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Studies on Transcription of 3′-Extended Templates by Mammalian RNA Polymerase II. Parameters That Affect the Initiation and Elongation Reactions†

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ABSTRACT: Addition of short sequences of dCMP residues to the 3′-OH end of duplex linear DNAs allows rapid and efficient transcription to be initiated at these sites by purified mammalian RNA polymerase II [Kadesch, T. R., & Chamberlin, M. J. (1982) *J. Biol. Chem. 257*, 5286–5295]. The use of such tailed DNA templates should allow biochemical studies on transcription elongation and termination with almost any desired DNA sequence. However, in vitro transcription with RNA polymerase II is aberrant in that the DNA template is not re-formed after transcription; rather, the DNA strands are separated, and most of the RNA product is found as a DNA–RNA hybrid. To better understand the factors that affect the process of transcription with these tailed DNA templates, we have varied a number of parameters that might be expected to play a role in the reaction. RNA polymerase II preparations from calf thymus, HeLa cells, and *Drosophila* all fail to displace the product RNA. However, RNA polymerase II from wheat germ gives only free RNA as a product, as does the *Escherichia coli* RNA polymerase. Hence, the displacement of the nascent RNA from a transcription complex seems to depend on some intrinsic property of the polymerase itself and not simply on the nature of the template. Variation of reaction conditions, or of the divalent metal ion, does not restore the renaturability of the DNA template. However, variation of the duplex 3′-terminal sequence of the template led to significant alterations. In general, GC-rich sites enhanced the displacement of the nascent RNA, while AT-rich sites enhanced formation of the DNA–RNA hybrid. With some terminal sequences, over 85% of the RNA produced by purified calf thymus RNA polymerase II is displaced. Of the DNA homopolymers tested, only dC allows efficient initiation when added to the 3′-OH terminus of a duplex template. This appears to be due in part to the inability of other homopolymer sequences to bind the polymerase efficiently and in part to a requirement for a pyrimidine at the start site for efficient chain initiation. The sites of RNA chain initiation with dC-tailed duplex DNA templates have been mapped by primer extension and S_1 nuclease digestion to the first six nucleotides on the dC tail immediately adjacent to the 3′ end of the template duplex. The sites of initiation are the same for different RNA polymerases and are not affected by whether the transcript is displaced or remains a hybrid.

Protein-coding genes in eukaryotes are transcribed by class II RNA polymerase. While this enzyme plays a vital role in the process of gene expression and has been the subject of much research, it has been difficult to obtain detailed information as to the biochemical processes involved in transcription. This is due to the complexity of the eukaryotic transcription machinery and the difficulty of recreating the transcription reaction in a well-defined in vitro reaction. Purified RNA polymerase II preparations do not use promoters efficiently but depend on nicks and single-stranded regions to initiate transcription. This usually results in random starts and elongation over heterogeneous sequences [reviewed in Lewis & Burgess (1982)]. Specific transcriptional initiation by RNA polymerase II can be achieved in vitro by using cell extracts (Weil et al., 1979; Manley et al., 1980), and fractionation of the components of these extracts that allow RNA polymerase II to recognize and initiate at authentic promoters is in progress (Parker & Topol, 1984; Matsui et al., 1980; Samuels et al., 1982; Davison et al., 1982; Dynan & Tjian, 1983). These systems have already been valuable in deter-

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mining the sequences that control the action of eukaryotic promoters in vitro (Manley, 1983).

Regulation of transcription in prokaryotic systems is not restricted to the steps involved in template binding and chain initiation. There are important regulatory processes that control elongation and termination of RNA chains (von Hippel et al., 1984), and similar mechanisms have been suggested to occur in mammalian systems (Shaw & Ziff, 1980; Hay et al., 1982). Studies of transcription in cell extracts and isolated nuclei have provided some information on the elongation phase of transcription. However, these reactions are inefficient with respect to the number of RNA polymerase molecules involved in specific transcription (Weil et al., 1979) and with respect to the rate of RNA chain elongation, as compared to transcription in vivo. In addition, true transcription termination reactions have not yet been documented in vivo or in vitro (Manley, 1983).

We have recently described an in vitro transcription system that achieves efficient transcription of defined templates by using purified mammalian RNA polymerase II (Kadesch & Chamberlin, 1982). When a linear DNA, such as that from phage T7, is modified by the addition of polydeoxycytidine "tails" on the 3'-OH ends, it becomes a highly active template for transcription by purified RNA polymerase II from calf thymus. We will refer to this as a tailed template or simply pCT7 DNA. Transcription with this tailed template initiates in the terminal regions of the T7 DNA and continues for at least several thousand nucleotides. All of the active enzyme rapidly initiates transcription. Consequently, it is possible to measure important biochemical parameters of the transcription reaction, such as the proportion of enzyme protein that is active, the rate of elongation, and the effect of different DNA sequences on the elongation process. While this mode of initiation bypasses the normal components needed in vivo, the efficiency and adaptability of the method make it an attractive system for studying the biochemical properties of the elongation and termination reactions of eukaryotic RNA polymerase II on defined templates.

The significance of such studies is compromised, however, by the unexpected finding that transcription of pCT7 DNA by calf thymus RNA polymerase II leaves a majority of the RNA produced hybridized to the template (Kadesch & Chamberlin, 1982). This phenomenon does not occur in vivo where the RNA transcript is complexed with proteins, processed, and transported to the cytoplasm [reviewed in Nevins (1983)]. The reaction is not simply a feature of transcription of pCT7 DNA templates, since hybrid formation does not occur when *Escherichia coli* core RNA polymerase is used. Hence, transcription by RNA polymerase II in vitro is aberrant and lacks essential features of the in vivo reaction.

In this report, we show that RNA polymerases purified from different sources vary in their ability to produce free RNA from a tailed template. To understand this phenomenon, and identify the factors that lead to normal transcription in vivo, we have studied the factors that affect transcription from tailed templates. While tailing seems to be a general method for activating linear DNA templates, it is dependent on the nucleotide used to tail. Furthermore, initiation occurs quite specifically on the tail near the terminal region of the molecule, and the production of free RNA can be a function of the DNA sequence at the tail–duplex junction for some RNA polymerases.

