Interactions between RNA Polymerase II, Factors, and Template Leading to Accurate Transcription*

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Accurate transcription by RNA polymerase II has been shown to require multiple factors in addition to the purified polymerase. In this study, we use a reconstituted transcription system, consisting of RNA polymerase II and three essential HeLa cell chromatographic fractions, to study events leading to transcription from the adenovirus major late promoter. A preincubation-pulse-chase protocol resolves the reaction into events occurring before and after nucleotide addition. Preincubation of template with a mixture of RNA polymerase II and factors allows formation of "activated" complexes, which are defined by the ability to rapidly commence accurate transcription when presented nucleotides. Maximal activation requires the polymerase, template, and each of the three HeLa fractions be present during preincubation. The activated complexes are template associated, as shown by their inability to exchange onto a second template added during further preincubation. Similar protocols are used to define functional intermediates leading to the activated complex. A template-associated functional complex is formed during the preincubation of template with just two of the HeLa fractions. Polymerase can associate with this intermediate complex in the absence of the third HeLa fraction. In the accompanying paper, we describe a direct analysis of initiation by "activated" complexes.

Synthesis of messenger RNA precursors in eukaryotic cells is catalyzed by RNA polymerase II (1). An understanding of site selection and transcription by this enzyme is crucial to the biochemical analysis of gene expression. Precise mapping of 5' termini for a variety of messenger RNAs in vitro has been shown to define faithful transcription in vitro. Purified RNA polymerase II does not faithfully initiate transcription on any gene so far tested. However, cellular extracts have been shown to carry out faithful transcription on a variety of genes (2-4). Specific transcription requires several factors present in cellular extracts in addition to purified RNA polymerase II (5). These factors have been resolved by chromatography and can be used to reconstitute a reaction similar to that observed in the initial extract (5-8). At the moment, at least four factors have been detected; as yet, none has been purified to homogeneity.

The quantitative assay for faithful transcription has been to measure total accumulation of a discrete length RNA product. Because efficient termination has not been observed in vitro, such studies generally use a cleaved DNA template. Accurate initiation followed by elongation to the end of the template produces a "runoff RNA" that migrates as a band upon electrophoresis. Even in the simplest conceivable scheme, many different events must occur to generate runoff RNA. Polymerase must bind to the initiation site; the strands of the helix must be locally separated to allow reading of the DNA; the two first nucleoside triphosphates must be properly positioned and joined to initiate transcription; finally, subsequent nucleotides must be added to complete synthesis of the runoff transcript (elongation). The requirement for multiple protein factors in addition to RNA polymerase II suggests that the transcription reaction could be considerably more intricate than this simple scheme.

Because runoff transcript accumulation depends on a complex series of events, a large change in rate for a step not near limiting would be undetectable. This is of particular importance in attempting to reconstruct regulatory effects and sequence dependences observed in vivo. For example, deletion of a DNA segment promoting polymerase binding might have no detectable effect on total accumulation if the relevant binding step is not limiting under the assay conditions used. A kinetic analysis of different steps in the reaction would contribute to interpretation of such studies, and could provide the desired biochemical assays for regulation and for upstream promoter elements.

The pathway leading to transcription by Escherichia coli RNA polymerase has been well studied (9). The initial [polymerase·promoter] complex, called the "closed" complex, must undergo an isomerization reaction to an "open" complex before initiating transcription. This isomerization is nucleotide independent and apparently involves an opening of the DNA helix around the initiation site (10). For some promoters, efficient formation of the open complex requires extra factors in addition to the polymerase holoenzyme. In the case of the lac promoter, an activator (catabolite activator protein) binds specifically to the template, facilitating subsequent transcription by polymerase (11, 12). A similar phenomenon occurs in transcription of S 5 genes by RNA polymerase III; in that case, the promoter recognition event appears to be mediated primarily by factor TFIIIA, a highly promoter-specific DNA-binding protein (13-16). The "preprimosome" formed during synthesis of replication primers on φX174 provides a more intricate example of sequence-specific events that can precede polymerase binding (17). In each of the
above cases, a functional complex can be identified on the template in the absence of polymerase. In the 5S and cabotolite activation examples, the interaction between factor and template plays a physiological role in regulating transcription.

