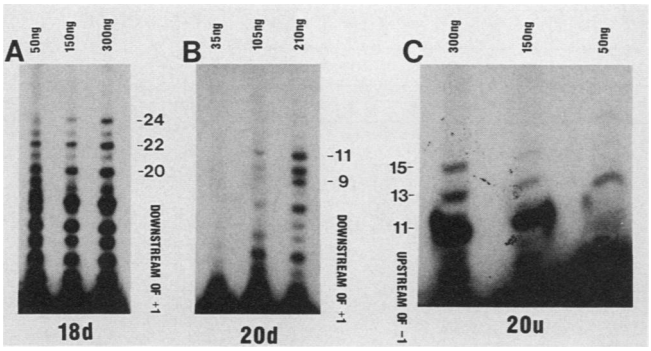

FIG. 1. Purified pSmaF-1 template DNA was digested with 80 μg of DNase I per ml for 2 min. The amounts of digested DNA, indicated at the top of the figure, were then hybridized to 1 ng of 18d (A), 20d (B), or 20u (C) primer; the hybrids were elongated with reverse transcriptase and electrophoresed. The extent of elongation upstream or downstream of the initiation site is given in the panel margins (size markers provided by dideoxy sequencing reactions, produced with the same primers; the markers were electrophoresed in adjacent lanes and are not shown).

the distance from the end of the DNase-truncated template to the transcription initiation site.) DNase digestion of protein-free DNA under these conditions left fragments that were large enough to support elongation of 18d to base +24, 20d to base +11, and 20u to base −15. This represents the addition of 14, 11, and 10 bases, respectively, to the primers. In many experiments (see Fig. 3 and 4) we saw fragments from the digestion of naked DNA support 18d elongation to base +25 or +26. The length and pattern of the control ladder was (except for the variation just noted) reproducible for each primer and was relatively independent of the amount of DNA added to the reaction. We noted in subsequent experiments that the intensity of the ladder obtained by primer extension on digests of naked DNA was often less than that of the apparent background ladder in lanes in which trimmed complex was used as a template for extension, in spite of our efforts to add the same amount of DNA in all reactions. Potential reasons for this problem are discussed below. Because of this variability, we routinely disregarded any extension products shorter than the longest DNAs obtained in our control extensions in which DNase-digested purified DNA was used as a template.

Having established the minimum meaningful elongation for each primer in the study shown in Fig. 1, we next wanted to explore protection beyond these points due to the various transcription complexes. As noted above, the use of 80 μg of DNase per ml for 2 min seemed an appropriate level of digestion for complexes 2 and 10; there remained, however, the question of how extensively complex 0 should be digested, since complex 0 is inactivated for RNA synthesis by DNase I digestion levels above 20 μg/ml for 2 to 3 min (3). We therefore followed the time course of DNase I digestion of complex 0 using the primer extension protocol (Fig. 2). Each numbered pair of lanes contained DNA from a single aliquot of complex 0 digested to the extent noted at the top of the figure. The purified DNA from each reaction was divided into two parts and hybridized to end-labeled 18d (Fig. 2, D lanes) or 20u (Fig. 2, U lanes) primer; the hybrids were elongated with reverse transcriptase. The downstream extension pattern that we obtained was complicated, particularly at low digestion levels. The longest DNAs extended to base +52, and there was a strong group of bands between bases +29 and +35. This pattern remained the same with increasing digestion up to 80 μg/ml for 2 min (Fig. 2, lane 4), at which point the longest bands abruptly dropped to bases +29 to +31. This shrinkage of protection coincided with the complete loss of transcriptional activity of complex 0 (3; data not shown). The upstream protection pattern was much simpler. Except for a set of very short extension products in the −11 to −15 range (which, as noted in Fig. 1, were produced in the absence of complex), essentially all the upstream extension proceeded to a set of bands between bases −40 and −45.

Based on the results shown in Fig. 2 and our previous work (3), we decided to continue the analysis of all of the early initiation complexes after DNase digestion at 80 μg/ml for 2 min. The initial results (Fig. 2) were reassuring, since the patterns of template protection that they revealed agreed with other data. Results of our earlier studies (3) indicated that the downstream edge of complex 0 DNase trimmed to the extent shown in Fig. 2, lane 2, was +30 to +45, as assayed by runoff RNA size. Furthermore, the sharp upstream protection boundary in Fig. 2 was in exactly the same position as the strong DNase I hypersensitive site which Sawadogo and Roeder (15) mapped at the upstream edge of transcription factor TFIID bound to the Ad2 ML promoter. It is necessary, however, to prove that the extension products in question resulted from protection by transcription complexes and not from some other protein-DNA interaction. To demonstrate this we assembled two batches of complex 0, one in the conventional manner and a second in

