In Vitro Transcription by Wheat Germ Ribonucleic Acid Polymerase II: Effects of Heparin and Role of Template Integrity†

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ABSTRACT: Double-stranded deoxyribonucleic acid (DNA) from bacteriophage λ is a good template for wheat germ DNA-dependent ribonucleic acid (RNA) polymerase II. We delineate conditions for obtaining maximum polymerase activity using as template both the relatively intact DNA extracted from the λ phage and DNA into which single-strand nicks have been introduced by deoxyribonuclease (DNase) I digestion. The deliberate introduction of nicks produces a modest increase in transcription. The NaCl and $MgCl_2$ optima are broader with the nicked template, so that higher concentrations of these salts are needed before polymerase activity begins to decline. Heparin inhibits initiation but not elongation by wheat germ polymerase. Polymerase can be protected against heparin inhibition by forming binary complexes with the template. The formation of these complexes is reduced at low temperature. The complexes, once formed, decay in the presence of heparin with a half-life of 10–20 min. The number of complexes is highly dependent on the degree of nicking of the template, suggesting that single-strand nicks are the predominant type of site where these heparin-resistant complexes are formed. Our data do not allow us to decide whether or not the presence of nicks plays as decisive a role in the absence of heparin.

The biologically correct synthesis of new RNA requires RNA polymerase to perform four functions: it must select a site at which to begin transcription, it must initiate formation of an RNA chain complementary in sequence to the DNA, it must elongate that RNA chain, and it must terminate transcription appropriately. A variety of RNA polymerases have been isolated from eucaryotes; in every case the enzymes are capable of incorporating labeled precursors into RNA, but not much is known about how they select an initiation site and begin transcription. It remains controversial whether RNA polymerase II, the polymerase which synthesizes mRNA precursors, is able to initiate transcription at all using an intact duplex template in vitro in the absence of accessory protein factors [reviewed by Roeder (1976)].

We have studied the transcription of phage λ DNA by wheat germ RNA polymerase II. Wheat germ polymerase is easily obtained in pure form and is similar in subunit structure and amanitin sensitivity to mammalian RNA polymerase II (Jendrisak & Burgess, 1975, 1977). We have previously reported that wheat germ RNA polymerase II is more active on native than on denatured calf thymus DNA when several potential artifacts are excluded (Dynan et al., 1977). Preliminary experiments showed that wheat germ polymerase was also active on several defined templates, including adenovirus 2, phage T7, and phage λ.

In this paper, we report the results of a search for transcription conditions where the activity of the polymerase on unnicked template is highest relative to its activity on a template into which nicks have been deliberately introduced by DNase I treatment. We have also characterized the action of the polyanionic inhibitor heparin, which inhibits initiation but not elongation by wheat germ polymerase. We have used heparin to study the properties of binary complexes formed by the polymerase and DNA.

We became interested in heparin because it has proven useful in probing the interaction of *Escherichia coli* RNA polymerase with various phage templates. Heparin binds to and inactivates free *E. coli* RNA polymerase, but not enzyme which is engaged in RNA synthesis (Walter et al., 1967). Furthermore, preinitiation complexes resistant to heparin inactivation are formed by polymerase at some, but not all, sites where RNA synthesis can begin (Schäfer et al., 1973). Heparin slowly attacks and inactivates the enzyme in these resistant binary complexes at a rate which is distinctive for each complex site (Pfeffer et al., 1977; Giacomoni et al., 1977; Miller & Burgess, 1978). Although the rate of attack is not always correlated with promoter strength in the absence of heparin, in some experimental systems incorrect or nonspecific binding appears to be especially sensitive to heparin (Schäfer et al., 1973; Reznikoff, 1976).

There have been a number of reports of heparin affecting transcription by eucaryotic RNA polymerases. Xenopus RNA polymerase III forms heparin-resistant binary complexes similar to those formed by bacterial enzyme (Long & Crippa, 1976). Human cell RNA polymerase III has also been reported to form heparin-resistant complexes (Hossenlopp et al., 1978). Recently, wheat germ RNA polymerase II was found to form binary complexes on adenovirus 2 DNA which are resistant to polyriboinosinic acid, a polyanionic inhibitor which may work by the same mechanism as heparin (Seidman et al., 1979). These complexes share many properties with the complexes reported here. Polymerase II from several sources has also been reported to form heparin-resistant ternary complexes after transcription is allowed to begin (Bitter & Roeder, 1978; Pays, 1978).

In choosing to study phage λ DNA as a model template, we are depending on the chance occurrence of sites similar to in vivo eucaryotic polymerase recognition sites. The DNA of certain animal viruses contains excellent bacterial polymerase promoters (Surzycki et al., 1976; Lebowitz et al., 1977); we argue that the reverse situation is likely to occur. Phage λ DNA has the advantages of being well characterized, readily available, and uniform. Its transcription by eucaryotic polymerase is of practical interest because phage λ derived cloning vectors may be useful for studying eucaryotic templates.

Materials and Methods

RNA Polymerase Purification. Enzyme was prepared as previously described (Jendrisak & Burgess, 1975). Briefly,

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wheat germ was homogenized in a buffer of moderate ionic strength. Polymerase was precipitated with Polymin P, eluted from the Polymin P precipitate, and precipitated with ammonium sulfate. Gradient elution from DEAE-Sepharose CL 6B (Pharmacia) was substituted for the DEAE-cellulose column step (Smith & Braun, 1978). Pooled fractions containing RNA polymerase were subjected to chromatography on phosphocellulose and DEAE-Sephadex A-25 (Pharmacia). The A-25 column was eluted with a gradient of ammonium sulfate to remove traces of polymerase III and to eliminate several other impurities which elute at a slightly higher ionic strength than polymerase II. Enzyme purified by this method contains no detectable DNase, RNase, or protease activity. After dialysis against storage buffer containing 50% glycerol, the enzyme was stored frozen at −70 °C (Jendrisak & Burgess, 1977). Concentration of the enzyme was determined by using the extinction coefficient $E_{280}^{1\%} = 7.7$ (Jendrisak et al., 1976).