We describe here a kinetic dissection of the RNA polymerase II transcription reaction. In particular, assays for events preceding initiation and an assay for subsequent elongation events are presented. These assays have been used to obtain information concerning the roles of template, polymerase, and the required protein fractions in events leading to transcription.

MATERIALS AND METHODS

Unlabeled nucleoside triphosphates purified by high pressure liquid chromatography were purchased from ICN. [α-32P]UTP and GTP were purchased from New England Nuclear. Purified human placental RNase inhibitor (18) (RNasin) was purchased from Biotec. Poly(dI-dC):poly(dI-dC) was purchased from Collaborative Research. HeLa chromatin fractions (7) and purified calf thymus RNA polymerase II (19) were prepared as described. Our preparations of RNA polymerase II had specific activities of 1-2 × 10^5 units/mg (7). Purity of the polymerase preparation (>95%) was determined by examination of denaturing polyacrylamide gels stained with silver nitrate.

**Transcription Reaction**—The following solution conditions were maintained throughout each reaction: 12 mM Hepes-NaOH, pH 7.9, 12% glycerol, 1 mM EDTA, 60 mM dithiothreitol, 60 mM KCl, 5 mM MgCl₂. To start preincubations, proteins (mixed at 4°C) were added to DNA; all incubations were at 30°C. A standard "complete" preincubation contained (except where noted) template DNA, RNA polymerase II (20 units), RNasin (15 units), and HeLa chromatin fractions; 1 μl [AB], 3 μl [CB], 2 μl [DB], and 2 μl [CD]. Preincubations were performed in 20 μl; in experiments with sequential preincubations, the first preincubation was in 15 μl while the second preincubation bringing volume up to 20 μl. DNA concentrations in the text all refer to a 20-μl preincubation. Pulses and chases each added 5 μl to total volume. Pulse nucleotide concentrations were 2 μM [α-32P]UTP or GTP (400 Ci/mmol) and 30 μM of the three unlabeled nucleoside triphosphates. Chase nucleotide concentrations were 1 μM of each of the four unlabeled triphosphates, with equimolar MgCl₂ added.

**RNA Analysis**—Extraction of RNA products, denaturation with glyoxal, and agarose gel electrophoresis followed a standard protocol (4). Procedures for quantitation of specific transcription by densitometry of preflassched film have been described in detail (7). Because recovery of peak area measurements from different experiments depended on autoradiography time and densitometer settings, ordinate values have been normalized to an (arbitrary) value of 10 for a "complete" reaction: a 60-min preincubation with 60 ng of the specific template, a 4-min pulse, and a 10-min chase.

RESULTS

The Reconstituted Transcription System—We have recently described a transcription system utilizing purified RNA polymerase II supplemented with partially purified transcription factors (7). Calf thymus RNA polymerase II was purified (>95% pure) by the method of Hodo and Blatti (19) and contained an equal mixture of IIA and IIB forms. A whole cell extract of HeLa cells (3), which is itself capable of directing faithful transcription by RNA polymerase II, was fractionated as shown in Fig. 1A. The purified polymerase and three of the HeLa fractions (designated [AB], [CB], and [DB]) were necessary and sufficient for production of accurately initiated runoff transcripts from the adenovirus major late promoter. Transcriptional activities in these fractions exhibited the following properties: 1) each was sensitive to mild heat treatment, 2) each sedimented with a peak of activity through sucrose gradients, and 3) each titrated line-

\[RNA \text{ Pol}ernase \text{ II} \]

![Fig. 1. Transcription components and template. A, scheme for the resolution of transcription factors contained in solubilized HeLa cell extracts. The details of the chromatography have been described (7). (S.S., single stranded.) B, the adenovirus late promoter, pBR322 recombinant used as template in these studies. Adenovirus 2 sequences (14.7-17.1 map units), denoted by a solid bar, were inserted between the BamHI and HindIII sites of pBR322. The adenovirus major late cap site (25) denoted by an arrow, defines position +1 of the plasmid; relevant restriction enzyme sites are shown.](http://www.jbc.org/)
pulse; and (c) the fraction of these chains that becomes fully elongated during the chase to yield full length runoff RNA.