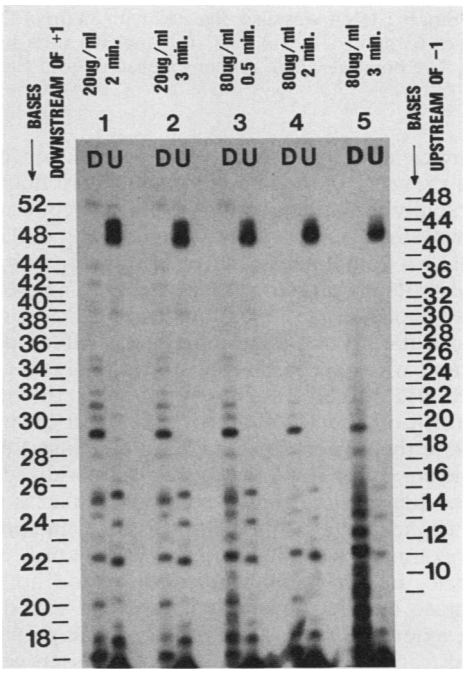

FIG. 2. Five 50-μl aliquots of complex 0 were incubated with DNase I to different extents, as indicated. DNA from each aliquot was purified, divided in half, and hybridized to 0.5 ng of end-labeled 18d (lanes D) or 20u (lanes U) primer; the hybrids were then extended with reverse transcriptase and the products were electrophoresed along with size markers provided by dideoxy sequencing reactions produced with the same primers; the markers were run in adjacent lanes and are not shown. Sizes of the D-lane extension products are given in the left margin and are expressed as the distance downstream of base +1 at which extension terminated; sizes for the U-lane extension products are given in the right margin and are expressed as the distance upstream of base −1 at which extension terminated.

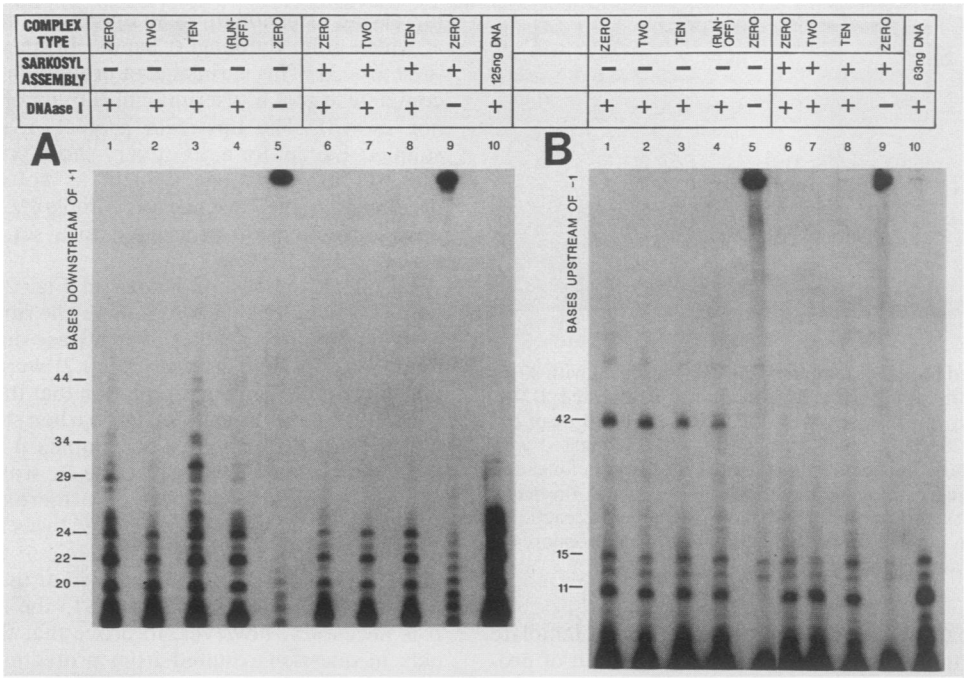

FIG. 3. Aliquots of 100 μl of normal complex 0 (lanes 1 to 4 in panels A and B) or 100-μl aliquots of complex 0 assembled with 0.025% Sarkosyl (lanes 6 to 8 in panels A and B) were digested with DNase at 80 μg/ml for 2 min. The DNAs purified from the transcription complex digests, along with DNA purified from 100-μl aliquots of nondigested normal and Sarkosyl-assembled complex 0, were each divided in half and hybridized to 1 ng of 18d (A) or 20u (B) primers; the hybrids were elongated with *E. coli* DNA polymerase Klenow fragment (see text). Purified pSmaF-1 DNA was also digested with 80 μg of DNase per ml for 2 min, and the resulting fragments (from 125 ng of DNA in lane 10 [panel A] or 63 ng of DNA in lane 10 [panel B]) were hybridized to 1 ng of 18d (lane 10 [panel A]) or 20u (lane 10 [panel B]) primer and extended. The point at which primer extension terminated, upstream or downstream of the initiation site, is given in the panel margins.