The enzyme used in these studies was further characterized for trace levels of endonuclease activity. Following RNA synthesis, standard transcription assay mixtures containing unnicked phage DNA were analyzed by alkaline agarose gel electrophoresis. No fragmentation of the template was observed.

E. coli RNA polymerase was prepared as previously described (Burgess & Jendrisak, 1975; Lowe et al., 1979).

Transcription Assays. Assays of enzyme activity were performed by measuring incorporation of [^{3}H]UTP into trichloroacetic acid (Cl_3AcOH) precipitable material as previously described (Dynan et al., 1977). The standard reaction mixture contained 10 μg/mL RNA polymerase, 10 μg/mL phage λ DNA, 50 mM NaCl, 5 mM $MgCl_2$, 0.4 mM ATP, GTP, CTP, and UTP, 20 μCi/mL [5-^{3}H]UTP (Amersham; supplied in 50% ethanol, 1 mCi/mL), 50 mM Tris-HCl, pH 7.9 at 25 °C, 0.5 mM EDTA, 0.1 mM dithiothreitol, and 20% (v/v) glycerol. Heparin (Calbiochem), when used, was usually present at 1 μg/mL. The volume of the standard reaction was 100 μL. After a 10-min preincubation at 37 °C with all assay components except the nucleoside triphosphates present, the nucleoside triphosphates were added and the reaction was allowed to proceed at 37 °C for 15 min before Cl_3AcOH precipitation. After the precipitates were collected on Whatman GF/C glass fiber filters, they were solubilized by adding 0.5 mL of a 95% Soluene-350 (Packard)–5% water mixture and incubating for 30 min at room temperature (Dynan et al., 1977). Incorporation of ^{3}H was determined by liquid scintillation counting. Results are averages of duplicate assays and are presented as disintegrations per minute above background, which was typically 30 dpm. Reactions were carried out in sterile plastic tubes (Falcon no. 2052), and all solutions except for DNA and enzyme were sterilized by autoclaving or filtration.

Template Preparation. Purified phage λ stored in isopycnic CsCl (gift of Dr. W. Szybalski) was dialyzed 3 times for 30 min against a buffer containing 0.3 M NaCl and 0.03 M Tris-HCl, pH 7.9. The phage was disrupted by adding EDTA to 10 mM and sodium dodecyl sulfate to 1% and heating to 65 °C for 15 min. The mixture was adjusted to a 0.2 M KCl concentration, chilled at 0 °C for 10 min, and centrifuged in a Beckman microfuge for 10 min. The supernatant was gently extracted 3 times with a mixture of equal volumes of chloroform and phenol containing 0.1% 8-hydroxyquinoline, spinning in the microfuge to separate the phases. The final aqueous phase was dialyzed extensively against buffer containing 125 mM Tris, pH 7.9 at 25 °C, 1.8 mM EDTA, and 100 mM NaCl. The dialyzed DNA was spun for 15 min at low speed, and the supernatant was heated to 65 °C for 5 min to disrupt concatemers. Further purification of the DNA by banding in CsCl had no effect on template activity.

Nicked DNA was prepared by incubation for 60 min at 14 °C of a mixture containing 140 mM Tris, pH 7.9 at 25 °C, 1 mM EDTA, 13 mM $MgCl_2$, 60 mM NaCl, 13 μg/mL bovine serum albumin (Miles), 30 ng/mL pancreatic DNase I (Worthington DPFF, RNase-free), and 150 μg/mL DNA. The reaction was stopped by adding one-half volume of 0.04 M EDTA, adjusted to 0.2% diethyl pyrocarbonate (Sigma Chemical Co.), and incubated at 37 °C for 2 h. Diethyl pyrocarbonate is an alkylating agent which reacts specifically with proteins (Ehrenberg et al., 1976). We followed the safety precautions recommended by Sigma.

Using alkaline agarose gel electrophoretic analysis, we estimated that our unnicked DNA preparations contained 0.1–0.5 nick/single strand. The standard digestion procedure introduced ~3–5 nicks/strand. Digestion with DNase I cleaves a single phosphoester bond, leaving 3′-hydroxyl and 5′-phosphate termini (Dreyer & Hausen, 1976).

Gel Electrophoresis. Electrophoresis in 0.5% agarose (Bio-Rad standard low M_r) was carried out in a horizontal slab gel apparatus as described by McDonnell et al. (1977). Neutral gels were run in a continuous buffer system containing 40 mM Tris-HCl, pH 7.9, 5 mM sodium acetate, and 1 mM EDTA. After electrophoresis for 18 h at 0.5 V/cm, gels were stained for 15 min in 1 μg/mL ethidium bromide and the UV fluorescent material was photographed.

For alkaline gels a pH 12.6 buffer containing 67 mM NaOH, 33 mM glycine, 33 mM NaCl, and 2 mM EDTA was used both in the gel and in the reservoirs. Samples were adjusted to 15% sucrose, 0.045% bromcresol green, and 0.2 M NaOH before loading. Electrophoresis at 0.5 V/cm was carried out for 18 h. It was important to maintain a sufficiently alkaline pH during electrophoresis. We regard the use of the glycine-containing buffer, an agarose wick gel apparatus, and buffer recirculation as essential. Gels were stained for 1 h in 0.2 M Tris, pH 7.9 at 25 °C, 0.05 M NaCl, and 1 μg/mL ethidium bromide. Kodak Tri-X film was used to photograph the UV fluorescent material, and the negatives were scanned with a Joyce-Loebl microdensitometer.

The precise mechanism of ethidium bromide staining of denatured DNA is not known. We found that this staining technique was at least semiquantitative, since relative areas under densitomer tracings of different samples or bands corresponded to the actual relative mass of DNA within a factor of 2. An estimate of the number of single-strand breaks per DNA strand was made by calculating the number-average molecular weight (Peterson et al., 1974), or for less nicked samples by monitoring the disappearance of unnicked strands and applying the Poisson distribution.

