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The interaction of RNA polymerase II with non-promoter DNA sites

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ABSTRACT

Various complexes formed between purified RNA polymerase II and simian virus 40 DNA have been characterized with respect to rates of formation, rates of dissociation, and initial velocity of RNA synthesis. Two different types of complexes can form on intact DNA templates. One of these is formed rapidly, but is quite labile; the other forms more slowly, but is moderately stable once formed. The introduction of a single strand break into DNA leads to rapid and stable complex formation, and thus is expected to create the favored binding site. The observed properties of these complexes provide a general framework for describing the interactions of RNA polymerase II at non-promoter DNA sites. This framework appears to be similar to that established for Escherichia coli RNA polymerase interactions, suggesting that the fundamental mode of non-promoter DNA binding is similar for the bacterial, plant, and mammalian enzymes.

INTRODUCTION

The mechanism by which RNA polymerase II recognizes, binds, and initiates transcription of eukaryotic DNA is largely unknown. This lack of knowledge is due in large part to lack of appropriate purified components of the transcription apparatus. Although RNA polymerase II has been isolated from many sources, in no case is it clear that a purified enzyme preparation is truly a holoenzyme and in several cases specific transcription has been shown to depend upon the addition of uncharacterized factors (1). Among the most studied purified enzymes are those from wheat germ and calf thymus. However, even for these enzymes there is considerable uncertainty concerning the general manner in which the enzymes interact with DNA.

Several studies have involved the determination of the stability of RNA polymerase-DNA complexes, a monophasic

dissociation curve yielding a unique half-life being taken as a property of a unique complex. Estimates for half-lives of complexes formed vary widely, from seconds to hours, even when similar enzyme-template combinations are studied (2-6). These varying estimates might arise from study of different types of complexes or from differences in experimental protocols used to obtain dissociation data.

Some, but not all, of these and other apparently conflicting observations may be explained on the basis of variations in template integrity. RNA polymerase II from wheat germ has been shown to bind preferentially near single strand breaks in DNA (7) and heparin-resistant transcription also arises preferentially from nick sites (5). Restriction cleavage of DNA may also lead to single strand breaks (8). Therefore, it is now apparent that it is crucially important to control and monitor the presence of nicks in DNA when studying RNA polymerase II. In this study we have used a simian virus 40 DNA template which, since it is a circular DNA, allows simple assay and control of the extent of nicking.

Below we show that although experimental conditions can be altered to yield widely varying apparent half-lives of polymerase-DNA complexes, the observed properties can be explained by postulating the existence of two distinct complexes formed at non-promoter sites. These two complexes are formed between un-nicked SV40 DNA and both the plant and animal enzymes. They are transcriptionally competent and their formation does not depend on DNA supercoiling. When the properties of the animal, plant and bacterial enzymes are compared interesting similarities emerge.

MATERIALS AND METHODS

Cells, SV40 virus, and supercoiled DNA were prepared as described previously (7). ^{3}H SV40 DNA was isolated from cells labeled by the addition of 10μ Ci of tritiated thymidine per ml of media 24 hours after infection. Specific activity varied from 30,000 dpm/μg to 240,000 dpm/μg in various preparations. Relaxed SV40 DNA was prepared from supercoiled DNA by the method of Champoux & Dulbecco (9) except that TC7 cells, a monkey kidney

cell line, were used as a source of relaxing enzyme instead of secondary mouse embryo cells. DNA nicked at a specific site was prepared from supercoiled SV40 DNA by incubation with restriction endonuclease Msp I in the presence of ethidium bromide as described previously. Randomly nicked SV40 DNA, used in transcription assays, was prepared by incubating 60 µg of supercoiled SV40 DNA for various times with 7.5 or 30 ng of DNase I, as indicated, in 1.4 ml of 0.01 M Tris-HCl pH 7.5, 1 mM EDTA and 7 mM $MgCl_2$ at 15°C. Samples were extracted with neutralized phenol, then with phenol/chloroform/isoamylalcohol (25:25:1) and several times with diethyl ether.