the presence of 0.025% Sarkosyl. At this concentration, Sarkosyl prevents the assembly of a transcriptionally competent, rapidly initiating complex (7, 8); we confirmed (data not shown) that our Sarkosyl-assembled complex 0 was indeed almost completely inactive in transcription, as demonstrated by its inability to synthesize 6- to 17-base RNAs in the presence of ATP, CTP, and limiting UTP. We incubated fractions of the normal and Sarkosyl-assembled complex 0 preparations with appropriate substrates to obtain complex 2 or 10; we also chased fractions of complex 10 with excess NTPs to run off the RNA polymerases. All of these complexes were then digested with 80 μg of DNase I per ml for 2 min; the surviving DNA was purified, hybridized to either the 18d or 20u primer, and extended with excess deoxynucleoside triphosphates (Fig. 3). In this case, the polymerase used for extension was the Klenow fragment of *E. coli* DNA polymerase. In our initial studies we tested both reverse transcriptase and Klenow polymerase and found that the resulting extension patterns, using the same preparation of protected fragments as a template, were essentially identical. Reverse transcriptase did, however, give fewer premature termination products in full-length extensions, so we used this enzyme exclusively in later experiments. For the normal complex 0, the extension pattern was essentially the same as that shown in Fig. 2 for the comparable level of digestion (compare lanes 1, Fig. 3A and B, with lanes 3 and 4, Fig. 2.) There was a strong downstream stop in elongation at base +29 and weaker stops out to approximately base +35 (Fig. 3A, lane 1). Consistent with results of our earlier work (3), downstream protection by complex 2 was less than that conferred by complex 0 (Fig. 3A, lane 2). The exact downstream boundary for complex 2 could not be determined in this experiment since it was indistinguishable from the pure DNA control (Fig. 3A, lane 10). Also consistent with results of our earlier work (3) was the downstream movement of the protection boundary as elongation proceeded to the complex 10 stage (Fig. 3A, lane 3); in this case there was a strong downstream stop at base +34 or +35 and weaker stops out to base +44. The downstream pattern for the complexes which were run off (Fig. 3A, lane 4) was basically the same as that of the purified DNA, except that a small fraction of the complex 10 pattern remained. This is consistent with a general loss of promoter protection coupled with the failure of a small portion of the paused complex 10 pool to elongate further (3, 10). In contrast to the downstream elongation patterns, the upstream patterns for (non-Sarkosyl) complexes 0, 2, 10, and runoff (Fig. 3B, lanes 1 to 4) were qualitatively very similar. The only significant elongation product beyond those produced by digested pure DNA (Fig. 3B, lane 10) was the band at about base −42. This signal was somewhat reduced in the complex 10 and runoff lanes, compared with that of complex 0. Most importantly, the Sarkosyl-assembled complex elongations, both upstream and downstream (Fig. 3A, lanes 6 to 8, and Fig. 3B, lanes 6 to 8), showed essentially no protection beyond that of the naked DNA controls. Primer extension on equal volumes of nondigested normal and Sarkosyl-assembled complex 0 showed that similar amounts of DNA were present in both complex 0 preparations (compare Fig. 3A, lanes 5 and 9, and Fig. 3B, lanes 5 and 9). Thus, we conclude that the protection that we observed beyond that conferred by the purified DNA controls is due to transcription complexes.

The data in Fig. 3 pose a number of important questions about changes in the configuration of the transcription com-

plex as initiation takes place. There is a large loss of downstream protection in complex 2 relative to that in complex 0. Since complex 2 is the least-elongated stable ternary complex, the question immediately arose as to which, if any, of the steps preceding the formation of the second bond would cause the same change. A related question concerns the rate at which the downstream change takes place. The complex 2 in Fig. 3 was incubated with substrates for 5 min before it was exposed to DNase. The results in Fig. 3 indicate that no movement of the upstream boundary (base −42) takes place at initiation, but upstream protection is gradually lost after initiation. What is the time course of this loss of protection and how many bonds must be made before this loss begins? To answer these questions we performed another experiment, for which the results are shown in Fig. 4. In this case complexes 0, 2, and 10 (as well as complex run off with excess NTPs) were incubated for various periods at 25°C before DNase digestion. Also, complex 0 was incubated with ATP alone or ATP analogs, to study the effect of satisfying the energy requirement for transcription initiation. The protected DNAs were used in primer extension reactions with the 18d (Fig. 4A), 20u (Fig. 4B), or 20d (Fig. 4C) oligonucleotides and reverse transcriptase in the manner described above.