Materials and Methods

Enzymes. Calf thymus RNA polymerase II was purified as described (Hodo & Blatti, 1977) except that the ammonium sulfate pellet was dialyzed against two changes of buffer D [50 mM tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl) (pH 7.9), 25% glycerol, 0.1 mM disodium ethylenediaminetetraacetate (Na_2EDTA), and 0.5 mM dithiothreitol] to the appropriate ionic strength in less than 2 h. The final product had a specific activity of 40–50 units/mg using the calf thymus DNA dependent assay described by Hodo & Blatti (1977), where 1 unit is 1 nmol of CMP incorporated into acid-insoluble material per 10 min in a 10-min reaction. RNA polymerase activity in the purified preparation was >99% sensitive to 1 μg/mL α-amanitin. Analysis by sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis (Laemmli, 1970) showed the preparation to be ≥90% pure. When polydeoxycytidine-tailed, linearized pBR322 DNA (pCpBR DNA) was used as template, 1 unit of activity was defined as the incorporation of 1 μmol of CMP per minute into trichloroacetic acid insoluble material in a 10-min reaction. This preparation typically had a specific activity of 30–50 milliunits/mg under standard conditions. The proportion of active RNA polymerase was estimated to be 11–15% by measuring the rate of CMP incorporation and assuming an elongation rate of 7–10 nucleotides per second (Kadesch & Chamberlin, 1982) and a molecular weight of 550 000 (Hodo & Blatti, 1977).

Wheat germ RNA polymerase II was purified by the method of Jendrisak & Burgess (1979) with suggested modifications (Dynan & Burgess, 1979). Analysis on SDS–polyacrylamide gels showed the enzyme to be ≥95% pure with the appropriate subunit composition. The specific activity of this preparation with pCpBR DNA was 50–80 milliunits/mg.

Escherichia coli RNA polymerase core enzyme was purified by the method of Gonzalez et al. (1977). Although a very slight contamination by the σ subunit could be seen overloading an SDS–polyacrylamide gel, the activity was over 100-fold more active on tailed DNA as compared to its activity with the same linearized template lacking tails. The specific activity of the core polymerase preparation with pCpBR DNA was 150–180 milliunits/mg.

Drosophila RNA polymerase II was prepared from cultured Kc cells (Echalier & Ohanessian, 1969) following the procedure used by Coulter & Greenleaf (1982). Cells were grown at 25 °C in T flasks and then transferred into a 4-L spinner culture at a cell density of 3×10^5 cells/mL. Growth was continued for two generations. Cells were harvested by centrifugation (1000*g*, 20 min) and then washed with 10 volumes of 10 mM sodium phosphate, pH 7.4, 0.1 M NaCl, and 5 mM $MgCl_2$. The final cell pellet (5.8 mL) was resuspended in buffer A (50 mM Tris-HCl, pH 7.9, 0.1 mM EDTA, 10% glycerol, 25 mM KCl, 5 mM $MgCl_2$, and 0.5% 2-mercaptoethanol) containing 1.5 mM phenylmethanesulfonyl fluoride, and cells were lysed by sonication. Purification was continued through chromatography on DE-52. The final preparation was judged to be about 20% pure by SDS–polyacrylamide gel electrophoresis. RNA polymerase activity in the final fractions was >98% sensitive to 1 μg/mL α-amanitin.

Calf thymus terminal deoxynucleotidyl transferase was the generous gift of Dr. Robert Ratliff (Los Alamos, NM). HeLa cell RNA polymerase II was the gift of Dr. William Dynan and was purified by the procedure of Dynan & Tjian (1983). Restriction enzymes *Pvu*II, *Pst*I, and *Sma*I were obtained from BRL. *Aha*III was obtained from New England Biolabs. Nuclease digestion conditions were those recommended by the supplier. Pancreatic ribonuclease A and deoxyribonuclease I were obtained from Worthington. Ribonuclease A was dissolved in glass-distilled water to 10 mg/mL and heated to

80 °C for 15 min to eliminate contaminating activities. Contaminating ribonuclease was removed from deoxyribonuclease I by treatment with bentonite as a substitute for macaloid (Maniatis et al., 1982). Avain myeloblastosis virus reverse transcriptase was obtained from Life Science, Inc. (St. Petersburg, FL).

DNA Templates. Plasmid DNA was prepared by NaOH/SDS lysis (Birnboim & Doly, 1979; Bethesda Research Laboratories, 1983), omitting ribonuclease treatment followed by banding in CsCl/ethidium bromide. Residual contaminating tRNA was removed after linearization of the plasmid by chromatography on Sepharose Cl-2B in TE (10 mM Tris-HCl, pH 8, and 1 mM EDTA). Tailed templates were prepared essentially as described previously (Kadesch & Chamberlin, 1982) except for the following modifications: The concentration of linearized plasmid DNA was adjusted to 25 nM ends, terminal deoxynucleotidyl transferase was present at 500 units/mL [1 unit = 1 nmol of dNTP/h (Chang & Bollum, 1971)], and the reaction was incubated at 25 °C. The extent and heterogeneity of addition of poly(dC) tails to pBR322 DNA linearized by *Pvu*II were examined by comparing tailed and untailed DNA digested with *Ava*I and *Pst*I on agarose gels. The two fragments from the tailed DNA containing the original *Pvu*II ends ran as heterogeneous populations more slowly than the corresponding untailed DNA, while the internal fragment ran as a tight band in each case. It was estimated that >95% of the DNA molecules were modified by the addition of tails. While the distribution of dC tail lengths varied from 0 to 300 nucleotides long, the average tail length was ~100 nucleotides. The DNA was purified by phenol/chloroform extraction, concentrated by ethanol precipitation, and dialyzed against several changes of TE.

^{32}P-Labeled inorganic phosphate was purchased from New England Nuclear and was converted to [α-^{32}P]CTP by the method of Symons (1977) and to [γ-^{32}P]ATP by the procedure of Johnson & Walseth (1979).

Abbreviations Used for DNA Templates. The nomenclature used to designate particular templates employs the following pattern: Purified pBR322 plasmid DNA which has been cut with *Pvu*II restriction endonuclease, followed by addition of 3′-poly(dC) tails, is designated as pCpBR DNA or as pCpBR/*Pvu*II DNA in cases where the nature of the restriction site is relevant. Similarly, pCpBR/*Aha*III is pBR322 DNA cut with *Aha*III restriction endonuclease followed by poly(dC) tailing, and pApUCl8/*Pst*I DNA is pUCl8 DNA cut with *Pst*I to which poly(dA) tails have been added.

RNA Polymerase Assays. Transcription reactions using tailed templates contained 70 mM Tris-HCl, pH 8.0, 50 mM $(NH_4)_2SO_4$, 20% glycerol, 6 mM $MgCl_2$, 5 mM spermidine, 0.15 mM dithiothreitol, 800 μM each of GTP, ATP, and UTP, 100 μM [α-^{32}P]CTP (1000 cpm/pmol), and 0.5 μg of DNA in 50 μL unless otherwise noted. Transcription reactions were carried out at 37 °C for 10 min and terminated by the addition of 200 μL of YEP (50 mM sodium pyrophosphate, 50 mM EDTA, and 0.5 mg/mL Sigma yeast RNA), and trichloroacetic acid insoluble radioactivity was determined as described by Chamberlin et al. (1979).