The various forms of SV40 DNA were quantitated and monitored using agarose gel electrophoresis (10,11). Typical conversions were 80% for formation of Msp-nicked DNA and 98% for formation of relaxed DNA. When quantitation was required, bands containing ^3H DNA were excised from agarose gels and placed in counting vials to be dissolved and counted. Pieces of acrylamide gels were crushed, 0.1 ml of 30% hydrogen peroxide and 0.05 ml of 70% perchloric acid were added, and samples were incubated at room temperature or at 70°C until the gel material dissolved, then neutralized and counted in toluene/triton X-100 (2:1) based scintillation fluid containing 2.7 g/liter 2,5-diphenyloxazole.

RNA polymerase II was purified from wheat germ using the procedure of Jendrisak & Burgess (12) with the modification of storage conditions described previously (7). The enzyme was devoid of significant contaminants appearing on sodium dodecyl sulfate-acrylamide gels, and had a high specific activity. Calf thymus RNA polymerase II was prepared by the procedure of Hodo and Blatti (13) with modifications introduced to minimize lipid contamination (14). The enzyme was approximately 70% pure and active with the major contaminant being a 46,000 dalton polypeptide.

Nitrocellulose filters were obtained from Schleicher & Schuell (BA 85). Filters were stored in 20 mM Tris pH 7.9, 20 mM EDTA at 4°C and were soaked in binding buffer for at least 1 hour before use. Bovine serum albumin (Pentex) was dissolved in water at 20 mg/ml, adjusted to pH 8.0 with sodium hydroxide and heated for 20 minutes to inactivate nuclease. Bovine serum albumin for

transcription assays was iodoacetate treated and dialyzed (15). DNase I was obtained from Worthington (Grade DPFF), dissolved at 5 mg/ml in 10^{-4} M HCl and stored in aliquots at -20°C. Dilute solutions were prepared immediately before use. Calf thymus DNA was obtained from Sigma and dissolved in 10 mM Tris-HCl pH 7.5 and 1 mM EDTA at 5 mg/ml. Partially degraded DNA was obtained by treatment with 25 ng/mls DNaase I for 15 minutes at 15°C.

Buffer for binding or transcription reactions contained 100 mM Tris-HCl pH 7.9, 7 mM thioglycerol, 0.1 mM EDTA, 24 mM ammonium sulfate, 2.6 mM manganese chloride and 0.4 mg/ml bovine serum albumin. DNA and RNA polymerase were added as indicated in the figure legends. Samples were diluted to 1 ml in binding buffer without bovine serum albumin and filtration was performed on a manifold with 1.3 cm filter holders (Millipore) which had been adapted by the addition of a silanized glass reservoir. Since this allows dilution and filtration in the same vessel, the manipulations may be performed rapidly and consistently. The filtration rate was about 6 ml/minute. Filters were rinsed with 2 drops of diluent buffer. For counting, filters were dried thoroughly and counted in toluene containing 0.5% 2,5-diphenyloxazole. Filters in experiments which required eluting DNA were not dried but placed quickly in 0.25 ml of 500 mM ammonium acetate, 0.1% (w/v) sodium dodecyl sulfate, 10 mM magnesium acetate and 0.1 mM EDTA. DNA was dissociated from bound protein and eluted from the filter by soaking for 30 minutes at 37°C. After the filter was removed, DNA was precipitated by the addition of 3 volumes of 95% ethanol and after precipitation, was dissolved in the appropriate solution for gel electrophoresis.

Transcription assays (0.14 ml) were performed in the same buffer used for filter binding with the addition of 0.1 mM ATP, CTP, and GTP, and 0.005 mM UTP with 1 μ Ci ^{3}H UTP in a final volume of 0.14 ml per reaction. Each reaction contained 0.43μ g of SV40 DNA, and was initiated with the addition of 2.0 μg wheat germ RNA polymerase II, unless otherwise indicated. Reactions were terminated by the addition of 1.4 ml of cold 7.5% trichloroacetic acid (w/v) containing 0.1% (w/v) sodium pyrophosphate. Precipitated material was collected on GFC

filters (Whatman). These were washed twice with cold 7.5% trichloroacetic acid, then twice with ethanol, dried and counted in toluene containing 0.5% 2,5-diphenyloxazole.

RESULTS

Figure 1 shows that form I SV40 DNA may be driven rapidly and quantitatively into complexes with both the plant and animal RNA polymerase II. This nitrocellulose filter-binding assay does not distinguish labile from stable complexes; all that is required is that the DNA be bound by RNA polymerase in a complex that is stable during the short time needed for filtration. The complex is retained due to affinity of the nitrocellulose for the protein.