The results (Fig. 4A, lanes 1 to 3) indicate that incubation of complex 0 at 25°C for up to 3 h does not eliminate the downstream contacts. Excision and scintillation counting of the bands at bases +28 to +32 showed that in lane 3 this signal dropped to 62% of the value in lane 1. Similar incubation of complex 10 (Fig. 4A, lanes 6 to 8) also left the extension pattern substantially unchanged. These results are consistent with results of our earlier study (3) in which we showed that the transcriptional activities of complexes 0 and 10 are resistant to prolonged incubation at 25°C. We were somewhat surprised to discover that the same inward movement of the downstream boundary seen at the complex 0 to complex 2 transition (Fig. 4A, lanes 4 and 5) could be obtained by satisfying the energy requirement for initiation (Fig. 4A, lanes 12 to 17). Exposure of complex 0 to ATP or dATP, either of which satisfies the energy requirement (14), led to an extension pattern like that of complex 2; however, AMP-PNP, which has a nonhydrolyzable β-γ bond and which does not fulfill the energy requirement (14), did not change the complex 0 pattern. It could be argued that initiation actually occurred in Fig. 4A, lanes 12 to 15, because of a minute amount of CTP contamination, thus allowing the initial phosphodiester bond to be made. Formation of the initial bond at Ad2 ML, however, is blocked by α-amanitin (D. S. Luse and G. Jacob, J. Biol. Chem., in press); α-amanitin has no effect on the ability of ATP to alter the downstream boundary (Fig. 4A, lane 16). Also, incubation with CTP alone left the complex 0 pattern unaltered (data not shown).

We could only determine an upper limit for the time required for downstream conformational change, since we digested the ATP-containing complex with DNase for 2 min. At the shortest ATP preincubation time tested (30 s; Fig. 4A, lane 12), the protection change was already complete. We digested complex 0 that had been incubated with ATP for 30 s with 80 μg of DNase per ml for 20 s (data not shown); analysis of the surviving DNA showed substantial (approx. 80%) loss of the complex 0 pattern. Based on these data we estimate that the conversion takes less than 60 s.

Upstream extension with primer 20u (Fig. 4B) on complex 0 showed that protection to approximately base −42 is predominantly retained even after 3 h of incubation at 25°C (Fig. 4B, lanes 1 to 3). In contrast to the result shown in Fig. 4A, however, upstream protection was almost completely lost in complex 10 preparations incubated for 1 h or more before DNase digestion (Fig. 4B, lanes 7 and 8), even though such complexes remain active in elongation of their nascent RNAs (3). This loss of upstream protection, like the change in downstream protection, could be caused by fulfilling the energy requirement for transcription initiation (Fig. 4B, lanes 12 to 17). Incubation of complex 0 with ATP or dATP for 10 min, even in the presence of α-amanitin, caused a substantial drop in the band at base −42 band; no change was seen if AMP-PNP was used instead of ATP. The upstream protection loss occurred much more slowly than the change in downstream protection (this point will be considered more fully below).

A disappointing aspect of the results in Fig. 4A is the fact that the loss of downstream protection on the addition of ATP or dATP on initiation could not be precisely mapped, since the elongation products in this case were no longer than products extended on digests of naked DNA (Fig. 1). This problem was resolved with the 20d primer, which gave extension only to base +11 by using purified DNA digests as template (Fig. 1). The use of the 20d primer gave results with complex 0 (Fig. 4C, lanes 1 to 3) that were identical to those obtained with the 18d primer. The inward movement of the boundary at initiation (Fig. 4C, lanes 4 and 5) or on exposure to ATP or dATP (Fig. 4C, lanes 12 to 16) produced (in either case) a new edge at base +25. A striking difference from Fig. 4A was the complete lack of any protection downstream of position +25 for complex 10. The ladder extending up to base +25 faded out with the incubation of either complex 10 (Fig. 4C, lanes 7 and 8) or the runoff complex at 25°C (Fig. 4C, lanes 10 and 11); extended incubation under runoff conditions (Fig. 4C, lane 11) left almost no protection beyond that conferred by a digest of purified DNA alone. The ladder also began to disappear on exposure of the complex to ATP for 10 min (Fig. 4C, lane 14). This is essentially the same time course of protection loss noted when the 20u primer was used to map the upstream region (Fig. 4B). A model encompassing the results presented in Fig. 4A to C is given below.

Two points concerning the findings in Fig. 4 deserve further comment. First, we noted that ATP alone triggers both an immediate shift in downstream protection and a slower change in upstream protection. We have shown previously (3) that the transcriptional activity of complex 0 falls off sharply after a 10-min incubation with ATP or dATP. It was thus of interest to determine whether the drop in upstream protection exactly paralleled the loss of transcriptional activity. To test this we performed a series of reactions similar to those done earlier (Fig. 4B, lanes 12 to 14). Complex 0 was incubated at 25°C (in this case, with 10 μM dATP), and samples were withdrawn at various times up to 32 min. Each sample was tested for upstream protection and for activity in the synthesis of 6- to 17-base RNAs in a UTP-limiting transcription assay relative to those of an unincubated control (see above). The results (Table 1) indicate that the loss of upstream contacts (half-time under our conditions, approx. 16 min) was much slower than the loss of transcriptional activity (half-time, approx. 2 min). It should also be noted that the decline in transcriptional activity with ATP or dATP was almost certainly not the result of a proteolysis artifact. A number of proteases have been described that are activated by ATP (9, 18). These enzymes also require Mg^{2+}; it is not clear whether they are activated by dATP. When we incubated complex 0 with 10 μM dATP