RNase A Sensitivity Assays. To determine the amount of RNase A sensitive transcript produced, a standard transcription reaction (40–160 μL) is prepared on ice. RNA polymerase is added, and a sample (10–50 μL) is removed to a tube containing 0.5 mL of cold stop/digest (10 mM Tris-HCl, pH 8, 100 mM NaCl, and 10 mM EDTA) to serve as a zero-time control. The remaining transcription mix is incubated for 10 min at 37 °C and stopped by cooling on ice. Samples of the same volume as the control are then removed to two more tubes containing 0.5 mL of cold stop/digest. RNase A is added to the third tube to a final concentration of 10 μg/mL (10 μL of 0.5 mg/mL RNase A). The second and third tubes are incubated at 37 °C for 20 min and then cooled to 4 °C. Nucleic acids are then precipitated from all samples by the addition of 200 μL of YEP and 2 mL of 12.5% trichloroacetic acid and are collected on Whatman GF/C filters as described previously (Chamberlin et al., 1979). Under the conditions of this assay, *E. coli* RNA polymerase holoenzyme transcripts initiated at T7 promoters are ≥95% sensitive to RNase A digestion, while *E. coli* core RNA polymerase transcripts from single-stranded M13 DNA are ≥98% resistant (Chamberlin & Berg, 1963).

Analysis of in Vitro RNA by S_1 Nuclease and Primer Extension Mapping. In vitro RNA was produced under standard conditions in 100–200-μL reaction values, and synthesis was followed by measuring trichloroacetic acid insoluble [α-^{32}P]CMP in a parallel reaction. DNA was removed by adding ribonuclease-free DNase I to 5 μg/mL and $CaCl_2$ to 2 mM and incubating 30 min at 37 °C. The solution was then adjusted to 20 mM EDTA and 20 μg/mL calf thymus DNA. Nucleic acid were purified by phenol/chloroform extraction followed by ethanol precipitation.

S_1 nuclease analysis was performed as described previously (Gilman & Chamberlin, 1983). The single-stranded DNA probe used was prepared from *Pvu*II-digested pUCl8 (Norrander et al., 1983). The DNA fragments were treated with calf intestinal alkaline phosphatase and 5′ end labeled with [γ-^{32}P]ATP as described (Gilman & Chamberlin, 1983). The strands of the smaller fragment (321-nucleotide base pairs) were separated on a 5% acrylamide gel and eluted from a gel slice and were identified by sequencing, using procedures described by Maxam & Gilbert (1980). Experiments were each done with 0.02–0.2 μg of in vitro RNA plus 20 μg of carrier yeast tRNA and 1000–5000 cpm of probe. Primer extension analysis was done under the conditions of McKnight & Kingsbury (1982). The single-stranded probe used was prepared from the 5′-end-labeled *Pvu*II-digested pUCl8 DNA described above. The DNA was further digested with *Hae*III, and strands were separated on an 8% acrylamide gel. Bands of approximately the correct mobility were eluted and identified by sequencing. In vitro RNA (0.02–0.2 μg) and 5000–10 000 cpm of probe were coprecipitated with ethanol and washed with 70% ethanol. The precipitate was resuspended in 10 μL of hybridization buffer, covered with mineral oil, heated to 90 °C for 2 min, and transferred without cooling to a 65 °C water bath for 2 h. The sample was then cooled to 4 °C, spun briefly, and then diluted to 33 μL to final reverse transcriptase reaction conditions. Primer extension was carried out for 1 h at 37 °C. Samples were then precipitated with ethanol, washed, and resuspended in 5–10 μL of 80% formamide, 1 mM EDTA, 10 mM NaOH, and 0.1% xylene cyanol and bromphenol blue, and analyzed by gel electrophoresis.

Results

RNA Polymerases from Various Sources Differ in Their Ability To Displace RNA Transcripts from Tailed Templates. In our initial studies on the use of pCT7 DNA as a template for calf thymus RNA polymerase II, we noted that the majority of the RNA produced with pCT7 DNA in vitro (~65%) remained in the form of an RNA–DNA hybrid (Kadesch & Chamberlin, 1982). This is not the normal product of transcription in vivo, and we were interested in discovering what factors affect this anomalous reaction in vitro. To follow the

Table I: Use of RNase A Sensitivity To Assay Transcript Displacement by Various RNA Polymerases from Different DNA Templates

enzyme	template	product pmol	product % RNase A sensitive
Experiment A[a]			
E. coli holo RNAP	T7 DNA	178	97
E. coli core RNAP	M13 DNA	40	2
	pCpBR	59	96
calf thymus RNAP II	pCpBR	60	39
wheat germ RNAP II	pCpBR	81	86
HeLa RNAP II	pCpBR	5.9	30
Drosophila RNAP II	pCpBR	19	52
Experiment B[b]			
wheat germ RNAP II	pCpBR	22.8	83
calf thymus RNAP II	pCpBR	18.9	40
wheat germ RNAP II/calf thymus RNAP II mixture	pCpBR	20.9	66.4

[a] Transcription reactions using *E. coli* holo RNA polymerase (RNAP) and T7 DNA and *E. coli* core RNA polymerase and M13 DNA were carried out for 10 min at 37 °C according to reaction conditions described by Chamberlin et al. (1979). Spermidine was added to the RNase A digestion reaction to 100 μM to stabilize RNA–DNA hybrids. All other reactions were done under the standard tailed DNA transcription conditions described under Materials and Methods. RNA polymerase II products were distinguished in partially purified preparations by measuring total and RNase-resistant products in parallel reactions in the presence or absence of 1 μg/mL α-amanitin. [b] Transcription reactions were carried out using 0.33 μg of pCpBR and 0.1 μg of wheat germ RNA polymerase II, 0.17 μg of calf thymus RNA polymerase II, or a mixture containing 0.085 μg of calf thymus RNA polymerase II and 0.05 μg of wheat germ RNA polymerase II.

effect of different conditions on displacement of the nascent RNA from its template strand, a procedure was used that takes advantage of the fact that single-stranded RNA is sensitive to digestion by pancreatic ribonuclease A (RNase A), while RNA hybridized to DNA is protected. Under the conditions of our assay, *E. coli* core RNA polymerase transcripts from single-strand M13 DNA, which are DNA–RNA hybrids, are 100% resistant to ribonuclease, while transcripts formed by *E. coli* holoenzyme with T7 DNA as template are over 95% sensitive to ribonuclease (Table I).

The ability of *E. coli* core RNA polymerase to produce free RNA from pCT7 DNA under the same conditions where calf thymus RNA polymerase II leaves ~60% RNA–DNA hybrid indicates that this phenomenon is not an inherent property of transcription from tailed templates (Kadesch & Chamberlin, 1982) but depends on the nature of the enzyme protein involved. To explore this situation further, we studied the nature of the RNA products obtained with a collection of eukaryotic RNA polymerase II preparations isolated from different sources (Table I). As template, we used dC-tailed pBR322 DNA, which had been linearized by cutting at the single *Pvu*II site (pCpBR). As in our previous studies with pCT7 DNA templates, purified calf thymus RNA polymerase II gives about 60% ribonuclease-resistant product, consistent with its identity as a DNA–RNA hybrid (Kadesch & Chamberlin, 1982). Quite similar results are found with RNA polymerase II preparations from human cells (HeLa) and *Drosophila* cells. However, the wheat germ RNA polymerase II gives almost exclusively free RNA. This confirms our earlier conclusion that the formation of DNA–RNA hybrids in such a reaction is controlled by the nature of the RNA polymerase protein involved and is not simply a general feature of transcription from tailed templates.