Results

Optimization of Reaction Conditions. Figure 1 shows the results of varying the enzyme/DNA ratio in the standard assay. As the amount of polymerase per assay is increased, the amount of transcription increases also, whether the template is nicked or unnicked DNA. At a weight ratio of 16 μg of enzyme per μg of DNA there is 1 enzyme molecule for every 50 base pairs of DNA, but even at this level no plateau is observed.

Glycerol stimulates enzyme activity, with the largest stimulation observed at higher temperatures, as shown in Figure 2. An extensive discussion of the possible effects of glycerol on transcription has been published by Buss & Stalter

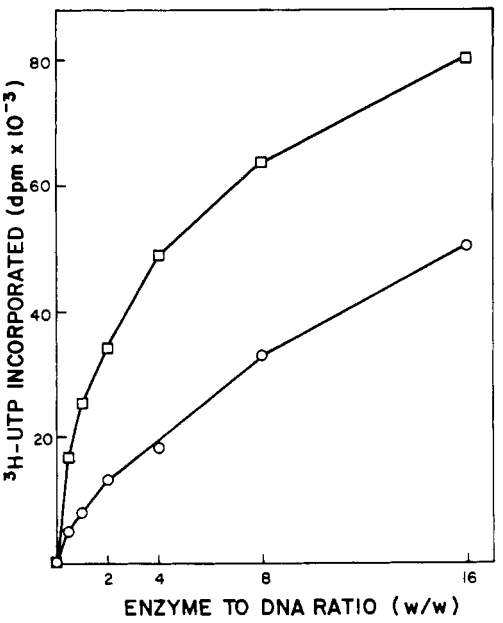

FIGURE 1: Effect of increasing enzyme/DNA ratio. Each assay contained 1 μg of nicked (□) or unnicked (○) phage λ DNA template prepared as described under Materials and Methods. Incorporation of [^{3}H]UTP was measured as described under Materials and Methods, except that each assay contained from 0.5 to 16 μg of RNA polymerase. Our standard amount of RNA polymerase used in most other experiments was 1 μg/100-μL assay.

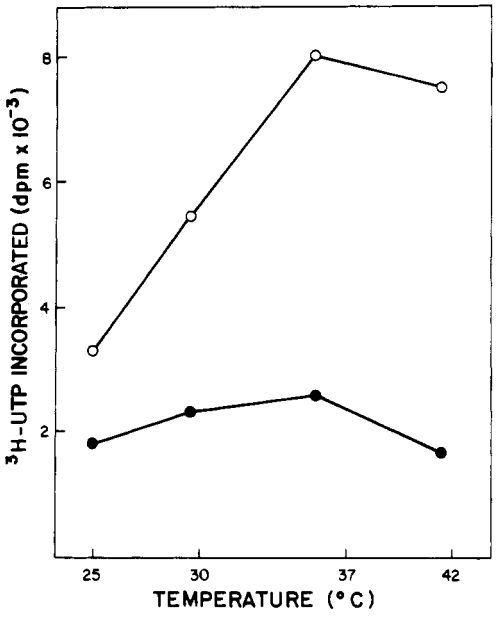

FIGURE 2: Effect of temperature. Assays were preincubated for 10 min at the temperature indicated, nucleoside triphosphates were added, and RNA synthesis was allowed to proceed for 15 min while continuing to incubate at the indicated temperature. Incorporation of [^{3}H]UTP was measured as described under Materials and Methods. Assays contained 1 μg of RNA polymerase, 1 μg of unnicked phage λ DNA, and either 20% glycerol (○) or 0.5% glycerol (●). Our standard temperature for transcription in other experiments was 37 °C, and our standard assay included 20% glycerol.

(1978). Glycerol causes *E. coli* polymerase to increase its level of transcription from certain weak promoters (Nakanishi et al., 1974). It has been suggested that this effect is mediated by destabilization of the secondary structure of the DNA. Since the stimulatory effect of glycerol on wheat germ RNA polymerase is strongest at higher temperatures, we suggest that the glycerol in our assays is working not by destabilizing the template but by protecting the polymerase against inactivation during the course of the assay.

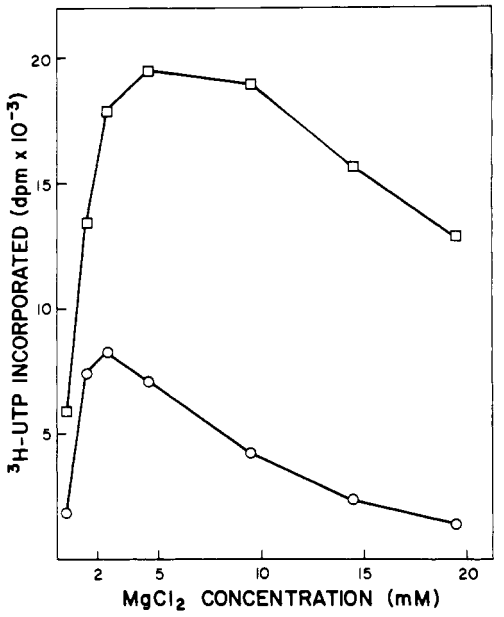

FIGURE 3: Effect of $MgCl_2$ concentration. Each assay contained 1 μg of RNA polymerase and 1 μg of nicked (□) or unnicked (○) phage λ DNA. Incorporation of [^{3}H]UTP was measured as described under Materials and Methods, except that assays contained a molar excess of $MgCl_2$ over EDTA ranging from 0.5 to 19.5 mM. Our standard amount of $MgCl_2$ used in other experiments was 4.5 mM in excess of EDTA.