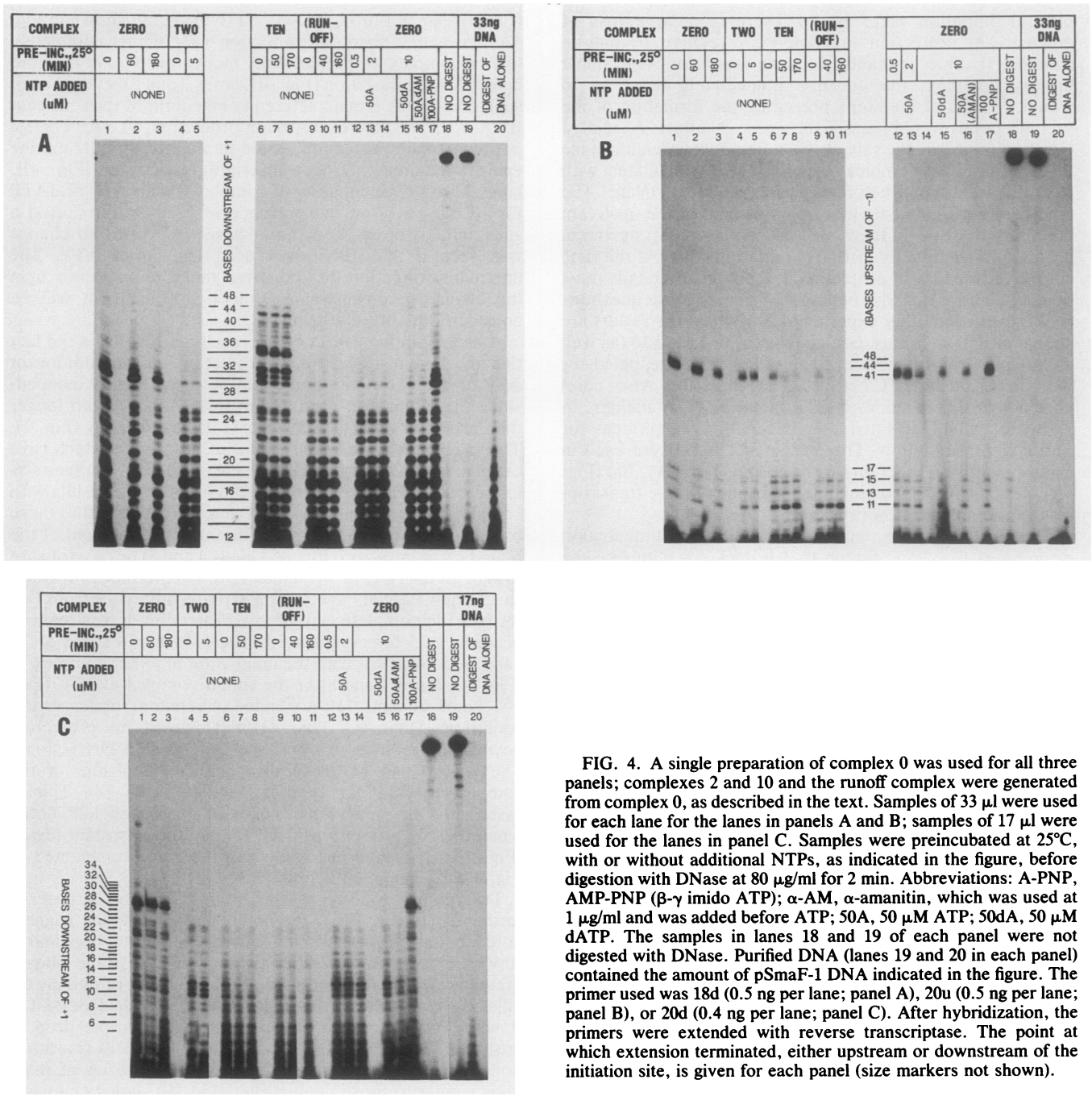

FIG. 4. A single preparation of complex 0 was used for all three panels; complexes 2 and 10 and the runoff complex were generated from complex 0, as described in the text. Samples of 33 μl were used for each lane for the lanes in panels A and B; samples of 17 μl were used for the lanes in panel C. Samples were preincubated at 25°C, with or without additional NTPs, as indicated in the figure, before digestion with DNase at 80 μg/ml for 2 min. Abbreviations: A-PNP, AMP-PNP (β-γ imido ATP); α-AM, α-amanitin, which was used at 1 μg/ml and was added before ATP; 50A, 50 μM ATP; 50dA, 50 μM dATP. The samples in lanes 18 and 19 of each panel were not digested with DNase. Purified DNA (lanes 19 and 20 in each panel) contained the amount of pSmaF-1 DNA indicated in the figure. The primer used was 18d (0.5 ng per lane; panel A), 20u (0.5 ng per lane; panel B), or 20d (0.4 ng per lane; panel C). After hybridization, the primers were extended with reverse transcriptase. The point at which extension terminated, either upstream or downstream of the initiation site, is given for each panel (size markers not shown).

and EDTA (in 0.5 mM excess over the Mg^{2+} concentration) or with 10 μM dATP and phenylmethylsulfonyl fluoride (0.1 mM), we observed the same decline in transcriptional activity noted in Table 1 (data not shown).