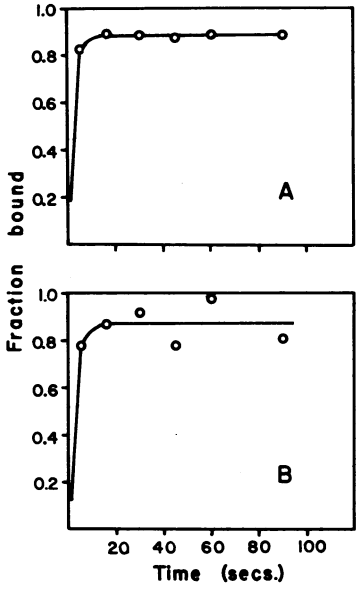

FIGURE 1 Association rates of fI-[^{3}H]SV40 DNA with wheat germ (A) or calf thymus RNA (B) polymerase II. Binding reactions were initiated by the addition of RNA polymerase (0.11 µg per time point) to 10 µl binding buffer containing 0.14 µg fI [^{3}H]SV40 DNA. Incubation was at 37°C for the times indicated. Samples were removed at various times and filtered. Background radioactivity was determined by filtering a reaction aliquot containing RNA polymerase II storage buffer but without RNA polymerase and was subtracted from each point.

In order to measure complex stability, RNA polymerase and labeled DNA were first pre-incubated for a short time, sufficient to allow the rapid complex formation as assayed above. An excess of unlabeled calf thymus chase DNA was then added. As the labeled DNA in complexes dissociates it cannot be re-bound by RNA polymerase since the enzyme is bound efficiently by the excess chase DNA. The free labeled DNA then passes through the filter. Therefore, the amount of labeled DNA which may be retained on filters is a measure of the amount of complex which has not yet dissociated at the time of filtration.

Figure 2 shows that the bulk of complexes formed during a one minute incubation with either the plant or animal enzyme are relatively unstable. Two types of unlabeled chase DNA were used in the assay, "native" calf thymus DNA and the same DNA which had

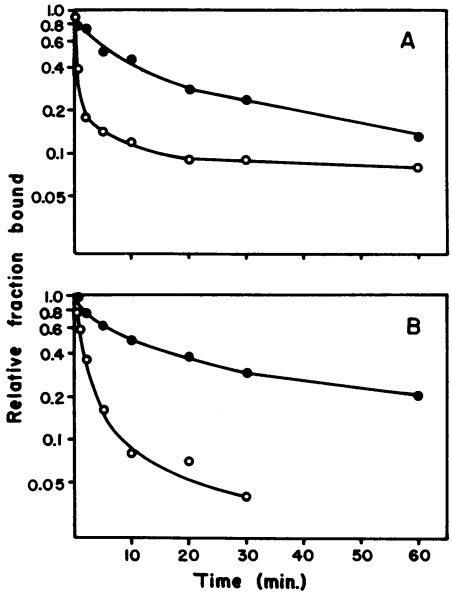

FIGURE 2 Dissociation rate of complexes formed during a one minute incubation (see Fig. 1) with fI-[^{3}H]SV40 DNA. A- wheat germ RNA polymerase complexes chased with either native (-●-) or partially degraded (-O-) calf DNA. B- calf thymus RNA polymerase complexes chased as in A. All data are plotted relative to the amount of SV40 DNA bound prior to addition of unlabeled chase DNA. This amount was 100%, 92%, 84%, and 71% of total input radioactivity in the four experiments shown (top to bottom).

been partially degraded with DNase I to provide a greater number of stable interaction sites for the dissociated RNA polymerase. For both enzymes the apparent stability is reduced in the presence of the low molecular weight DNA chase. This indicates that the chase DNA is not merely binding free RNA polymerase but is also disrupting the complexes formed on radioactive SV40 DNA. This inference was supported for both types of chase DNA by measuring the amount of DNA in complexes as a function of the concentration of chase DNA. Increasing amounts of both types of chase DNA led to a reduction in the level of complex formation (data not shown). Thus, both the nature and concentration of the chase DNA may affect the observed complex stability. Therefore, true stability is not determined in these experiments. All that may be concluded is that the complexes formed during a one minute incubation are somewhat unstable and easily disrupted.