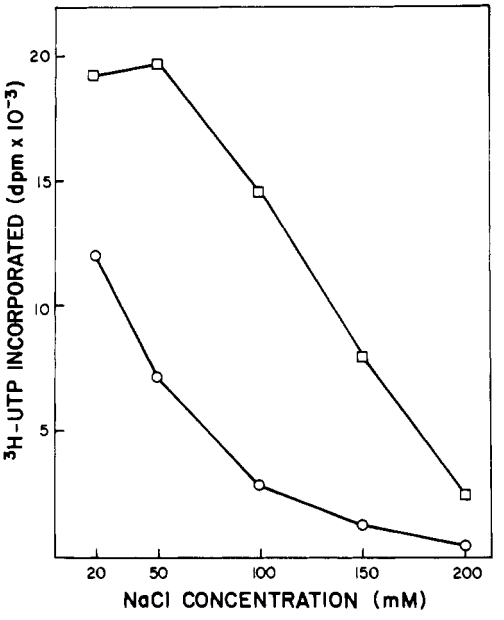

FIGURE 4: Effect of NaCl concentration. Each assay contained 1 μg of RNA polymerase and 1 μg of nicked (□) or unnicked (○) phage λ DNA. Incorporation of [^{3}H]UTP was measured as described under Materials and Methods, except that assays contained NaCl concentrations ranging from 20 to 200 mM. Our standard amount of NaCl used in other experiments was 50 mM.

We have observed that the wheat germ enzyme loses a fraction of its activity upon dilution from storage buffer into buffer lacking DNA or glycerol and that this activity is not restored by subsequent incubation in buffer containing DNA and glycerol. Abrupt dilution out of glycerol has been reported to have deleterious effects on RNA polymerase from *E. coli* (Williams & Chamberlin, 1977; Gonzalez et al., 1977). We include 20% glycerol in all our subsequent experiments. Standard procedure is to dilute enzyme into 50% glycerol buffer, add DNA, and then add the other reaction components.

Figures 3 and 4 show the effect of different $MgCl_2$ and

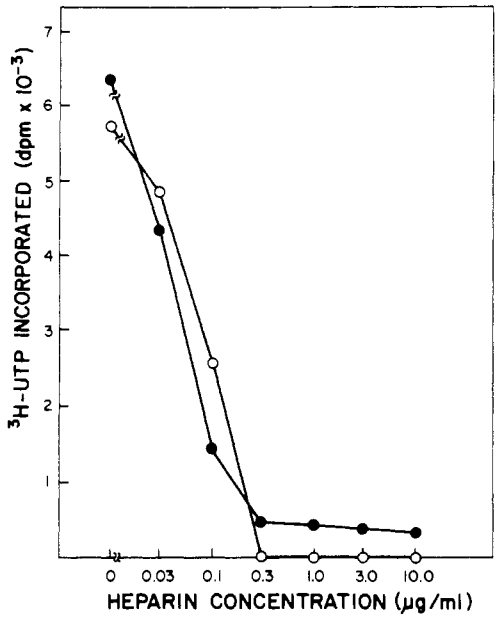

FIGURE 5: Inhibition of transcription by heparin. Each assay contained 1 μg of RNA polymerase and 1 μg of unnicked phage λ DNA. Heparin was either added to RNA polymerase prior to the addition of DNA (○) or was added simultaneously with nucleoside triphosphates after RNA polymerase and DNA had been allowed to preincubate at 37 °C for 10 min (●). Incorporation of [^{3}H]UTP was measured as described under Materials and Methods.

NaCl concentrations on the activity of wheat germ polymerase. Moving to higher concentrations of either salt suppresses transcription from unnicked DNA before beginning to affect transcription of nicked DNA. Because the polymerase activity we measure is a composite of site selection, initiation, elongation, and termination reactions, it is not possible to interpret this result in terms of initiation at different sites on nicked and unnicked DNA. It is possible, for example, that enzyme is initiating at nicks on both templates but that low salt concentrations somehow facilitate the process of searching out rare nicks in a largely unnicked template population. The practical implication of the results in Figures 3 and 4 is that it may be more rewarding to search for initiation on intact duplex DNA at low NaCl and $MgCl_2$ concentrations since higher concentrations of either salt would bias the investigation toward finding transcription only at nicks.

The maximum polymerase activity on unnicked DNA in these experiments was 12000 dpm, or 110 pmol of UTP, incorporated in 15 min by 1 μg of enzyme using 1 μg of DNA. With phage λ DNA containing less than one nick per duplex molecule, this level of transcription represents more than 3300 nucleotides of UTP incorporated per nick. If wheat germ polymerase is indeed limited to initiation at rare nicks in the unnicked template population, then it is using them efficiently.

Inhibition by Heparin. The inactivation of polymerase by heparin is shown in Figure 5. This experiment has been carried out according to two protocols. In the first, polymerase and heparin were preincubated together for 10 min, and then DNA and the other reaction components were added and the whole mixture was allowed to preincubate further before RNA synthesis was measured. Heparin completely inhibits polymerase activity under this protocol when it is present in an amount approximately equimolar to the enzyme. Heparin and DNA are both polyanions. Wheat germ polymerase binds both to heparin and DNA affinity columns; pretreatment with heparin reduces binding to a DNA column (J. Jendrisak, personal communication; and our laboratory, unpublished results). A likely mechanism for heparin inhibition of transcription is that it is competing for the DNA-binding site of the polymerase. The amount of heparin used to inhibit wheat germ polymerase is similar to the amount required to inhibit *E. coli* RNA polymerase: one molecule of heparin per enzyme protomer (Walter et al., 1967). The concentrations of heparin needed to inhibit Xenopus, rat liver, and MOPC RNA polymerases were higher than those used in our experiments (Long & Crippa, 1976; Bitter & Roeder, 1978).

The second protocol in the experiment in Figure 5 was to preincubate the complete assay mixture lacking only the nucleoside triphosphates and then simultaneously add the heparin and triphosphates. Heparin is again observed to inhibit the bulk of the polymerase activity when added at low concentration, but now a small fraction of the activity is resistant even to much higher heparin concentrations. Although the resistant fraction is only ~5% of the total activity, it is reproducible and well above the background of unincorporated substrate. Furthermore, both the total activity and the heparin-resistant fraction are reduced 98% in the presence of 1 μg/mL α-amanitin. The heparin-resistant transcription therefore is an activity of RNA polymerase II and not an activity of a minor enzymatic contaminant of the polymerase II preparation.