A second point of interest in Fig. 4 concerns the proportion of total DNA involved in transcription. Since the Bio-Gel column used in the preparation of complex 0 would not be expected to separate complex 0 from noncomplexed DNA, and since the assembly of RNA polymerase II transcription complexes is inefficient (3), it was anticipated that only a fraction of DNA in the complex 0 preparation would be active in transcription. Each panel in Fig. 4 contains one lane (lanes 18) in which complex 0 DNA was used directly for extension, without DNase digestion. In other lanes, in which the complex was digested, extension beyond the length in the purified DNA control should have occurred only for that subset of DNAs protected by complex. This is most easily seen in Fig. 4B, in which extension of the 20u primer occurred to essentially a single band. The ratio of the intensity of the −42 band in, for instance, Fig. 4B, lane 1, to the intensity of the extension product made on undigested complex 0 DNA (Fig. 4B, lane 18) should give the fraction of the total DNA which bears a transcription complex. We assumed that all protection was due to the transcription complex. This seems reasonable in view of the fact that both upstream and downstream protection patterns changed in response to NTPs and that the protection patterns were absent in complexes assembled in low levels of Sarkosyl that

TABLE 1. Survival of transcriptional activity and upstream protection for complex 0 on incubation with dATP[a]

Time (min)	Percentage of:	
	Upstream protection	Transcriptional activity
0	100	100
0.5	103 ± 6[b]	69[c]
1	99 ± 13	67 ± 8
2	82 ± 23	51 ± 16[b]
4	90 ± 11	33 ± 15
8	76 ± 8	18 ± 8
16	52 ± 17	15 ± 4
32	28 ± 9	14 ± 6[b]

[a] Details of the procedure are given in the text. At least three separate experiments were run for each point, unless indicated otherwise, and means ± standard deviations are given.

[b] Only two measurements were made for these points. For these measurements the mean ± range of the two measured values is given.

[c] Only one measurement was made for this point.

had no transcriptional activity. Independent determinations of the fraction of active templates (discussed below) were in agreement with this assumption. The major band at −42 (Fig. 4B, lane 1) had 20.2% of the counts of the full-length extension band (Fig. 4B, lane 18), as determined by scintillation counting. The amount of total DNA per volume of complex 0 was determined by comparing the intensity of the nondigested complex 0 extension product (Fig. 4B, lane 18) with the intensity of the extension product made on a known amount of purified pSmaF-1 DNA (Fig. 4B, lane 19). Results of preliminary studies in which DNA purified from complex 0 was coelectrophoresed on agarose gels with intensity standards indicated that our average complex 0 preparation contained about 1 ng of DNA per μl of complex. On the basis of this observation, the amounts of purified DNA used in the experiment shown in Fig. 4 were chosen. Scintillation counting of the extension products in Fig. 4B, lanes 18 and 19, gave an apparent DNA concentration of 0.97 ng/μl.

We wished to confirm our estimates of the fraction of template in complex independently by directly measuring the number of complexes that were active in RNA synthesis. This was done by allowing a sample of complex 0 to synthesize 6- to 17-base RNAs under severely UTP-limiting conditions (3, 10). The short RNAs were purified, resolved on 20% polyacrylamide gels, and excised and quantitated by scintillation counting. Note that, by definition, this assay involves only a single round of transcription, since the RNA polymerases have paused within 17 bases of the initiation site. We then determined the DNA content in a sample of the complex by extracting the DNA with phenol-chloroform and electrophoresing it on an agarose gel with intensity standards. We were surprised to find that this approach showed approximately twice the percentage of DNA in complex (41.5% for the complex 0 used in the experiments shown in Fig. 4), as we expected. This discrepancy was resolved when we examined more closely our method for extracting DNA from the transcription complexes. In all of the figures, DNA was recovered from the digested complexes by phenol-chloroform extraction (see above). We found that treatment with proteinase K (100 μg/ml at 37°C for 30 min, in the presence of 0.1% sodium dodecyl sulfate) before phenol-chloroform extraction gave a DNA recovery (as assayed either by primer extension or electrophoresis on agarose gels) that was about twice that obtained with phenol-chloroform extraction alone. Unfortunately, it was not possible to repeat the DNA determination on the complex 0 used in the experiments illustrated in Fig. 4. A different preparation of complex 0, however, was assayed in both ways. Comparison of the results of 20u primer extension on phenol-chloroform extracted DNA from either complex 0 or complex 0 treated with DNase indicated that 19.2% of the DNA was in complex. When we measured total DNA after proteinase K treatment and phenol-chloroform extraction and total complex by the amount of RNA that was synthesized, the value obtained for percent complex was 20.1%. Since these values agree quite well, we are confident that the failure to extract DNA completely with phenol-chloroform applied essentially equally to DNA that was in complex and to undigested free DNA. Thus, we have no reason to suppose that any substantial subpopulation of transcription complexes escaped our analysis. It does, however, seem likely that very short DNA fragments are efficiently extracted by phenol-chloroform alone. This would explain why we observed a weaker background extension pattern in the case of naked DNA versus transcription complex (Fig. 4). Compare, for example, lanes 11 and 20 in Fig. 4B. The extended-incubation runoff complex (Fig. 4B, lane 11) almost certainly represents free DNA, but the background ladder (−11, −13, and −15 bands) was much darker than that seen with extension of digested free DNA (Fig. 4B, lane 20). We presume that this is due to the fact that Fig. 4B, lane 11, actually contained about 66 ng of DNA (from 33 μl of complex), whereas only 33 ng of purified DNA was used in Fig. 4B, lane 20 (see also Fig. 1).