In order to characterize the elongation reaction, a time course of chase was performed with fixed periods for the preincubation (32 min) and pulse (2 min) (Fig. 2A). The template for this experiment was an equimolar mixture of PvuII-cut pFLBH and TthI-cut pFLBH (see Fig. 1B). The elongation rate during the chase was measured by observing the differential appearance of the two resulting transcripts. The 841-nucleotide PvuII runoff transcript appeared between 1 and 2 min, while the 2364-nucleotide TthI runoff transcript appeared between 4 and 8 min. From this and similar experiments, an average elongation rate between 400 and 600 nucleotides/min was calculated for the chase phase. This is similar to the values observed in pulse-chase experiments with purified RNA polymerase II elongating from the ends of a phage T7 template (22).

During the course of the chase, a number of bands shorter than the full length runoffs appeared and subsequently decreased. These are evidently pause sites during elongation. These pause sites could be intrinsic to RNA polymerase II (such pause sites were observed by Kadesch and Chamberlin; Ref. 22) or could represent binding sites for proteins that interfere with elongation.

Using the above pulse-chase protocol, the specific activity (in counts/transcript) should be similar for the two different length runoff transcripts, since all radioactivity is incorporated near the 5' end. If elongation during the chase is efficient, and the transcripts are stable, then signals from an equimolar template mixture should reach plateaus of equal intensity after a long chase. This was the case for the 841- and 2364-nucleotide late promoter runoffs. An 8-min chase was sufficient for maximal accumulation of both runoffs (Fig. 2A).

Fig. 2B shows the time course for the pulse phase of the reaction, with (60 min) preincubation and a constant (10 min) chase (A). Each point represents radioactive incorporation into the specific runoff transcript, as measured by densitometer tracing of autoradiograms. With the 60-min preincubation, the observed signal increased linearly for pulse times up to 32 min. This would be expected if a population of polymerase molecules initiated and began elongation at the beginning of the pulse. Two points from this experiment are worth special note. First, no signal was observed when the pulse was for zero time (i.e. labeled pulse and excess unlabeled chase nucleotides added together). This indicates that label was effectively diluted during the chase phase. Second, even with

Fig. 2. Time courses for preincubation, pulse, and chase. Preincubations contained the complete protein mix described under "Materials and Methods" and the indicated template. Pulse and chase conditions are described in the text. Graphs show the radioactive incorporation into the specific runoff RNA, quantitated by densitometry as described (7). A, preincubation was for 60 min with a mixture of 60 ng of PvuII-cut pFLBH and 60 ng of TthI-cut pFLBH. After a 4-min pulse, chases of the indicated length were performed. B, A, preincubation for 60 min with 120 ng of PstI-cut pFLBH was followed by a variable pulse and constant 10-min chase. B, same except without preincubation. Inset shows expansion of early time points. C, a variable (0-180 min) preincubation with 120 ng of PstI-cut pFLBH, a 4-min pulse, and 10-min chase. C, same with template omitted from preincubation and added to pulse.
the longest (32 min) pulse, synthesis of full length runoff RNA still depended upon a chase (data not shown). This is the result of a slow elongation rate (<20 nucleotides/min) under the limiting nucleotide conditions of the pulse.

A different time course of pulse labeling was observed when the preincubation was omitted. In this case, incorporation was not observed at early time points, but eventually occurred after a lag (Fig. 1B, Δ). Hence, events in the preincubation must be required for transcription to begin immediately when nucleotides are added.

A time course of preincubation with constant pulse (4 min) and chase (10 min) phases is shown in Fig. 2C (○). The observed signal intensified as preincubation time was increased, reaching a plateau with approximately 1 h of preincubation and staying relatively constant over the next several hours. A very small amount of the specific runoff transcript was observed with no preincubation (<1% of maximum). By keeping the pulse phase short, this specific background was minimized, thus maximizing the dependence on the preincubation.