Wheat germ RNA polymerase II and calf thymus RNA polymerase II differ somewhat in subunit composition (Hodo & Blatti, 1977) which suggested that transcript displacement might require a subunit, present in the plant enzyme, that has been damaged or lost during the purification of the calf thymus enzyme. However, if such a subunit does exist in the wheat germ RNA polymerase II, it is apparently unable to complement the deficient activity of the calf thymus RNA polymerase II. When equal activities of purified wheat germ RNA polymerase II and calf thymus RNA polymerase II are mixed together, the RNA product resulting from transcription of pCpBR behaves as would be predicted from the two independent reactions (Table I).

In addition, we have tested the RNase A sensitivity of RNA transcripts produced from pCpBR by calf thymus RNA polymerase II fractions obtained from the various steps in the purification procedure (Hodo & Blatti, 1977). RNA polymerase II activity was distinguished by doing parallel reactions with and without α-amanitin. There is no change in the amount of RNA displaced from the template for any of the final three fractions in the purification. This result, together with the fact that the HeLa cell polymerase gives the same level of RNA displacement but involves different purification procedures, suggests that the failure of RNA polymerase II to displace is probably not due to enzyme damage.

The involvement of another component in the RNA displacement reaction was suggested by the behavior of transcripts produced by calf thymus RNA polymerase II present in the salt extract of material precipitated from the cell extract by poly(ethyleneimine) (F_2) (Hodo & Blatti, 1977). While transcripts from pCpBR DNA formed in fraction F_2 were largely resistant to RNase A, *E. coli* core RNA polymerase transcripts produced from pCpBR DNA in the presence of fraction F_2 and α-amanitin were also largely resistant to RNase

Table II: RNase A Sensitivity of Transcription Products from Crude Calf Thymus RNA Polymerase II Preparations[a]

enzyme sample	untreated pmol of [^{32}P]CMP calf thymus	untreated pmol of [^{32}P]CMP *E. coli*	untreated % RNase A sensitive	SDS/phenol extracted pmol of [^{32}P]CMP calf thymus	SDS/phenol extracted pmol of [^{32}P]CMP *E. coli*	SDS/phenol extracted % RNase A sensitive
E. coli core RNAP		2.3	96		2.3	96
calf thymus RNAP II	16.6		32	14.6		53
F_2	1.4		6	1.3		68
E. coli core RNAP + F_2 + 1 μg/mL α-amanitin		1.2	54		0.8	83

[a] Transcription reactions were carried out in 170-μL final volume under standard conditions given under Materials and Methods using pCpBR as a template. Aliquots of 30 μL were withdrawn for zero-time controls, transcription reactions were incubated 10 min at 37 °C and then cooled, and samples were withdrawn as for a standard RNase A sensitivity assay (see Materials and Methods). To the remaining reaction mix was added 20 μL of 5% SDS, and the samples were extracted 2 times with phenol/chloroform and 1 time with chloroform. Aliquots of 37.5 μL were then assayed for total and RNase A sensitive product under standard conditions. Transcription reactions using purified RNA polymerase contained 9 ng of *E. coli* core RNA polymerase or 0.32 μg of calf thymus RNA polymerase II. The experiments using the crude calf thymus RNA polymerase II fraction contained 16 μg of F_2 protein or 16 μg of F_2 protein and 9 ng of *E. coli* core RNA polymerase. RNA polymerase II activity in the F_2 was distinguished by parallel reactions in the presence or absence of 1 μg/mL α-amanitin. *E. coli* RNA polymerase activity was distinguished in the F_2 mixture by parallel reactions in the presence or absence of 20 μM rifampicin.

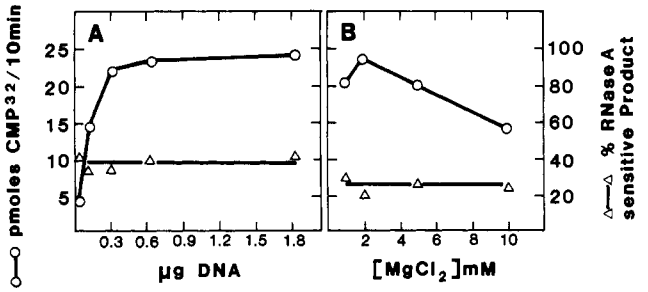

FIGURE 1: Effect of reaction parameters on calf thymus RNA polymerase II activity and the proportion of RNase A sensitive product. (A) Effect of enzyme to DNA ratio. Transcription reactions were done in 170 μL. Aliquots (50 μL) were removed containing 0.1 μg of calf thymus RNA polymerase II and the indicated amount of pCpBR for analysis as described under Materials and Methods. The molar ratio of total enzyme to pCpBR ranges from 0.3 to 5.4. (B) Effect of $MgCl_2$ concentration. Transcription reactions were done in 100 μL, and 30-μL aliquots containing 0.1 μg of RNA polymerase were removed for analysis.

A (Table II). Since *E. coli* core RNA polymerase transcripts are normally >95% sensitive to RNase A digestion, this suggested that something present in the extract protects nascent RNA from RNase A. If the transcription products were extracted with phenol/SDS before being assayed for RNase A sensitivity, F_2 RNA polymerase II transcripts became sensitive to RNase A, and *E. coli* core RNA polymerase transcripts made in the presence of F_2 components returned to a level more indicative of free RNA (Table II). Treatment by SDS/phenol extraction does not affect the sensitivity of the purified *E. coli* core RNA polymerase transcript to RNase A. While there is an increase in the RNase A sensitivity of the transcripts from purified calf thymus RNA polymerase II following SDS/phenol extraction, it is not sufficient to explain the level seen with the F_2 RNA polymerase II. These results suggest that RNA polymerase II in fraction F_2 is capable of producing free RNA, but these transcripts are protected from RNase A digestion by complexing with some other component of the extract. We have not been able to obtain a preparation of RNA polymerase II by any fractionation method we have tried that is competent in the displacement of RNA from tailed DNA templates. However, we have identified a protein factor that acts, together with purified calf thymus RNA polymerase II, to facilitate production of free RNA, and this factor has been extensively purified from HeLa cell extracts (Kane & Chamberlin, 1985).