DISCUSSION

The molecular dynamics involved in transcription initiation by RNA polymerase II are not well understood, in part because a complete list of the participating proteins has not been made. We have begun to investigate this problem by studying protection of the template from extensive DNase I attack as initiation proceeds. This approach lacks the high resolution of conventional footprinting, but it has the important advantage of being applicable in a system in which only a minority of the DNA molecules bear transcription complexes (since noncomplexed DNA should be completely digested and provide no protection pattern). We are confident that the protection (beyond that conferred by digests of naked DNA) which we observed with our various transcription complex preparations actually reflects protein-DNA interactions in transcription complexes for several reasons. First, no protection was observed for mock complexes assembled in the presence of 0.025% Sarkosyl, which prevents the formation of a transcriptionally active complex (8). It should be noted that template commitment is not known to be inhibited by this level of Sarkosyl (8), so it is possible that one or more transcription factors could have bound to the template and remained bound through chromatography on Bio-Gel. However, we have consistently observed very little protection above that afforded by naked DNA digests when we used complex 0 assembled with Sarkosyl for primer elongation (Fig. 3). These low levels of protection, which were visible with long autoradiographic exposures, appear to be proportional to the very low but detectable RNA synthetic capability of the Sarkosyl-assembled complexes. The residual protection obtained with Sarkosyl-assembled complexes has always responded to ATP or NTP incubation in exactly the same way as the much stronger protection patterns given by normal complexes (data not shown). Second, the fraction of DNA in the transcription complex, as suggested by the results of the primer extension studies,

agrees well with the same value computed directly from measurements of moles of RNA synthesized per mole of template. Finally, both the movement of the downstream protection boundary in response to ATP, CTP, and limiting UTP (i.e., the complex 0 to complex 10 conversion) and the loss of protection in response to excess NTPs are the expected changes in template protection for a transcription complex.

The preinitiation complex, complex 0, protected a large stretch of template (about bases −42 to +31), even when it was digested with DNase at levels that rendered it transcriptionally inactive. At lower digestion levels, which preserved activity, protection reached from approximately bases −44 to +52 (Fig. 2, lanes 2 and 3). The protection edges of complex 0 at high DNase levels were almost exactly the same as those obtained by Sawadogo and Roeder (15) when they incubated only the TATA box-binding transcription factor TFIID with the Ad2 ML promoter. The similarity of the complex 0 protection pattern with that of TFIID extends to certain details of the patterns. In both cases unbroken protection was seen over the upstream promoter sequences, including the TATA box. TFIID protects the region from bases −23 to −33, not only from DNase I but also from methidiumpropyl-EDTA · Fe(II), which is a much smaller reagent (15). Downstream of base +1, TFIID binding induces alternate regions of protection and enhanced cleavage. The downstream cleavage pattern that we observed for complex 0 included a defined edge but also featured several strong internal cutting sites and some cutting at almost every position (Fig. 4C). Since the low DNase (activity retention level) protection pattern for complex 0 extended downstream considerably beyond the protection conferred by TFIID, it is tempting to speculate that higher digestion levels destroy activity by disrupting the interaction of a component other than TFIID with the template.

Probably the most significant of our results is the observation that incubation with ATP (or dATP) alone, without transcription initiation, causes a profound change in template protection by complex 0. There were two aspects to this change. The downstream protection edge immediately retreated from bases +31 to +25, and upstream protection decayed with a half-life of about 16 min (as opposed to >3 h without prior ATP incubation). At this point we cannot say which components shifted within the complex to cause this change in protection. It is important to note that the downstream boundary of complex 0 after ATP exposure was clearly closer to the initiation site than would be expected from the extent of TFIID template protection. Both this point and the loss of upstream (probably TFIID-specific) protection are consistent with a model in which ATP destabilizes TFIID binding specifically by disrupting its downstream contacts. Thus, a single event could be the source of both the rapid downstream loss and the slower upstream loss of template protection. It is also interesting that complex 0, which was first digested with DNase at 80 μg/ml for 2 min, exposed to ATP, and then further digested for 2 min, yielded a downstream protection pattern that extended only to base +25 (data not shown). Therefore, DNase inactivation of the transcriptional activity of complex 0 does not occur because the complex can no longer respond to ATP.