Figure 6 shows a time course of transcription. The [γ-^{32}P]GTP label used in this experiment is incorporated only into the 5′ end of each RNA molecule, so that only initiation, not elongation, is measured. The figure legend details a number of modifications which had to be made in the standard transcription assay to increase the incorporation of label into RNA above the high and variable background which accompanies the use of the γ-^{32}P label. When heparin is added to the reaction 5 min after RNA synthesis has begun, there is a rapid shutoff of initiation.

Incorporation of internal label into RNA is almost unaffected during the first 2 min after heparin is added to the transcription reaction, as shown in Figure 7. Unlike α-amanitin, which shuts off transcription immediately, heparin allows some transcription to continue even 15 min after it is added. These results demonstrate that heparin inhibits initiation, but not elongation, by wheat germ RNA polymerase.

The data in Figures 5–7 suggest that when polymerase and DNA are preincubated, a fraction of the polymerase molecules binds tightly to the template and becomes resistant to heparin inactivation. When heparin and nucleoside triphosphates are added simultaneously, the free enzyme is rapidly inactivated, but the enzyme bound to the template in heparin-resistant complexes is able to synthesize long RNA chains during the course of the standard 15-min assay. The amount of transcription in the presence of heparin is determined by the number of heparin-resistant complexes present initially and by the average length of the RNA chains which are synthesized.

Properties of Heparin-Resistant Complexes. Introducing a small number of single strand nicks into the template by a controlled digestion with DNase I produces a large increase in the amount of heparin-resistant transcription. Figure 8 shows that a 60-min digestion increases the heparin-resistant transcription 20-fold, although stimulation of total transcription in the absence of heparin is less than 3-fold. The increase in heparin-resistant transcription is roughly proportional to the increase in the number of nicks per strand.

The formation of heparin-resistant complexes, both on unnicked DNA and on DNA into which nicks have been deliberately introduced, is a temperature-dependent process. Table I summarizes the results of an experiment where

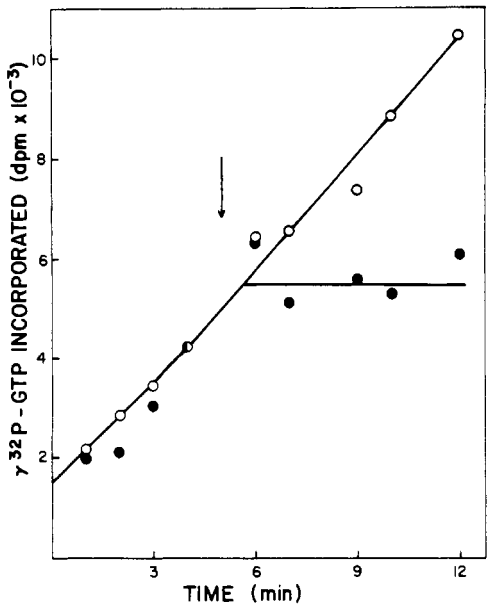

FIGURE 6: Time course of [γ-^{32}P]GTP incorporation. Assays were performed as described under Materials and Methods, except that a number of modifications were made to increase the fraction of label incorporated and to reduce the background of unincorporated label. Aliquots of 50 μL were withdrawn at the indicated times from each of two large assay mixtures, one with no heparin (O) and the other with heparin added to a final concentration of 20 μg/mL (●) 5 min after transcription had begun (arrow). Each 50-μL aliquot contained 10 μg of RNA polymerase, 5 μg of unnicked phage λ DNA template, 25 μCi of [γ-^{32}P]GTP and 2.5 μCi of [5-^{3}H]UTP. The concentration of unlabeled GTP was reduced to 0.1 mM. Transcription was arrested by pipetting each 50-μL aliquot into a tube containing 0.5 mL of 0.02 M EDTA and 0.01 M ATP and incubating at 0 °C. At the end of the experiment, 3 mL of a solution containing 5% Cl_3AcOH, 0.025 M sodium pyrophosphate, and 1 M KCl was added and the tubes were further incubated at 0 °C for at least 15 min. The precipitated nucleic acids were collected by filtration as described under Materials and Methods. To reduce background, the GF/C filters were presoaked overnight in 2% Cl_3AcOH, 0.01 M sodium pyrophosphate, and 0.01 M ATP. Additionally, the 2% Cl_3AcOH rinsing solution was supplemented with 1 M KCl. The background of unincorporated label retained on the filters was estimated to be 5×10^{-5} fraction of the input label, or 2000–3000 dpm/50-μL aliquot. Background has not been subtracted from the data shown here. Incorporation of [^{3}H]UTP in this experiment (not shown) was qualitatively similar to the incorporation shown in Figure 7.

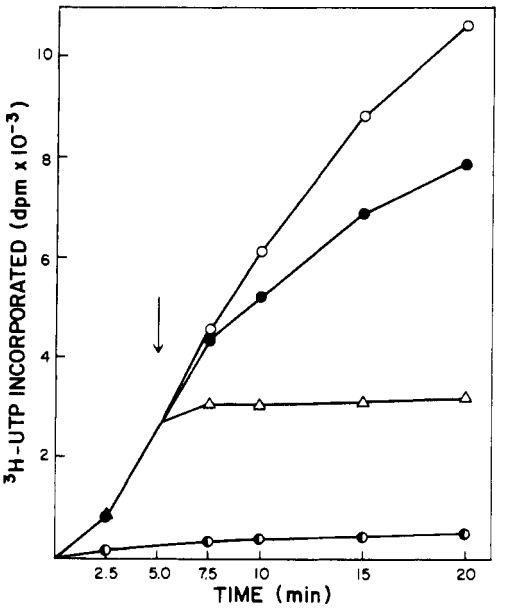

FIGURE 7: Time course of [^{3}H]UTP incorporation. Assays were performed as described under Materials and Methods by using unnicked phage λ DNA template. Aliquots of 100 μL were withdrawn at the indicated times from each of four large assay mixtures and pipetted into tubes containing 0.1 mL of 0.01 M EDTA. At the end of the experiment, the contents of each tube were Cl_3AcOH precipitated and filtered as described under Materials and Methods. Assay mixtures contained no inhibitor (O), α-amanitin (△) added to a final concentration of 1 μg/mL 5 min after transcription was begun (arrow), or heparin added to a final concentration of 1 μg/mL either simultaneously with the nucleoside triphosphates (◐) or 5 min after transcription was begun (●).