Reaction Conditions Do Not Influence RNA Displacement by Mammalian RNA Polymerase II. The substrate concentrations and ionic conditions we have employed for transcription with purified calf thymus RNA polymerase II were selected to give optimal rates of transcription. It seemed possible that, under some altered set of conditions, transcript displacement might take place. To explore this possibility, we followed transcript displacement by calf thymus RNA polymerase II under several different reaction conditions. There was no alteration in the fraction of nascent RNA which was resistant to ribonuclease A as the ratio of enzyme to DNA was varied from 0.30 to 5.4 (Figure 1A). Similarly, variation of the Mg^{2+} ion concentration over the range from 1 to 10 mM does not alter the nature of the product (Figure 1B). Similar results are obtained when the concentrations of glycerol (5–20%), $(NH_4)_2SO_4$ (0–100 mM), or spermidine (0–5 mM) are varied over the noted ranges. There was also no alteration of the state of the product when the pH of the reaction was varied (pH 7.5 or 8.0), at a reaction temperature of 20 or 37 °C, or if Mn^{2+} (2.5 mM) replaced Mg^{2+} as the divalent metal ion. Hence, the failure of purified mammalian RNA polymerase II to displace the major fraction of its RNA transcripts from the template is not changed by wide variations in in vitro reaction conditions.

Table III: Effect of Duplex DNA Termini on Displacement of RNA during Transcription of Tailed Templates[a]

template	terminal sequences	RNase A sensitivity of product (%) *E. coli*	RNase A sensitivity of product (%) CT pol II
pCT7	TCT—CCTC$_n$ C$_n$AGA—GGA	95	~40
pCpBR/*Pvu*II	CTG—CAGC$_n$ C$_n$GAC—GTC	93 (37)	38 (54)
pCpBR/*Aha*III	AAA—TTTC$_n$ C$_n$TTT—AAA		20 (6)
pCpUC4K	G—C TGCAC$_n$ C$_n$ACGTC$_{12}$—G$_{12}$		70 (11)
pCpBEU50/*Sma*I	GGG—CCCC$_n$ C$_n$CCC—GGG		85 (29)
pCpUC18/*Sma*I	GGG—CCCC$_n$ C$_n$CCC—GGG	93 (54)	86 (7)

[a] Each template was tailed with 50–100 dCMP residues as described under Materials and Methods; C_n denotes these sequences at the 3′-OH terminus of the DNA duplex. Enzymes are as follows: CT pol II, calf thymus RNA polymerase II; *E. coli*, *E. coli* core RNA polymerase. The numbers in parentheses indicate the picomoles of [α-^{32}P]-CMP incorporated for each experiment. The values for pCT7 DNA were obtained from Kadesch & Chamberlin (1982).

Nature of the Duplex Sequence at the End of the Template Can Enhance RNA Displacement. In the course of our investigation into the nature of the RNA transcript by calf thymus RNA polymerase II, it was discovered that when a plasmid (pBEU50) (Uhlin et al., 1983) was cleaved with *Sma*I and tailed with polydeoxycytidine (pCpBEU50), the resulting template allowed calf thymus RNA polymerase II to produce RNA largely sensitive to RNase A (Table III). This suggested that alterations in the base composition of the end of the DNA duplex could substantially alter the subsequent elongation and displacement of RNA chains.

To investigate the influence of the terminal sequence of the duplex DNA further, three other templates were selected with quite different terminal duplex sequences and tailed with deoxycytidine (Table III). The first was made by cleaving pBR322 with *Aha*III (pCpBR/*Aha*III) which leaves a series of A-T pairs at a blunt end. The second template was made from the ~1400 base pair *Pst*I fragment containing the kanamycin resistance gene from pUC4K (Viera & Messing, 1982) (pCpUC4K). This fragment was inserted into pUC4K by the addition of oligodeoxyguanosine residues and results in a string of ~12 G-C pairs at the end of the DNA duplex separated from the poly(dC) tail by the four residues that extend from the 3′ end after *Pst*I digestion. The final template was prepared from pUC18 linearized with *Sma*I (pCpUC18/*Sma*I).

When these various templates are transcribed with purified calf thymus RNA polymerase II, substantial variations are seen in the proportion of free RNA produced with the different tailed templates (Table III). Hence, the sequence of the duplex DNA at the tailed terminus influences the ability of calf thymus RNA polymerase II to displace its RNA transcript. In general, GC-rich regions appear to favor transcript displacement. Furthermore, tailing at a *Sma*I terminus apparently allows calf thymus RNA polymerase II to produce nearly as much free RNA as wheat germ RNA polymerase II or *E. coli* core RNA polymerase.

To determine whether this is purely the influence of the *Sma*I sequence, we inserted a *Sma*I linker (GCCCGGGC)

Table IV: Influence of the *Sma*I Restriction Sequence on Displacement of the Transcription Product by Various RNA Polymerases[a]

template	terminal sequences	RNase A sensitivity of product (%)			
		E. coli	WG pol II	CT pol II	DM pol II
pCpBR	CTGCCTCGCG—CTTTACCGCAGC$_n$ C$_n$GACGGAGCGC—GAAATGGCGTC	93	90	38	52
pCpBR/*Sma*I	GGGCCTGCCTCGCG—CTTTACCGCAGGCCC$_n$ C$_n$CCCGGACGGAGCGC—GAAATGGCGTCGGG	93	93	62	68
pCpUC18/*Sma*I	GGGGATCCTCT—CTCGGTACCCC$_n$ C$_n$CCCCTAGGAGA—GAGCCATGGG	92	94	86	83

[a] Each template was tailed with ~100 dCMP residues as described under Materials and Methods. Transcription reactions contained 0.4–1 μg of template DNA in 100 μL. Aliquots of 30 μL were removed for RNase A sensitivity analysis. Enzymes are the following: WG pol II, wheat germ RNA polymerase II; DM pol II, *Drosophila* RNA polymerase II; *E. coli*, *E. coli* core RNA polymerase; CT pol II, calf thymus RNA polymerase.

Table V: Transcription of Linear pBR322 Tailed with Different Deoxynucleotides[a]

enzyme	template activity (pmol of [^{32}P]CMP/10 min)				
	pBR322/*Pvu*II	pCpBR	pTpBR	pGpBR	pApBR
E. coli core RNAP	0.44	25	30	0.40	4.9
calf thymus RNAP II	0.17	19.2	2.4	0.18	0.07

[a] DNA templates were made from pBR322 linearized with *Pvu*II (pBR322/*Pvu*II) and tailed with dCTP (pCpBR), dTTP (pTpBR), dGTP (pGpBR), or dATP (pApBR). Average tail lengths (in nucleotides) were pCpBR = 80, pTpBR = 487, pGpBR = 68, and pApBR = 26. Reactions were done with 0.45 μg of DNA template in 50 μL with 0.086 μg of calf thymus RNA polymerase II or with 0.045 μg of *E. coli* core RNA polymerase.

into the *Pvu*II site in pBR322. The resulting plasmid was then linearized with *Sma*I and tailed with deoxycytidine (pCpBR/*Sma*I). When this template was transcribed with calf thymus RNA polymerase II, there was a significant increase in the proportion of free RNA produced as measured by RNase A sensitivity (Table IV). However, the level of free RNA does not increase to that seen with the other templates produced by *Sma*I cleavage for calf thymus RNA polymerase II. Therefore, the influence of the terminal duplex DNA sequences is not simply confined to the three residues left by *Sma*I cleavage but probably involves internal residues as well. The behavior of *Drosophila* RNA polymerase II resembles that of calf thymus RNA polymerase II with each template. Moreover, in no instance did the different duplex end cause either *E. coli* core RNA polymerase or wheat germ RNA polymerase II to lose the ability to displace nascent RNAs (Table IV).