**DNA Requirement during Preincubation**—Preincubation of proteins in the absence of the DNA template did not stimulate subsequent incorporation (Fig. 2C, ○). Thus, DNA-protein interactions are crucial in the preincubation. This experiment did not address the nature of the preincubation DNA requirement. Indeed, the role of DNA could either be the formation of a specific pretranscription complex (“activating” the DNA as template), or a less direct role: modifying the transcriptional apparatus or removing inhibitors so that transcription could begin more rapidly upon nucleotide addition. In order to examine these possibilities, experiments involving two DNA templates were performed. Selection of a specific template during preincubation would suggest the formation of a protein-template complex.

Plasmid pFLBH, cleaved uniquely by either *Pvu*I or *Pst*I, generates two templates yielding runoff transcripts of 841 and 974 nucleotides, respectively. After preincubating a mixture of these two templates (3 μg/ml each) with polymerase and factors for 60 min, a standard 4-min pulse and 10-min chase yielded the two expected runoff RNAs (Fig. 3, lane 1). If one of the two templates was added in the preincubation and the other was added at the beginning of the pulse, only RNA originating from the preincubated DNA was observed (Fig. 3, lanes 2 and 3). Thus, the preincubated DNA had been assembled into some transcriptionally activated structure, distinguished from the second template.

If the preincubation dependence represents time required to form an “activated” complex on the template, then the second added template should be subject to the same lag (see Fig. 2C) as was observed in single template experiments. It is also conceivable, however, that preincubation with the first template could have rendered the protein mix unable to transcribe any subsequently added template. To address this possibility of “interference,” the second template was allowed a further preincubation before pulse and chase. After preincubation of transcription components for 60 min with template I (*Pvu*I pFLBH; 3 μg/ml), the second template (*Pst*I pFLBH; 3 μg/ml) was added and allowed an additional preincubation. Incorporation into the two runoff RNAs after the standard short pulse (4 min) and chase (10 min) was plotted versus time of the second preincubation (Fig. 4A). Under these conditions, utilization of template II increased with increasing time of further preincubation, ultimately reaching levels comparable to that of template I. Thus, prior preincubation with low concentrations of the first template did not significantly alter the transcriptional competence of the protein mix.

Higher concentrations of the first template, however, did interfere with transcription of the second template. As shown in Fig. 4B, a prior preincubation with 10 μg/ml of template I blocked template II utilization, even with 60 min of further preincubation. Template I remained activated throughout this further preincubation. In the control reactions (where both templates were added together to the second preincubation), the two resulting runoffs appeared in parallel (Fig. 4, C and D). Hence, the observed interference depended on prior prein-

![Fig. 3. Activation is specific to the preincubated template.](http://www.jbc.org/)

Plasmid pFLBH was cleaved with *Pvu*I to generate template I and *Pst*I to generate template II. Lane 1 shows the product of a complete (60 min) preincubation with 60 ng of each template followed by a pulse (4 min) and chase (10 min). Lane 2 is the same, except that only template 2 is preincubated and template 1 is added at the start of the pulse. Lane 3 shows the converse, with template 1 preincubated and template II added at the start of the pulse. The runoff transcripts from the two templates, DNA1 (841 nucleotides) and DNA2 (974 nucleotides) are indicated.

*M. Samuels and A. Fire, unpublished observations.*

![Fig. 4. Sequential activation of two templates.](http://www.jbc.org/)

*A, 60 ng of *Pvu*I pFLBH (template I) was allowed complete preincubation (60 min) after which 60 ng of *Pst*I pFLBH (template II) was added and allowed a further (second) preincubation of 0–60 min. Radioactive incorporation into the two resulting runoff transcripts (DNA1, 841 nucleotides (○); DNA2, 974 nucleotides (●)) after a 4-min pulse and 10-min chase was quantitated as described under “Materials and Methods.” B, same as A except with 200 ng of each template. C, same as A except that the first preincubation contains neither template; both templates are added to the second preincubation. D, same as C with 200 ng of each template.*
cubation of the interfering DNA (template I). A similar interference with template II was observed if template I was replaced by pBR322 (data not shown); thus, late promoter sequences were not absolutely required in the interfering DNA.