When either enzyme is incubated with SV40 DNA for a longer time before addition of chase, the observed stability is increased for both enzymes (figure 3). Each dissociation curve is clearly biphasic with a well-defined long-lived component. Apparently, during, the 30 minute incubation prior to addition of chase, some complexes of increased stability have formed (compare figures 2 and 3; note change in time axis). Thus, complexes of moderate stability can indeed form, but they form much more slowly than labile complexes. This is true for both enzymes although the calf enzyme complexes can still be disrupted by the low molecular weight chase DNA.

In order to confirm this result and exclude the possible participation of nicked DNA, we assayed directly the formation of these slow-forming, moderately stable complexes. Wheat germ RNA polymerase II was mixed with labeled SV40 DNA at zero time to initiate complex formation. At various times thereafter, aliquots were diluted into excess unlabeled chase DNA and the incubation continued for several minutes. This prevents further formation of complexes with labeled DNA and also leads to dissociation of most of the unstable complexes with the labeled DNA (see figure 2). The sample is then filtered to retain the fraction of DNA which had been driven into moderately stable complexes at the time of addition of chase. The DNA is eluted

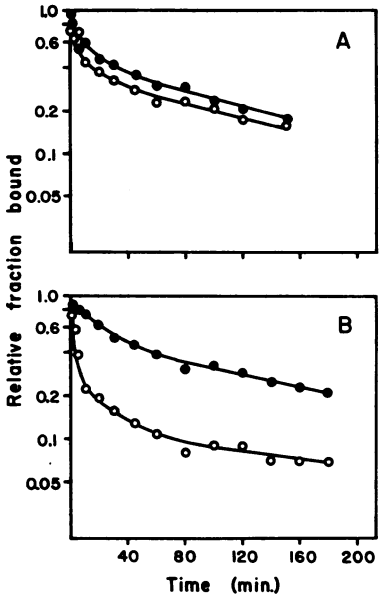

FIGURE 3 Dissociation rate of complexes formed (see Fig. 1) during a 30 minute incubation with fI [^{3}H]SV40 DNA. A- wheat germ RNA polymerase complexes chased with either native (-●-) or partially degraded (-○-) calf DNA. B- calf thymus RNA polymerase complexes chased as in A. All data are plotted relative to the amount of SV40 DNA bound prior to addition of unlabeled chase DNA. This amount was 90%, 94%, 73% and 81% of total input radioactivity in the four experiments shown.

from the filter and subjected to agarose gel electrophoresis to separate DNA forms. Since the DNA is tritium labeled, the amount of un-nicked DNA in complexes as a function of incubation time may be determined. This is done simply by excising and counting the appropriate un-nicked (form I) DNA species from the agarose gel.

Using this assay the amount of form I DNA participating in moderately stable complexes was shown to increase systematically with incubation time over a 1 hour time period (Figure 4). This confirms directly that moderately stable complexes form slowly on DNA which does not contain a nick. The half-time for complex formation cannot be determined very accurately from this experiment since it cannot be determined whether 100% complex formation has occurred. This is because there is a low recovery

of DNA through the many steps of this protocol and also because the free RNA polymerase is losing activity during the very long binding assay. From Figure 4 one can estimate only that the half-time is greater than 5 minutes. If the binding at 1 hour does not represent a true saturation value, as seems likely, then the half-time would be longer. Thus, 5 minutes very likely represents the minimum half-time for the formation of these moderately stable complexes at the specified concentrations.

At this point two distinct complexes between intact SV40 DNA and RNA polymerase II have been identified. Labile complexes (termed "B") form very rapidly (figure 1) and also dissociate relatively rapidly (figure 2). Moderately stable complexes (termed B*) form more slowly (figure 4) and also dissociate more slowly (figure 3). However, although B and B* complexes are clearly distinct, absolute estimates of association and dissociation rates cannot be made due to complications such as direct displacement by chase DNA and multiple binding sites on SV40 DNA.