Activation of Linear Double-Stranded DNA Templates Depends on Poly(dC) Tails. Our use of deoxycytidine tails to activate linear duplex DNA templates (Kadesch & Chamberlin, 1982) was initially based on the properties of purified RNA polymerase II using homopolymer templates (Blatti et al., 1970; Dezélée et al., 1974). To investigate the ability of the other deoxynucleotides to activate linear duplex DNA and the properties of such molecules as templates, pBR322 was linearized with *Pvu*II, and samples were tailed individually with dCTP, dGTP, dTTP, or dATP. Transcription of these templates by calf thymus RNA polymerase II showed that only templates with pyrimidine tails are activated compared to untailed DNA (Table V). Moreover, there is a further discrimination between pyrimidines, with poly(dC) stimulating transcription about 100-fold while poly(dT) stimulation is only about 10-fold. *E. coli* RNA polymerase transcription is also preferentially stimulated by pyrimidine tails (Table V). However, the bacterial enzyme shows no preference for poly(dC) over poly(dT). Additionally, poly(dA) tails can activate the linear template for the *E. coli* enzyme.

The dependence of template activation on pyrimidine tails could be due either to preferred binding of calf thymus RNA polymerase II to these sites or to a strong preference of the enzyme to initiate transcription with a purine ribonucleotide. To distinguish between these possibilities, pUC18 DNA was linearized with *Sma*I, which leaves a blunt end, or with *Pst*I, which leaves four bases protruding from the 3′ end, and these DNAs were tailed with either dCTP or dATP. The 3′ protrusion that results from *Pst*I digestion contains one each of the four bases (~TGAC 3′-OH), providing pyrimidines in the initiation region of the single-strand tail (see below), regardless of which nucleotide is used in the remainder of the tail. With the template tail at a blunt end, calf thymus RNA polymerase II showed a 30-fold increase in activity with poly(dC) addition (pCpUC18/*Sma*I), while poly(dA) addition (pApUC18/*Sma*I) again showed no increase in activity when coopared to untailed DNA (Table VI). However, with the *Pst*I linearized template, the addition of poly(dA) tails stimulates transcription about 7-fold, suggesting that when the pyrimidine residue is provided in the initiation region, calf thymus RNA polymerase II can utilize a purine tail, though still much less efficiently than the corresponding poly(dC) tailed molecule (Table VI). It is interesting to note that the four-base 3′ "tail" provided by *Pst*I digestion alone is not sufficient to activate the DNA for efficient transcription by calf thymus RNA polymerase II.

The role of the homopolymer tail in the binding of calf thymus RNA polymerase II was investigated further by mixing pCpUC18/*Sma*I and pApUC18/*Sma*I DNAs together before the addition of enzyme. If RNA polymerase II bound to the poly(dA) tail but was unable to initiate transcription because there was no pyrimidine in the template strand, we would expect to see a decrease in the amount of transcription compared to transcription of pCpUC18/*Sma*I alone. However, under these conditions, there is no inhibition of RNA polymerase activity by the template mixture compared to transcription of the same amount of pCpUC18/*Sma*I (Table VI)

Table VI: Transcriptional Activity of pUC18 Linearized with *Sma*I or *Pst*I with Poly(dA) or Poly(dC) Tails[a]

template	activity[b]	template	activity	template mixture	activity
pUC18/*Sma*I	0.35	pUC18/*Pst*I	0.38	(pUC18/*Sma*I)/(pCpUC18/*Sma*I)	12.0
pApUC18/*Sma*I	0.30	pApUC18/*Pst*I	2.5		
pCpUC18/*Sma*I	12.6	pCpUC18/*Pst*I	18.5	(pApUC18/*Sma*I)/(pCpUC18/*Sma*I)	13.6

[a] Linearized pUC18 was tailed with dATP (pApUC18) or dCTP (pCpUC18) such that ~100 deoxynucleotides were added per duplex end. Transcription reactions were done under standard conditions in 50 μL with 0.065 μg of calf thymus RNA polymerase II. [b] Picomoles of [^{32}P]-CMP/10 min.

(neither template is limiting in any of these experiments). Furthermore, while the activation of linear duplex DNA is due to the addition of single-strand poly(dC) tails, exogenously added single-stranded homopolymer DNA has very little effect on the activity of calf thymus RNA polymerase II when transcribing pCpBR. When poly(dC) or poly(dA) is mixed with pCpBR before addition of calf thymus RNA polymerase II, there is little inhibition of transcription even when the single-stranded DNA is present in a 3-fold excess by mass over the tailed template [this represents a 120-fold excess over the single-stranded poly(dC) present as tails] (data not shown). These results suggest that purified RNA polymerase II does not bind tightly to the homopolymer tail even when it is poly(dC), nor does an excess of single-stranded homopolymer greatly interfere with the utilization of the tailed template.

Initiation on Tailed Templates Occurs Specifically at the Tail–Duplex Junction. Since linear double-stranded DNA templates are activated by the addition of poly(dC) tails exclusively at the ends of the molecule (see Materials and Methods), it might be expected that transcription would initiate somewhere near the terminal region of the tailed template. When runoff transcripts from pCpBR truncated with restriction enzymes were examined by electrophoresis through agarose gels and autoradiography, a sharp band was observed roughly the size of the tailed DNA fragment (data not shown). This is in contrast to the heterogeneous nature of the lengths of poly(dC) added to template in the tailing reaction. These observations suggested that transcription initiates at a reasonably discrete site near the tail–duplex junction. To determine the point of initiation more precisely, the 5′ ends of the transcripts synthesized from pCpUCl8/*Sma* DNA by calf thymus RNA polymerase II, wheat germ RNA polymerase II, and *E. coli* core RNA polymerase were analyzed by S_1 nuclease and primer extension mapping.

Analysis by S_1 nuclease mapping showed an identical cluster of protected fragments for all enzymes tested (Figure 2, lanes 10–12) centered around the *Sma*I site when compared with the sequence ladder and corrected for mobility differences that arise from the different 3′ ends of S_1 nuclease fragments and the chemically modified sequencing ladder. These polymerase enzymes do not appear to have major initiation sites any significant distance within the duplex DNA. Since any RNA originating from the tail will not be complementary to the S_1 probe, any DNA protected past the *Sma*I site must represent some interference in the action of S_1 nuclease near the end of the RNA–DNA hybrid.