The above results define two assays for activities involved in template-specific events preceding transcription: I. Activation: the ability of a template complex to rapidly begin transcription when presented nucleotides defines activation (see Fig. 2B). Operationally, the activation of a preincubated template is quantitated by measuring incorporation into the product (and will not exchange onto) new template added during the further preincubation (see Fig. 4B). A positive result (utilization of the first but not the second template) indicates the following: 1) during the first preincubation, some stable functional complex must have formed on the first template and 2) such a complex can no longer form (and will not exchange onto) new template added during the further preincubation. Assays for interference can detect stable complexes or structures whose formation may precede full template activation.

**RNA Polymerase II and Factor Requirement during Preincubation**—An analysis of requirements for the observed activation and interference phenomena proved suggestive concerning the roles of the different fractions.

The contribution of each fraction to template activation was examined using parallel sets of preincubation-pulse-chase reactions; in each set, one of the required protein fractions was omitted from the preincubation and was added to the subsequent pulse phase, to the chase phase, or not at all (Fig. 5). Lane 1 shows a positive control with all components present in the preincubation. A background of 10% was observed in the absence of any added polymerase II (lane 2) and probably resulted from polymerase contamination in other fractions. No significant stimulation above this background was observed if polymerase was added during the pulse or chase phase (lanes 3 and 4). Similar results were obtained with fractions [AB] and [DB]: to significantly stimulate the signal above a low background, each of these fractions had to be present during the preincubation (lanes 5–7 and 11–13). Strikingly different results were obtained when the same experiment was performed for fraction [CB]. Addition of [CB] during the pulse resulted in 20–50% of the positive control signal; this should be compared to a background of less than 1% observed in the absence of [CB] (lanes 8 and 9). Addition of [CB] during the chase failed to stimulate transcription above this background (lane 10). The unique level of stimulation obtained when [CB] was added to the pulse suggested that [CB] could act late relative to the other components.

To extend the results of Fig. 5 and obtain a time course of action for each of the protein fractions in the observed activation, a two-period preincubation protocol was used. The first template (I) was preincubated for 1 h with three of the four necessary protein components. A second template (II) was then added with the omitted component. The reactions were allowed an additional preincubation of 0–60 min, after which activation of the two templates was measured using the standard short pulse (4 min) and chase (8 min). Activation of each template was plotted versus the time of additional preincubation (solid lines, Fig. 6). Negative controls (never adding the indicated fraction) are shown with dotted lines; Fig. 4 provides a positive control (complete preincubations).

Initially, these experiments were done at 3 μg/ml of each DNA (Fig. 6, A–D). In the case of polymerase and [CB], the results were straightforward extensions of those in Fig. 5. When the [CB] was added in the second preincubation, both templates were activated (lanes 2, 4, and 6). However, no significant stimulation above this background was observed if polymerase was added during the pulse or chase phase (lanes 8 and 10). The unique level of stimulation obtained when [CB] was added to the pulse suggested that [CB] could act late relative to the other components.

To investigate the behavior of the system under higher template (interference) conditions, a similar series of experiments was performed with 10 μg/ml of each template (Fig. 6, E–H). One should first note that, under these conditions, a time-dependent activation of template I was observed when each fraction was added. Again the activation occurred most rapidly in the case of [CB]. At this intermediate template concentration, activation of template I in the case of [DB] was only partial (Fig. 6G). As was shown in Fig. 4B, a complete preincubation with 10 μg/ml of template I interfered with the ability of the mix to activate a second added template. This interference was also observed when either [CB] or polymerase II were omitted from the first preincubation (Fig. 6, F and H). However, no

**Fig. 5. Factor requirements during preincubation.** Lanes 1 and 14 show a complete preincubation (60 min; with 60 ng of PstI-cleaved pFLBH) followed by a 2-min pulse and 8-min chase. Lanes 2, 5, 8, and 11 show the reaction with the indicated component omitted. Lanes 3, 6, 9, and 12 show equivalent reactions with the indicated component added back to the pulse; lanes 4, 7, 10, and 13 show equivalent reactions with the indicated component added back to the chase. The arrow indicates the position of the 974-nucleotide late promoter runoff.
that [AB], [DB], and DNA are necessary to establish interference. Preincubation of template I (10 pg/ml) with just [AB] and [DB], followed by a second preincubation after a 4-min pulse and 8-min chase was quantitated as described under "Materials and Methods." The dotted lines represent identical protocols except that the indicated component was never added. A-D were performed with 60 ng of each template; E-H were performed with 600 ng of each template.