Table I: Effect of Preincubation Temperature on Heparin-Resistant Transcription[a]

		[^{3}H]UTP dpm incorporated		
		with 1 μg/mL heparin		without heparin
enzyme	template	0 °C	37 °C	37 °C
wheat germ	unnicked	109	379	6705
wheat germ	nicked	436	11725	21429
E. coli	unnicked	4289	32866	51012

[a]Transcription reactions contained 1 μg of *E. coli* or wheat germ RNA polymerase and 1 μg of nicked or unnicked phase λ DNA. Components of the reaction mixture were as described under Materials and Methods, except that 50 mM Hepes buffer, pH 7.8 at 20 °C, was substituted for Tris-HCl. Reactions were preincubated for 10 min at either 0 or 37 °C, transcription was started by addition of nucleoside triphosphates and in some cases heparin, and the reactions were immediately shifted to 37 °C. After 15 min, reactions were stopped by the addition of Cl_3AcOH, and incorporation of [^{3}H]UTP was determined as described under Materials and Methods. A background equivalent to 36 dpm has been subtracted from all samples. In the absence of heparin, changing preincubation temperature had little effect (not shown).

complexes were allowed to form during preincubation either at 0 °C or at 37 °C. Heparin and nucleoside triphosphates were added, and the reaction was shifted immediately to 37 °C. The entire experiment was carried out in Hepes buffer, pH 7.8 at 20 °C, in order to reduce the shift in pH which accompanies a shift in the temperature of Tris buffer. Heparin-resistant transcription of nicked DNA was sharply reduced when preincubation was at 0 °C. The same effect was seen to a lesser extent with the unnicked template. *E. coli* RNA polymerase holoenzyme, which initiates transcription at the in vivo λ promoters, was included for comparison. Like the wheat germ polymerase, it showed a large drop in heparin-resistant activity at the lower temperature. With *E. coli* polymerase, transcription of nicked and unnicked templates was similar (not shown). In this experiment, wheat germ polymerase initiating at nicks mimics the behavior of *E. coli* polymerase initiating at the in vivo λ promoters.

The decay of heparin-resistant complexes in the presence of heparin is shown in Figure 9. In this experiment, after complexes were first allowed to form, heparin was added and the reaction was incubated for various lengths of time prior to the addition of nucleoside triphosphates. As might be expected, the longer the period between the addition of heparin and the beginning of RNA synthesis, the less RNA is made. Despite a 25-fold difference in the amount of synthesis initially resistant to heparin, the decay curves for the nicked and unnicked templates are similar. Some of the complexes on unnicked DNA are less stable than the complexes on nicked DNA; it takes twice as long for half of the resistant activity to disappear when the template contains nicks.

No experiment we have performed has revealed an unequivocal difference between heparin-resistant complexes formed on nicked and unnicked templates. Both sorts of complexes are equally sensitive to α-amanitin and equally resistant to high concentrations of heparin, up to 100 μg/mL.

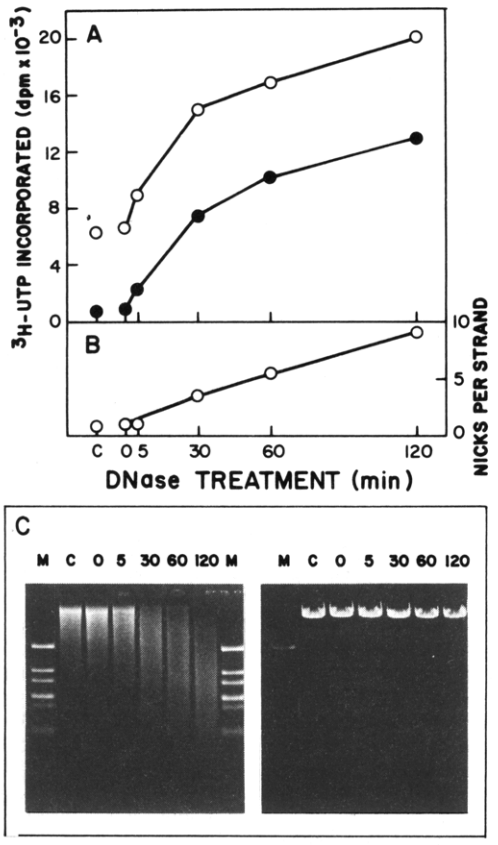

FIGURE 8: Effect of DNase I digestion of phage λ DNA. Each sample of DNA was incubated with 60 ng/mL DNase I for the length of time indicated under the conditions described under Materials and Methods. The reactions were terminated with EDTA followed by diethyl pyrocarbonate, and the digested DNA samples were analyzed for template activity and structural integrity. Digestion for 30 min under the conditions described here produces a template equivalent to the nicked phage λ DNA used in other experiments. (A) Stimulation of transcription following DNase I treatment. Incorporation of [^{3}H]UTP was measured as described under Materials and Methods. Assays contained either no heparin (○) or 1 μg/mL heparin added simultaneously with the nucleoside triphosphates (●). Control assays using template DNA to which no DNase was added are designated as "C" in the figure. (B) Number of nicks per strand introduced by DNase I digestion. A photographic negative of the alkaline gel shown in Figure 8C was scanned, and the approximate number of nicks per strand was calculated as described under Materials and Methods. (C) Agarose gel electrophoresis of the same samples of digested DNA assayed in Figure 8A. Electrophoresis was carried out in alkaline (left) and neutral (right) 0.5% agarose gels as described under Materials and Methods. Tracks labeled "C" are control samples of DNA to which no DNase was added, and tracks labeled "M" are molecular weight markers produced by restriction endonuclease *Ava*I digestion of λ DNA (Rosenvold & Honigman, 1977). Other tracks are labeled to show digestion time in minutes.