S_1 nuclease mapping cannot locate the start site precisely if transcription starts outside the duplex DNA, because the probe used does not contain the poly(dC) sequences. Therefore, the analysis of the start site was complemented by primer extension analysis. The results shown in Figure 2, lanes 3–5, show that primer extension on the transcripts from all of these RNA polymerases results in a cluster of bands corresponding to initiation sites on the poly(dC) tail just outside the tail–duplex junction. If we consider the first deoxycytidine residue added to be number 1, calf thymus RNA polymerase II transcripts produce bands corresponding to residues 1–5 on the poly(dC) tail with positions 3 and 4 being favored. Wheat germ RNA polymerase II transcripts produce bands corresponding to residues 3–7 with positions 4–6 being favored. Finally, *E. coli* core RNA polymerase transcripts produce major bands at residues 5 and 6, but the pattern suggests that initiation can occasionally occur as far out as the 12th residue. The bands that arise in regions within the duplex DNA must represent pausing or premature termination by the reverse

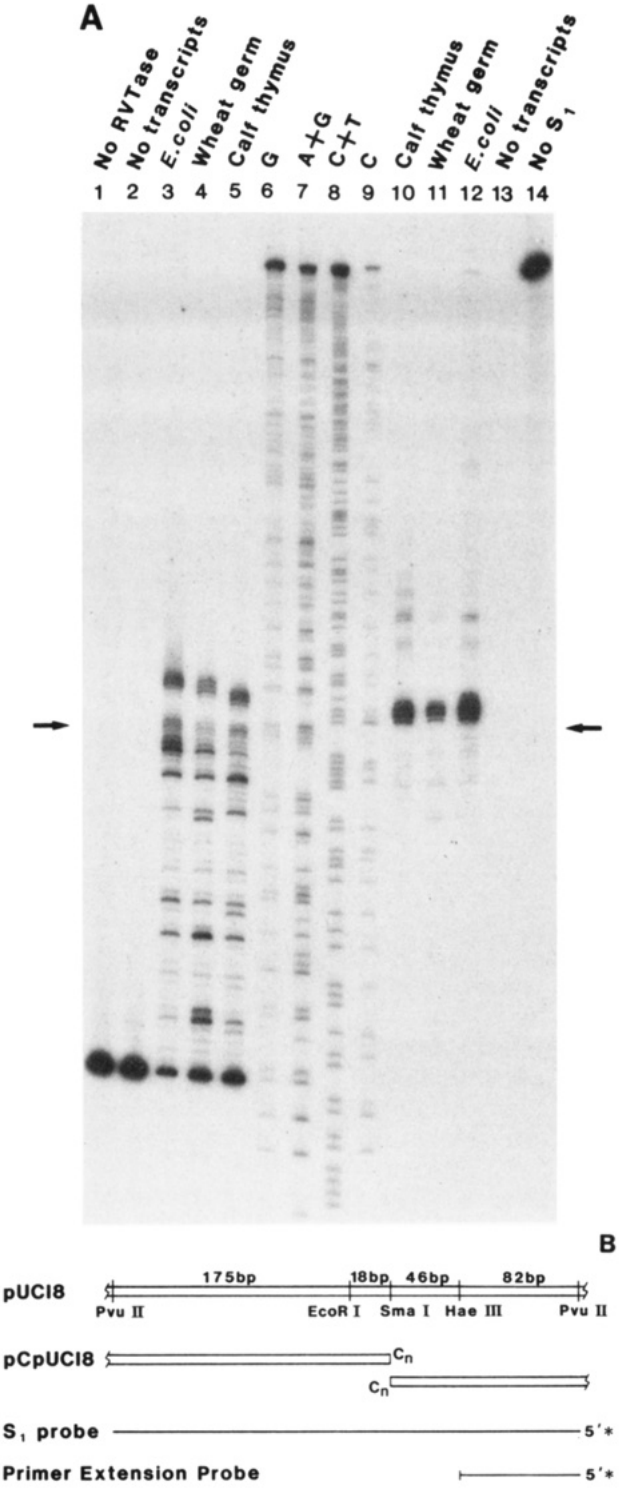

FIGURE 2: Primer extension and S_1 nuclease mapping of the transcriptional initiation site on pCpUCl8/*Sma*I. (A) Lanes 1–5 are primer extension analysis of in vitro transcripts: (1) no reverse transcriptase; (2) 20 μg of yeast tRNA; (3) *E. coli* core RNA polymerase transcripts; (4) wheat germ RNA polymerase II transcripts; (5) calf thymus RNA polymerase II transcripts. Lanes 6–9 are a sequence ladder of the S_1 probe (Maxam & Gilbert, 1980): (6) G; (7) A + G; (8) C + T; (9) C. Lanes 10–14 are S_1 nuclease analysis of in vitro transcripts: (10) calf thymus RNA polymerase II transcripts; (11) wheat germ RNA polymerase II transcripts; (12) *E. coli* core RNA polymerase transcripts; (13) 20 μg of yeast tRNA; (14) no S_1 nuclease. The procedures for primer extension and S_1 nuclease analysis are given under Materials and Methods. Samples were electrophoresed on an 8% acrylamide/8.3 M urea sequencing gel. The arrows indicate the position of the *Sma*I cleavage site with respect to the sequencing ladder. (B) Schematic representation of the preparation of $pCpUCl_8$/*Sma*I and the 5′-end-labeled probes used for primer extension and S_1 nuclease analysis.

transcriptase used in the analysis since there are no strong corresponding signals in the S_1 nuclease analysis.

Taken together, these data suggest that all of these RNA polymerases initiate transcription on pCpUC$_{18}$/*Sma*I quite specifically on the poly(dC) tails three to six residues outside the duplex DNA. There appear to be slight variations in the preferred site of initiation for each of the three enzymes. Furthermore, *E. coli* core RNA polymerase is apparently the least exact in its positioning at the tail–duplex junction. Clearly, there is no strong correlation between the initiation site and the ability of an RNA polymerase to displace its transcript from a tailed template. Primer extension and S_1 nuclease analysis of the initiation sites of these three enzymes on pCpBR, where the differences in strand displacement are more pronounced than on pCpUC18/*Sma*I, show identical patterns and preferences (data not shown).

Discussion

A detailed study of the transcription reaction carried out by RNA polymerase II has been difficult because of the lack of specificity of the purified enzyme and the complexity and inefficiency of the systems where promoter recognition is possible. We have begun to study the enzymatic properties of purified eukaryotic RNA polymerase II using a novel template that allows efficient and specific initiation of the purified enzyme at the end of a well-defined, homogeneous population of DNA molecules. The flexibility and the universality of linear DNA template activation by the addition of poly(dC) tails make this an attractive method for studying elongation and termination of RNA chains by a variety of RNA polymerases on nearly any desired DNA sequence.