Prior preincubation of template I with just [AB] and [DB] was sufficient to render this template resistant to poly(dI-dC:dI-dC) (lanes 5 and 6). This demonstrates that formation of a functional template-associated complex occurs in the presence of just [AB] and [DB]. If either [AB] or [DB] was omitted from the first preincubation and added with poly(dI-dC:dI-dC) to the second preincubation, no transcription was observed (data not shown). Note that poly(dI-dC:dI-dC) did not block the action of polymerase II or [CB]; these two fractions were added simultaneously with inhibitor in lane 6; their omission from the reaction (lanes 7 and 8) resulted in a loss of signal.

The time courses in Fig. 6 suggested that polymerase might associate directly with template complexes formed by incubation of template with [AB] and [DB]. Experiments to test this directly involved two parallel preincubations, differing only in the point of polymerase addition (Fig. 7B). Two mixes containing different templates were preincubated separately for 1 h, each mix containing [AB] and [DB]. At the beginning of the pulse, the two separate preincubations were mixed and [CB] was added. Polymerase was added to either of the two preincubations (lanes 3 and 4), to the pulse (lane 1), or not at all (lane 2). Addition of polymerase to the pulse produced a low level signal from both templates (lane 1). The signal was stimulated 3-fold when polymerase was present during preincubation; only the [template:AB:DB] mixture preincubated with polymerase was subject to this stimulation (lanes 3 and 4). This suggests that polymerase forms a functional association with a template complex in the absence of [CB].

A similar protocol was used to test whether the stimulation...
Fig. 7. Protein-DNA interactions preceding activation. A, inhibition by poly(dI-dC)·poly(dI-dC) defines a stable pre-initiation complex. Lane 1, complete preincubation (60 min) with 60 ng of template I (PstI-cleaved pFLBH) and 60 ng of template II (PstI-cleaved pFLBH) followed by a 5-min pulse and 10-min chase. Lane 2, same as lane 1 with 200 ng of poly(dI-dC)·poly(dI-dC) added at the beginning of the preincubation. Lanes 3–8 were all two-stage preincubations with template I present in the first (60 min; 15 μl) preincubation and template II added at the start of a second (60 min; 25 μl) preincubation. These were followed by a 5-min pulse and 10-min chase. 200 ng of poly(dI-dC)·poly(dI-dC) were added with the second template in lanes 4, 6, 7, and 8. Lanes 3 and 4, all protein components present during the first preincubation, lanes 5 and 6, only [AB] and [DB] present during the first preincubation. Lanes 5 and 6, only [AB] and [DB] present during the first preincubation; [CB], [CD], RNA polymerase II, and RNase added with the second template. Lanes 7 and 8, same as lane 6 except that polymerase (lane 7) or CB (lane 8) was never added. B, polymerase associates with template in the absence of CB. For each lane, templates I and II (200 ng each) were preincubated separately (60 min; 15 μl) each with [AB], [DB], and RNase. The two preincubations were mixed at the start of the pulse (5 min) and chased (10 min). Polymerase (40 units) and [CB] were added as follows: lanes 1–5, [CB] in pulse; lane 6, no [CB]; lane 7, [CB] in template I preincubation only; lane 8, [CB] in template II preincubation only; lane 1, polymerase in pulse; lane 2, no polymerase; lane 3, polymerase in template I preincubation only; lane 4, polymerase in template II preincubation only; lanes 5–8, polymerase in both preincubations. (Addition of polymerase during the pulse stimulated the signal above the negative control with no polymerase to 5% of maximum. This experiment differs from that in Fig. 4, lanes 11 and 12, in that the pulse was longer, more polymerase was used, and the [CB] was added during the pulse.)