Since DNA supercoiling has been implicated in complex formation (2,3,16), we asked whether the formation of B or B* complexes requires supercoiled DNA. Relaxed closed circular DNA,

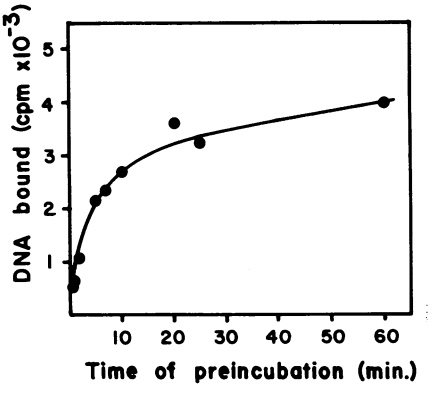

FIGURE 4 Rate of formation of moderately stable complexes of supercoiled SV40 DNA and wheat germ RNA polymerase. ^{3}H-SV40 DNA was extracted from such complexes (see text and fig. 1 legend for details) and subjected to gel electrophoresis. The fI DNA was excised, dissolved, and counted.

which lacks supercoils, was prepared for this purpose. The life time of complexes formed with wheat germ RNA polymerase II was measured as detailed above. The resulting curves are biphasic and consistent with the formation of two types of complexes. Moreover an increased fraction of stable complexes is observed upon prolonged incubation. These results (figure 5) demonstrate that the formation of both stable and unstable complexes does not require DNA supercoiling.

Previously, we reported that wheat germ RNA polymerase II binds principally to nicks when present in SV40 DNA (7) and that the complex formed is sufficiently stable to provide protection from nuclease attack. Figure 6 shows a direct measure of stability of a complex formed by the wheat germ enzyme at a nick introduced at 0.725 map units on the SV40 genome. This complex (termed "N") is highly stable and decays with a half-life of several hours. Such complexes form very rapidly (data not

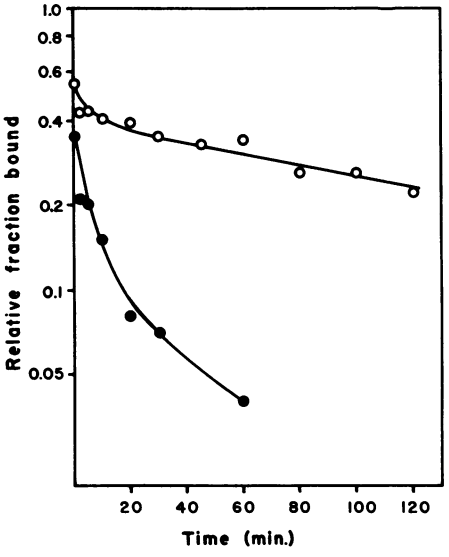

FIGURE 5 Dissociation rate of complexes formed (see fig. 1 for amounts) between relaxed SV40 DNA and wheat germ RNA polymerase. Complexes were formed during either a one minute (-●-) or a 30 minute (-O-) incubation. Data are plotted relative to the amount of DNA bound prior to addition of unlabeled partially degraded calf DNA; this amount was 47% and 33% of input radioactive SV40 DNA.

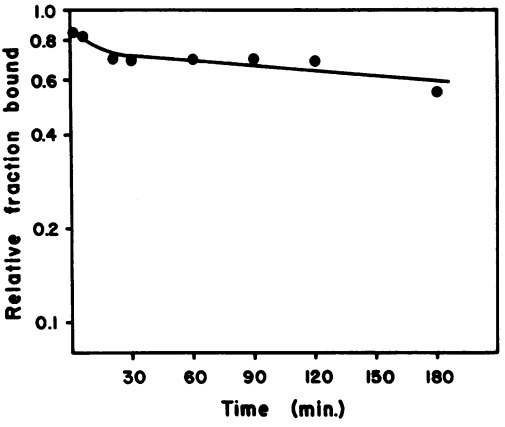

FIGURE 6 Dissociation rate of complexes formed between nicked SV40 DNA and wheat germ RNA polymerase. These complexes were formed for two minutes using MspI-nicked SV40 DNA and were chased with partially degraded calf DNA. The binding prior to addition of chase DNA was 99% of input.

shown). Thus when nicked DNA is present the polymerase would be expected to distribute rapidly to the stable N sites at nicks at the expense of the slower-forming B* sites elsewhere, consistent with our previous observations.