Minor differences in the stability and temperature dependence of formation of complexes on nicked and unnicked DNA have been discussed above. Our results are consistent with a model where most of the heparin-resistant complexes on so-called unnicked DNA are formed at the rare nicks in this template population, but where some of the complexes are formed either on intact duplex DNA, at ends, or at short gaps.

Discussion

We have characterized the interaction of wheat germ RNA polymerase II with phage λ DNA. The introduction of nicks into the template by controlled DNase I digestion produces a moderate stimulation of polymerase activity, a stimulation which is more marked when transcription reactions contain high concentrations of $MgCl_2$ and NaCl. Because the total incorporation of labeled precursor into RNA is a function of the rates of several steps involved in transcription, these results cannot be interpreted in terms of what kinds of sites can be used for initiation of RNA synthesis.

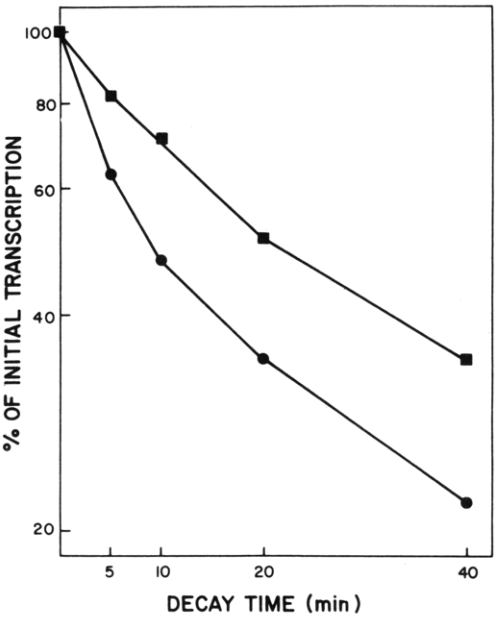

FIGURE 9: Decay of heparin-resistant complexes in the presence of heparin. Assays containing unnicked (●) or nicked (■) phage λ DNA template were preincubated for at least 10 min at 37 °C in the absence of heparin and nucleoside triphosphates. Heparin was added at various times, the incubation was continued, and finally transcription was begun by adding nucleoside triphosphates. Incorporation of [^{3}H]UTP was measured as described under Materials and Methods. The concentration of heparin was 1.25 μg/mL during the decay period and 1 μg/mL during transcription. The level of transcription when heparin was added simultaneously with nucleoside triphosphates, shown as 100% in the figure, was 9981 dpm with the nicked template and 409 dpm with the unnicked template.

We focussed on initiation by employing heparin, which blocks binding and initiation of polymerase but allows elongation of nascent RNA to proceed. Wheat germ polymerase becomes protected against heparin inactivation by entering into binary complexes with the template. These complexes are similar to complexes formed by *E. coli* RNA polymerase at promoters in that their formation is suppressed at 0 °C and they decay slowly in the presence of heparin. The complexes are dissimilar to those formed by *E. coli* RNA polymerase in that they form predominantly at nicks in the template.

The amount of heparin-resistant transcription observed using our unnicked template preparations is consistent with initiation limited to rare nicks. In the dual-label time course experiment shown in Figure 6, the ratio of [^{3}H]UTP to [λ-^{32}P]GTP incorporated approached 250 after heparin was added. The amount of label incorporated in the presence of heparin using unnicked DNA, containing 0.1–0.5 nick/strand, is typically 4 pmol of [^{3}H]UTP. If heparin-resistant complexes form at every nick and if all transcripts initiate with GTP, this level of transcription can be accounted for by the incorporation of 120–600 nucleotides of UTP per transcript, an amount which is in good agreement with the dual-label experiment. The high background accompanying the use of the γ-^{32}P label precludes direct measurement of initiation from the few heparin-resistant binary complexes which form on unnicked DNA.

The assumption that all transcripts initiate with GTP is based on our finding that wheat germ polymerase incorporates [γ-^{32}P]GTP in preference to [γ-^{32}P]ATP (unpublished experiments). We know, however, that wheat germ polymerase sometimes uses 3′-OH groups at a nick as a primer (M. K.

Lewis and R. Burgess, unpublished experiments). When a transcript is made using a primer, no γ-^{32}P label is incorporated into it, so that the occurrence of priming leads to an underestimate of the number of transcripts being synthesized. A further consideration in calculating the average RNA chain length is that some transcripts may be too short to be Cl_3AcOH precipitable. These short transcripts would go undetected in the experiments reported here.

We believe transcription beginning at the ends of the template molecules does not play a major role in wheat germ polymerase transcription of phage λ DNA. Our analysis of in vitro transcripts using the technique of Southern (1975) shows no apparent overrepresentation of sequences near the ends of the template, whether or not heparin is included in the transcription reaction (data not shown). Although there is evidence that single-stranded 3′ ends of DNA may serve as initiation sites for yeast and wheat germ polymerases (Nohara et al., 1973; Lilley & Houghton, 1979), the sticky ends of phage λ DNA are found at the 5′ ends of the strands [discussed by Lewin (1977)].

The idea that eucaryotic polymerase is able to use nicks in vitro has been explored previously. Wheat germ polymerase binds tightly to nicks introduced into SV40 DNA (J. Gralla, personal communication). Dreyer & Hausen (1976) studied the structure–template activity relationship for several kinds of enzymatically introduced nicks. They concluded that a 3′-hydroxyl end, such as is present in nicks made by DNase I, stimulates rat liver RNA polymerase II activity. Despite their results and others showing an apparent role of nicks in vitro, the presence of nicks does not seem to be an obligate feature of selective transcription in vivo. The DNAs of the animal viruses SV40 and adenovirus 2, for example, are not known to contain nicks at specific sites. The in vitro data raise the question of whether nicks or other interruptions in the DNA duplex merely facilitate binding and initiation by polymerase II or whether they are essential.