A significant drawback in the use of such templates with RNA polymerase II arises from the fact that most of the RNA transcribed from a pCT7 DNA template remains hybridized to the DNA template (Kadesch & Chamberlin, 1982). This phenomenon is not a general feature of transcription on tailed templates nor is it due to the particular reaction conditions we have chosen. Instead, it appears that the inability of calf thymus RNA polymerase II to displace the nascent transcript is an intrinsic feature of the polymerase protein itself. *E. coli* core RNA polymerase and wheat germ RNA polymerase II both readily produce free RNA with any template we have tested. On the other hand, RNA polymerase II preparations from calf thymus, *Drosophila*, and human (HeLa) cells are unable to displace more than half of their product from pCpBR/*Pvu*II DNA. All five of these enzymes share many characteristics: (1) they are all multisubunit complexes; (2) they are unable to utilize promoter sequences by themselves but require nicks or single-stranded regions to initiate; (3) the sites of initiation on tailed DNA templates are nearly identical, at least for the enzymes from *E. coli*, wheat germ, and calf thymus. It is noteworthy that the three enzymes from animal sources share the inability to displace the transcript and are more antigenically similar to each other than to the homologous enzyme purified from plants (Weeks et al., 1982). The displacement phenomenon may be an indicator of some mechanistic differences between the class II RNA polymerases from these two diverged eukaryotic kingdoms.

While we have some evidence that there may be other components in addition to RNA polymerase II involved in the process of transcript displacement, it is apparent that purified calf thymus enzyme alone can produce free RNA from a tailed template if there is an appropriate DNA sequence at the duplex DNA terminus. Using different template termini, we have been able to increase the proportion of RNA displaced from an initial 35% of the total transcription product on pCpBR/*Pvu*II DNA or pCT7 DNA to 86% on pCpUC18/*Sma*I. Our experiments indicate that the terminal sequence that affects RNA strand displacement involves more than the last 3 base pairs, and, in general, increased G-C content enhances the ability of the enzyme to displace the nascent transcript. This correlation between increased G-C content of the tailed terminus and increased RNase A sensitivity of the calf thymus RNA polymerase II transcription product is most evident when one compares the activity of template bearing an *Aha*III terminus ($\cdot\overset{TTT}{AAAC}_{n}3'OH$) with similar templates bearing a *Sma*I terminus ($\cdot\overset{GGG}{CCCC}_{n}3'OH$).

The relationship between the G-C content of the template terminus and the ability of animal class II RNA polymerase to produce free RNA suggests that the relative free energies of AT and GC base pairs formed between the nascent RNA and the DNA template strand may be important in the displacement reaction. There are large differences in the relative stability of DNA–RNA hybrids and the homologous DNA duplexes for AT and GC homopolymer pairs (Chamberlin, 1965). Thus, dA–rU duplexes, such as would be formed at the start site with pCpBR/*Aha*III, are much less stable than the equivalent dA–dT duplex. In contrast, rG–dC complexes, such as would be found at the start site of pCpBR/*Sma*I, are much more stable than the equivalent dG–dC duplex. However, the relationship between RNA displacement and the G-C content of the template terminus cannot be explained by any simple model involving relative stabilities of the DNA duplex and the RNA–DNA hybrid. If there were a simple correlation in which unstable RNA–DNA hybrids were preferentially released, pCpBR/*Aha*III transcripts should be released preferentially and pCpBR/*Sma*I transcripts preferentially retained. This is the inverse of what is found. Furthermore, the apparent correlation between displacement of nascent RNA and formation of a more stable DNA–RNA hybrid at the start site is also not sufficient to explain our data. When GTP is replaced by ITP during transcription of pCpBR/*Pvu*II DNA by calf thymus RNA polymerase II, an rI–dC hybrid is formed at the start site. This is much less stable than the rG–dC equivalent and also less stable than the dG–dC template helix (Chamberlin & Patterson, 1965). However, essentially all of the RNA formed in this reaction is displaced (R. Dedrick, unpublished results). While the relationship between RNA displacement and G-C content of the template terminus remains unclear at this point, it may be possible to further define the role of terminal sequences and to increase the amount of released RNA by using defined, chemically synthesized oligonucleotides.

Optimal initiation at the tailed terminus apparently requires both a certain amount of single-stranded polypyrimidine "platform" and also pyrimidine nucleotides, preferably C, in the template strand. The dependence of calf thymus RNA polymerase II on pyrimidine tails for transcriptional activation of a linear template, and maximal stimulation by poly(dC) tails, correlates with its activity on homopolymer templates (Blatti et al., 1970) which is similar to other class II RNA polymerases (Dezélée et al., 1974; Lentfer & Lezius, 1972). The 100-fold stimulation commonly seen with poly(dC)-tailed templates compared to the untailed molecule makes this modification preferable for in vitro studies to the much more modest ~10-fold activation seen with poly(dT) tails.

The requirement for a pyrimidine sequence in the tail is partly due to a requirement for a pyrimidine nucleotide in the initiation region. Thus, addition of poly(dA) to the *Pst*I 3′ extension allows a ~10-fold transcriptional stimulation, though poly(dA) alone on a blunt end is ineffective. However, the

fact that the pApUC18/*Pst*I modification does not lead to full (100-fold) activation for calf thymus RNA polymerase II indicates that there may well be a preference for polymerase in binding to poly(dC) over poly(dA) as well. Despite this apparent preference for binding to poly(dC), there is no evidence for formation of very stable RNA polymerase II–poly(dC) complexes. Thus, excess single-stranded poly(dC) does not interfere with transcription of the duplex DNA, nor do heparin-resistant binary complexes readily form between tailed DNA and calf thymus RNA polymerase II (Kadesch & Chamberlin, 1982).

The location of the sites of transcriptional initiation with tailed templates by the various RNA polymerases we have tested suggests that the polymerase molecule positions itself quite precisely at the end of the duplex region of the tailed template with the active site immediately downstream from the 5′-phosphoryl terminus of the nontranscribed DNA template strand. RNA polymerase II apparently also interacts in a very similar manner with the DNA template when initiating from nicks. Wheat germ RNA polymerase II is known to bind precisely to nicks and cover an area of about 40 base pairs (Chandler & Gralla, 1980). Furthermore, RNA polymerase II from both calf thymus and wheat germ can synthesize RNA covalently attached to the DNA when initiating from nicks (Lewis & Burgess, 1980; Lavialle et al., 1982), suggesting that the 3′-OH end of the nick can become positioned in the active site of the enzyme. From our experiments probing the initiation site on tailed template, it is evident that a similar positioning of the enzyme occurs at the tail–duplex junction where only the 5′ side of the "nick" is present.

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Registry No. RNAP, 9014-24-8; poly(dC), 25609-92-1; RNase, 9001-99-4.

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