DISCUSSION

An immediate goal in the resolution and purification of the factors in a multicomponent enzyme system is to determine the sequential steps in which each factor participates. Specific transcription by RNA polymerase II, assayed on the adenovirus major late promoter, has been shown to involve at least five separable components, including the polymerase (5–8). Because the purified polymerase carries out nonspecific initiation and elongation on denatured DNA templates (1), this enzyme is probably responsible for initiation and elongation during specific transcription as well. To date, no role has been assigned to the factors. The inability of purified polymerase to initiate accurately and its low activity on double-stranded templates suggest that some of the factors could play a role in directing the polymerase to initiate at the proper site, or in allowing transcription from double-stranded regions.

In order to address these possibilities, some knowledge of the sequence of events leading to initiation is needed. Using a three-stage transcription protocol, we have identified an "activated" DNA-protein complex whose formation is nucleotide independent and precedes initiation of transcription. The criterion defining this "activated" complex was its ability to rapidly commence faithful transcription when presented nucleotide precursors. Formation of the "activated" complex required preincubation of both polymerase and factors with the DNA template. Controls involving two DNA templates were used to show that the observed activation was both DNA dependent and specific to the preincubated template. In addition, the "activated" complex was resistant to concentrations of a synthetic DNA which would completely inhibit the reaction when added simultaneously with factors and polymerase.

A number of intermediate complexes can also be detected using activation and interference assays. First, neither polymerase nor fraction [CB] (containing two of the identified transcription factors) is necessary for the formation on template of a complex resistant to inhibition by excess DNA. The other two factors ([AB] and [DB]) are sufficient to mediate this step, forming an intermediate complex which is stably associated with the preincubated template. Second, polymerase II will associate with this intermediate complex in the absence of the required activities in fraction [CB]. Third, some component in [CB] interacts with the template complex in the absence of nucleotides to allow maximal activation. Thus, [CB] activities can apparently act late in the formation of an "activated" complex. These data suggest the model pathway shown in Fig. 8.

Several important questions are not addressed by these experiments. First, the fractions used in these experiments are not purified activities and, thus, results can be complicated
by the presence of extraneous proteins. Several transcriptional factors that act at different stages of the reaction may be present in any one fraction and thus complicate the analysis. However, the general conclusions that template selection can occur in the absence of polymerase and that at least one factor can act after polymerase has associated with the complex would not be negated by this complication. Second, although various fractions are required for formation of intermediate and activated complexes, components from these fractions may not be present in the complexes. In the case of RNA polymerase II, presence in the activated complex is strongly indicated by association of enzymatic activity with the DNA template. Finally, the reaction order suggested in Fig. 8 is a permitted order; these results do not establish that a given step need depend mechanistically on previous steps.

One can speculate that stable complex formation by [AB] and [DB] represents promoter recognition. Although our interference results demonstrate that this complex is template associated, we have presented no direct evidence for binding at the promoter site. Using a similar protocol, Davison et al. (24) have tested interference by a series of specific DNA fragments around the conalbumin promoter. Their results define a complex which appears equivalent to that defined by resistance to high concentrations of DNA in these studies. All DNA fragments tested by Davison et al. (24) interfered with transcription of a second added template. A quantitative comparison using the different conalbumin fragments revealed a correlation between interference activity and the presence of functional “TATA” sequences. This suggested that the TATA consensus sequences play a role in stable complex formation.

At this stage of analysis, the multicomponent processes leading to specific transcription by RNA polymerases II and III appear similar. Transcription of 5 S genes requires three fractions in addition to RNA polymerase III; these factors are distinct from the RNA polymerase II factors (5, 15). A 5 S specific transcription factor has been purified and shown to bind to internal sequences in the gene (13, 14). A similar analysis of sequence-specific recognition events for the RNA polymerase II system will be possible with further purification of factors. Our present hypothesis is that the relevant factors are present in fractions [AB] and [DB].

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