Next, we wished to determine whether the various complexes could support transcription. The assay used was to measure the initial velocity of RNA synthesis by wheat germ RNA polymerase II on various forms of SV40 DNA (see legend to Figure 7). RNA synthesis was measured by conversion of ^{3}H-UTP into acid-insoluble radioactivity. Figure 7 shows that under conditions where B complexes predominate (initial velocity conditions), both supercoiled and relaxed SV40 DNA support transcription. The super coiled DNA is the better template (figure 7, 1.0 vs. 0.23 relative rates); this may simply reflect the greater number of B complexes formed on this template (compare figures and legends 2A and 5).

Next, the effect of the introduction of nicks into DNA on transcriptional competence was investigated (see also references 5,8 and 17). Form I DNA was treated with varying amounts of DNase I to introduce a varying number of nicks. The initial

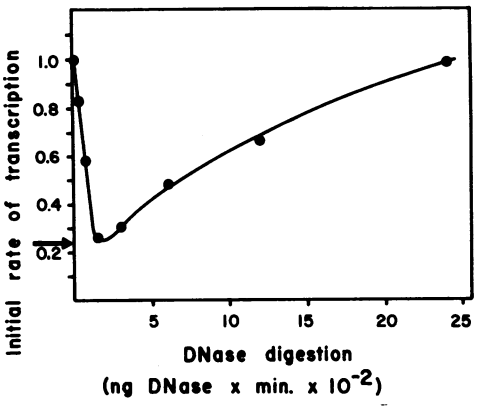

FIGURE 7 Initial rates of transcription of SV40 DNA templates by wheat germ RNA polymerase. Reaction conditions and DNA template preparation were described in materials and methods. The plotted values are the rates of transcription determined by the initial slope of the time course of transcription. The rate of incorporation was linear for approximately two minutes. Values are normalized to the initial slope of the time course for fI DNA which was 4500 dpm/min. For nicked DNA templates, the number of nicks was estimated from ethidium bromide stained agarose gels. The extent of nicking was varied by varying the reaction time and the concentration of DNase I. Control reactions showed that rates were similar for DNA prepared in reactions containing different amounts of DNase I when the product of DNase I concentration and time were equal. An average of one nick per SV40 occurred at (ng DNase I x minutes of reaction) of 75. The transcription rate observed using relaxed SV40 DNA as template is marked with an arrow. The curve is normalized to the rate observed using supercoiled DNA.

velocity of RNA synthesis was then determined as a function of the number of nicks introduced. Figure 7 shows that as more of the fI DNA molecules become nicked the transcriptional competence falls. Thus, nicked DNA is a poorer template for transcription than supercoiled DNA. RNA synthesis reaches a minimum at a level equivalent to that exhibited by transcription of relaxed DNA (marked with an arrow), suggesting that the introduction of nicks cannot make the DNA a poorer tem plate than non-supercoiled, relaxed DNA. As, on the average, more than one nick is introduced into the template, the template activity increases showing that N complexes are also transcrip tionally competent.

The above experiments were done under initial velocity

conditions where B complexes predominate over B*. B* complexes will form if the enzyme is pre-incubated with DNA prior to initiation of RNA synthesis. Figure 8 shows that the initial rate of transcription increases if the enzyme is first pre-incubated with DNA. Since this pre-incubation increases the fraction of DNA participating in B* complexes, B* complexes appear to support more rapid transcription than B complexes.

DISCUSSION

These data have shown that RNA polymerase II can form three distinct transcriptionally competent types of complexes with SV40. These complexes differ in their stability, rate of formation, and transcriptional competence. Therefore, it seems likely that the divergent estimates (see introduction) in the reported lifetimes of RNA polymerase -DNA complexes may be accounted for by divergent conditions leading to a study of different complexes. The principal conditions investigated here were incubation time, nature and extent of competition by