Our observation is that for a given amount of nicking, the stimulation of heparin-resistant transcription is much greater than the stimulation of total transcription. Possibly the presence of heparin suppresses initiation on intact duplex DNA, limiting initiation to nicks which may be present in the template. Alternatively, initiation may always be limited to nicks, with this limitation being more apparent when the presence of heparin prevents initiation sites from being used more than once.

One approach to studying the potential ability of RNA polymerase to initiate on intact duplex DNA is to reduce the number of nicks in the template population until it is arbitrarily close to zero. The phage DNA used in the experiments reported here had less than 1 nick/100 000 bases of single-stranded DNA. Using a covalently closed circular template and exercising care in its preparation, it should be possible to reduce the number of nicks still further. Binding and transcription of SV40, polyoma, and plasmid templates have been studied by using calf thymus and wheat germ RNA polymerases II (Mandel & Chambon, 1974; Lescure et al., 1978; Lilley & Houghton, 1979; J. Gralla, personal communication). Interpretation of these results is complicated by the presence of single-stranded regions generated by superhelical strain and by the inevitable presence of some nicked template molecules.

Another approach is to identify in vitro transcripts which initiate at specific internal sites on the template DNA. Polyacrylamide gel electrophoresis shows that most of the RNA is heterogeneous in size and so has not been useful in assessing the selectivity of initiation in vitro by wheat germ polymerase using phage λ DNA template (unpublished experiments). We have analyzed the population of in vitro transcripts by the technique of Southern (1975). While not all segments of the genome appear to be equally represented in the RNA population, no simple pattern of selectivity has emerged. This finding was not unexpected, given the qualitative nature of this technique and given the likelihood that any selective transcription must also be accompanied by a background of transcription from random nicks.

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On the Structure and Conformational Dynamics of Yeast Phenylalanine-Accepting Transfer Ribonucleic Acid in Solution†

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ABSTRACT: The solution structure of yeast $tRNA^{Phe}$ was investigated by using ethidium as a fluorescent probe in the D loop and the anticodon loop. For this purpose the dihydrouracils in position 16/17 and wybutine in position 37 were substituted by ethidium. The lifetimes and the time-dependent anisotropy of ethidium fluorescence were measured by pulsed nanosecond fluorometry. The kinetics of the transitions between different states of the $tRNA^{Phe}_{Etd}$ derivatives were determined by chemical relaxation measurements. It was found that the ethidium label irrespective of its position exhibits three different states called T_1, T_2 and T_3 characterized by lifetimes τ_1 = 30 ns, τ_2 = 12 ns, and τ_3 = 3 ns. The lifetime differences are due to different accessibilities of ethidium for solvent quenching in the three states. Thus, there are three different defined structural environments of the ethidium in both the anticodon and the D loop. The distribution of the three states was measured as a function of Mg^{2+} concentration and temperature; it was found that state T_3 is favored over states T_2 and T_1 by both increasing Mg^{2+} concentration and increasing temperature. The chemical relaxation kinetics exhibit a fast transition between T_1 and T_2 (10–100 ms) and a slow transition between T_2 and T_3 (100–1000 ms). The rates of both transitions depend likewise on Mg^{2+} concentration and temperature. The equilibrium and kinetic data clearly show the presence of strong and weak interactions between Mg^{2+} and tRNA. A cooperative model accounting for this behavior is developed. The ethidium probe behaves identically when located in different regions of the tRNA regarding both its distribution of states and its transition kinetics. This suggests that the different spectroscopic states report different conformations of the tRNA structure. The dependence of the three states on Mg^{2+} and spermine indicates that conformation T_3 is closely related to or identical with the crystal structure. The rotational diffusion constants indicate that of all three states T_3 is most extended while T_2 is most compact. The thermodynamic analysis reveals that the strongly bound Mg^{2+} ions reduce both the activation entropy and enthalpy of all transitions. The weakly bound Mg^{2+} ions increase both the activation enthalpy and entropy of the slow transition between T_2 and T_3. It is suggested that the breaking of several intramolecular bonds, e.g., hydrogen bonds, is involved in this transition.

A substantial amount of information has been accumulated from the crystal structure of yeast $tRNA^{Phe}$ regarding the role of conserved regions of tRNA in the folding of a conformation of tRNA which could be crystallized in the presence of Mg^{2+} and spermine (Kim et al., 1971; Ladner et al., 1972). Although a detailed picture of the tertiary folding of $tRNA^{Phe}$ (Kim et al., 1974; Ladner et al., 1975) now is available, the information regarding the functional role of this tRNA structure is very limited.

Studies of the interaction of $tRNA^{Phe}$ with ribosomal RNA and ribosomes suggest that the TΨCG sequence, which in the crystal structure is hydrogen-bonded to the D loop, can become available for hydrogen bonding (Erdmann et al., 1973; Richter et al., 1974; Schwarz et al., 1976). Thus, it is not unreasonable to assume the existence of tRNA conformations in solution which are different from the crystal structure.

The presence of such conformations is indicated by a variety of observations. Experiments in which the binding of oligonucleotides to tRNA was investigated indicate interactions between oligonucleotides and complementary sequences in the D loop (Uhlenbeck, 1972; Cameron & Uhlenbeck, 1973) as well as in the the anticodon loop (Uhlenbeck, 1972; Eisinger & Spahr, 1973) which are not expected to take place if the crystal structure prevails in solution.

The results of degradation studies of tRNA with exonucleases have been explained by the existence of at least two tRNA conformations which differ at or near the 3′ end (Thang et al., 1971) and at the 5′ end (Hänggi et al., 1970). Similar conclusions have been drawn from experiments in which the translational diffusion of $tRNA^{Phe}$ was investigated under various ionic conditions (Olson et al., 1976). NMR experiments have revealed two magnetic environments of the nucleoside T in $tRNA^{Phe}_{yeast}$ (Kan et al., 1977), indicating two

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