We saw no evidence of downstream boundary movement for the complex 0 with ATP to complex 2 transition (Fig. 4A, lane 4 versus lane 12). The protection boundary did move downstream when elongation proceeded to the complex 10 stage (Fig. 4A, lane 1 versus lane 6). Since the RNA polymerase is the one component of the complex which we know must translocate downstream, it is reasonable to assign to RNA polymerase movement the downstream protection that is unique to complex 10. Such movement can also explain the apparent loss of downstream protection for complex 10 when it was assayed with the 20d primer (Fig. 4C). Recall that the 18d primer, which detects complex 10 movement, hybridizes to bases −8 to +10 of the anticoding strand, while the 20d primer, which shows loss of downstream protection, hybridizes to bases −20 to −1 of the anticoding strand. Thus, if elongation from complex 2 to complex 10 revealed a strong cleavage site on the anticoding strand near position −5, the 20d primer would no longer be able to hybridize to fragments that could support downstream elongation. It should be noted that the acquisition of a cutting site at approximately −5 is not due to the loss of TFIID, since complex 10 samples that were not incubated extensively at 25°C retained considerable upstream protection but failed to give any downstream elongation products with the 20d primer (compare lanes 6 in Fig. 4B and 4C). It is clear that more than 1 h of incubation at 25°C abolishes all upstream protection for complex 10, and yet this complex remains fully active for elongation (3). Thus, the upstream contacts (and, by inference, the presence of TFIID) are not required for further transcriptional activity by complex 10. One could argue, by extension of the point made above, that loss of upstream protection is not due to the loss of TFIID binding, as we suppose, but to cutting at or near base −5 on the coding strand; this site would presumably be slowly revealed as a result of exposure to ATP. We cannot absolutely disprove such a model, but the results of downstream elongation with primer 20d argue against it. If TFIID remains stably bound for long periods of time even when upstream protection, as assayed by elongation of the 20u primer, is lost, then one would expect the downstream protection conferred by this factor to persist also. However, downstream protection, as assayed by 20d elongation, decays, when complex is exposed to ATP, at about the same rate as upstream protection (Table 2). The simplest explanation for this coordinate loss of upstream and downstream protection is the dissociation of TFIID from the template.

We do not know, in the case of complex 0 exposed to ATP, whether the presumed loss of TFIID binding coincides with the general disruption of the complex. The fact that transcriptional activity decays much more rapidly than template protection (Table 1), however, is consistent with the loss of one or more required components considerably before the dissociation of TFIID. Alternatively, TFIID may be required for the initiation step itself (as opposed to promoter recognition). One could then suppose that the action of ATP not only destabilizes TFIID binding to DNA but also destabilizes a necessary interaction of TFIID with the rest of the complex, leading to the decay of transcriptional activity with a half-life of about 2 min.

If we are correct in assigning the loss of upstream protection to the loss of TFIID, then one final point deserves further comment. Because of the stability and extent of its interaction with promoter DNA, factor TFIID (in conjunction with other core transcription factors, such as TFIIA [12, 13]) provides a good candidate for a component whose long-term promoter binding would form a stable transcription complex, thereby continuously committing the associated gene to an active state (1). We did not detect any long-term (i.e., >1 h) residual association of proteins with the Ad2 ML promoter after transcription initiation occurred. Upstream contacts which were not necessary for elongation, however, were not immediately lost at initiation but per-

TABLE 2. Time course of loss of upstream protection and downstream protection after exposure to ATP

Complex and lane no. in Fig. 4	Time (min) of exposure to ATP	% protection[a]	
		Downstream	Upstream
Addition of ATP alone			
13	0.5	100.0	100.0
14	2	88.7	80.9
15	10	75.5	55.3
Complex 10			
7	10	52.6	43.7
8	60	16.2	15.0
9	180	16.7	11.0
Runoff complex			
10	20	33.9	20.0
11	60	26.7	6.8
12	180	17.2	4.2

[a] Relative protection values were determined by using the gels shown in Fig. 4B and C. For downstream protection, bands on the autoradiogram from bases +12 to +25 were scanned with a microdensitometer; for upstream protection the region of the gel from bases −40 to −45 was counted by scintillation.

sisted for, on average, 15 min. A protein factor (termed upstream stimulatory factor, or USF, by Sawadogo and Roeder [15]; see also references 5 and 11) has already been described which binds upstream of TFIID at the Ad2 ML promoter and which stabilizes the binary complex of TFIID with the promoter. Since Sawadogo and Roeder (15) observed some transcriptional stimulation caused by USF in nuclear extracts prepared identically to ours (6), it is likely that USF was also present in our experiments. We did not, however, assay USF levels in our system. Given the importance of determining the molecular basis for committing a promoter to long-term activity and the established ability of purified USF to stabilize the TFIID-DNA interaction, it will be interesting to test the effects of the deletion of the USF binding site on the ATP- or initiation-induced destabilization of protection of the TFIID-binding region.

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