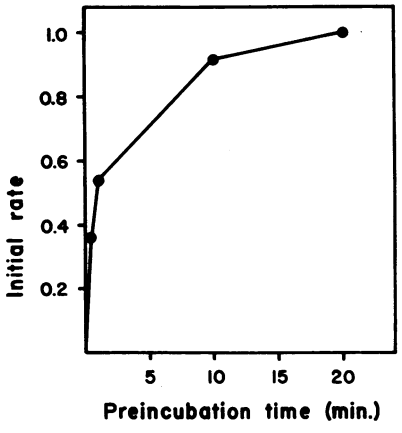

FIGURE 8 Initial rate of transcription after various times of preincubation. Transcription rates of fI DNA were determined as described in Figure 7 except that a 20 fold poly dAT chase and [^{32}P]CTP label were employed. The chase DNA was dissolved in the nucleotide triphosphate mix containing 0.200 mM ATP, GTP, UTP and 0.02 mM CTP with 0.001 mCi [^{32}P]CTP per reaction time point. No incorporation was observed when the chase was added before RNA polymerase II. Rate is normalized to that observed in the absence of chase.

heterologous DNA, and the form of the target DNA template. Clearly other conditions such as temperature, divalent cations, and ionic strength may also alter the distribution of complexes formed (2,8,18). Below, we summarize briefly some properties of the complexes detected and compare them to those described for E.coli DNA-RNA polymerase complexes.

The primary distinguishing characteristic of B complexes formed with RNA polymerase II is lability. Such complexes form quite rapidly and although they exist only transiently, they do assist in binding DNA to filters and can support transcription. These complexes resemble the class B complexes formed between E. coli RNA polymerase and DNA in several respects. Those complexes are inferred to form rapidly, be very labile, and exhibit no obvious sequence preference in association with DNA (19,20). We have also detected a lack of sequence preference for RNA polymerase II B complexes (14). Although these similar properties are intriguing, the extent of the analogy is not completely clear. Experiments have not been reported which ascertain whether E.coli B complexes have the transcriptional competence or ability to bind filters efficiently at polymerase excess observed for the eukaryotic complexes reported here. Such experiments would require the use of supercoiled, prokaryotic templates for strict comparability. However, whether or not the complexes are strictly analogous, the presumptive effect on promoter binding ought to be similar. Since the number of non-specific B sites on DNA likely greatly exceeds promoter sites, the rate of promoter binding may be significantly reduced as a consequence of the lowered amount of free enzyme (19,26).

The primary distinguishing characteristic of N complexes is, of course, that their formation depends on the presence of single strand breaks (nicks) in DNA. The site of nicking is in fact specifically bound and protected from nuclease digestion by RNA polymerase II (7). N complexes form rapidly, are very long-lived, and are , on the average, transcriptionally competent. These properties are very similar to those exhibited by E.coli RNA polymerase interacting with nicked DNA. The prokaryotic enzyme protects DNA at nicked sites in an identical manner (7). Nicks create "tight" binding sites for E.coli RNA

polymerase as tested by a filter-binding assay; the rate of complex formation has not been determined directly, but is inferred to be rapid (21). As is the case for the eukaryotic enzyme, E.coli core RNA polymerase appears to transcribe more efficiently from nicked DNA than from un-nicked DNA.

B* complexes are also distinguished easily since they form much more slowly than N or B complexes and exhibit intermediate stability. Recently, a new type of complex involving E.coli RNA polymerase has been described (22). These TB (tight binding) complexes resemble the B* complexes we have described for RNA polymerase II. That is, they form relatively slowly at non-promoter sites, and exhibit moderate stability once formed. TB complexes exhibit some sequence preference using an electron microscopy assay; using a less sensitive assay we have been unable to detect highly preferred binding of restriction fragments in B* complexes (14). Other E.coli RNA polymerase complexes also resemble these B* complexes superficially. For example, the moderate stability of B* complexes is exhibited by complexes formed between E.coli core enzyme and DNA. The slow rate of formation also has its counterpart in that less efficient E.coli promoters display this property (23-25). Thus the B* complexes resemble complexes which have simple, but important deficiencies with respect to optimal promoter complexes.

We conclude that the three types of complexes formed in the absence of transcription factors between plant and animal RNA polymerase II and SV40 DNA bear a reasonable resemblance to various non-promoter complexes described previously involving a bacterial polymerase. Thus the general mode of DNA binding by the two enzymes appears to be rather similar. Since historically, knowledge of these non-promoter E.coli complexes has assisted greatly in understanding the requirements for formation of proper promoter complexes, we anticipate that these studies will provide a similar assist in understanding eukaryotic promoter binding.

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