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Selectivity of RNA Chain Initiation in Vitro. 3. Variables Affecting Initiation of Transcription†

Jacqueline S. Miller and Richard R. Burgess*

ABSTRACT: The effects of salt, temperature, enzyme to DNA ratio, and heparin challenge on both total RNA synthesis and synthesis from specific promoters are examined using DNA from bacteriophages λcb2 and T7. Determination of synthesis from specific promoters is carried out by the fractionation and quantitation on polyethylenimine-cellulose thin-layer chromatograms of the 5′-terminal oligonucleotides produced by digestion of the RNA products with T1 RNase. The major findings of this work are that (1) λcb2 promoters are more salt sensitive than T7 promoters and the salt concentration affects individual promoters differently, (2) T7 promoters initiate maximally at 37 °C but the transition temperatures of promoters vary and may be dependent on the salt concentration, (3) increasing the enzyme to DNA ratio results in increasing initiations at the promoters on T7 DNA without causing measurable initiation at non-promoters, and (4) T7 and λcb2 promoters show differences in stability when challenged with heparin.

An important controlling point in regulation of gene expression is the selectivity of transcription at the level of initiation. *E. coli* RNA polymerase is capable of selectively initiating RNA synthesis in vitro at many promoters on bacterial and phage DNA. Selective initiation involves recognition by holoenzyme of a promoter region of the DNA, tight binding of the polymerase to form a stable, binary complex with the DNA, and initiation of RNA chain synthesis whereby the initiating ribonucleoside triphosphate binds to the polymerase and forms a phosphodiester bond with a second bound nucleotide (see Chamberlin, 1974).

Many factors influence the selectivity of initiation of transcription on a DNA template. Altering reaction conditions may cause either the suppression or enhancement of biologically incorrect transcription or the suppression or enhancement of a particular set of biologically correct transcripts.

Variables which have been implicated in altering the selectivity of transcription at the level of initiation include protein factors (Scaife, 1973; Losick & Pero, 1976), temperature (Walter et al., 1967; Chamberlin & Ring, 1972; Mangel & Chamberlin, 1974c), monovalent cations (Millette et al., 1970; Schäfer & Zillig, 1973; Mangel & Chamberlin, 1974b; Matsuge, 1972; Dausse et al., 1976), divalent cations (Nakanishi et al., 1975; Chamberlin & Ring, 1972), enzyme concentration (Takanami et al., 1970; Brody et al., 1970; Chamberlin & Ring, 1972; Dausse et al., 1972), organic solvents (Nakanishi et al., 1974; Küpper et al., 1976; Brody & Leutey, 1973), and the configuration of the DNA (Hayashi & Hayashi, 1971; Botchan et al., 1973; Wang, 1974; Richardson, 1975).

In this paper, the effects of several of these variables on both total RNA synthesis and synthesis from specific promoters are examined. DNAs from bacteriophage λcb2 and T7 were used as templates since their in vitro transcription patterns have been studied extensively and characterized. The method used for determining synthesis from specific promoters involves fractionation and quantitation of the 5′-triphosphate ends of the RNA following digestion by T1 RNase, as described in the preceding papers (Miller & Burgess, 1978a,b). The usefulness of this method in the rapid and quantitative analysis of changes in initiation of transcription is demonstrated.

Experimental Procedure

Preparation of the DNA, RNA Polymerase, and [γ-^{32}P]-ATP and GTP. DNA from λcb2 and T7 was isolated as previously described (Miller & Burgess, 1978a). RNA polymerase was purified by the method of Burgess & Jendrisak (1975). [γ-^{32}P]ATP and GTP were prepared as described by Miller & Burgess (1978a).

Synthesis of RNA. A standard reaction mix for RNA synthesis (0.25 mL) contained the following: 0.03 M Tris-HCl (pH 7.9 at 25 °C), 0.1 mM dithiothreitol, 0.1 mM EDTA, 0.5 mM KPO_4, 0.12 mM of [γ-^{32}P]ATP and GTP, 0.24 mM of the remaining nucleoside triphosphates, 0.15 M KCl, 30 μg of DNA, and 30 μg of RNA polymerase. The specific activity of the [γ-^{32}P]NTP was 1000 to 30 000 dpm/pmol depending

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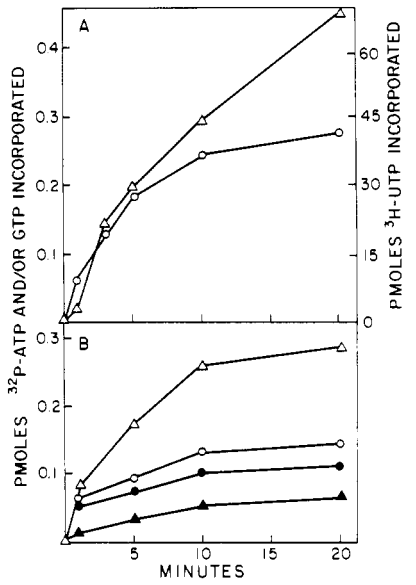

FIGURE 1: Time course of RNA synthesis on T7 DNA. (A) Total synthesis of RNA on T7 DNA was carried out under standard conditions as described in Experimental Procedures. Specific activities were 330 dpm/pmol for [^{3}H]UTP and 10 000 dpm/pmol each for [γ-^{32}P]ATP and [γ-^{32}P]GTP. Aliquots (10 μL) were added to 0.5 mL of ice-cold water at the times indicated and were precipitated and filtered as described. (△—△) Picomoles [^{3}H]UTP incorporated; (○—○) [γ-^{32}P]ATP and GTP incorporated. (B) Incorporation of [γ-^{32}P]NTP into the 5′ ends of RNAs initiated at individual promoters. Portions (250 μL) from a 1200-μL reaction mix containing both [γ-^{32}P]ATP and GTP at 10 000–30 000 dpm/pmol were added to 5 μg of rifampicin in a chilled test tube at the times indicated. The RNAs were isolated and digested and the 5′-terminal oligonucleotides fractionated and quantitated as described in Experimental Procedures. The results are the average of three experiments and indicate the pmol of ^{32}P present in each spot from one-fifth of each time point. (●—●) Spot 1, 5′-terminal oligonucleotide from the A3 promoter; (○—○) spot 2, 5′-terminal oligonucleotide from the A1 promoter; (△—△) pppGp, from A2 promoter; (▲—▲) spot 4, an unidentified spot.

on the nature of the experiment. When determination of total RNA synthesis was desired, [^{3}H]UTP was added at a specific activity of 330 dpm/pmol. The reaction mixture was preincubated for 10 min at 37 °C. The synthesis was started by the addition of $MgCl_2$ to 10 mM and allowed to continue for 10 min. Variations in conditions are indicated in the figure legends.

Determination of RNA Synthesis and Initiation. Total RNA synthesis and initiation were determined by measuring the amount of ^{3}H and ^{32}P label incorporated into Cl_3CCOOH-precipitable RNA. After 10 min of RNA synthesis, samples were diluted on ice with 0.5 mL of sterile double-distilled water. Fifty microliters of 1% (w/v) bovine serum albumin was added and the samples were precipitated with an equal volume of 20% Cl_3CCOOH, 0.01 M $NaPP_i$. The samples were filtered on Whatman GF/C glass fiber filters which had been presoaked in 2% Cl_3CCOOH, 0.001 M $NaPP_i$, 10 mM ATP. In order to reduce the background radioactivity, the filters were washed three times with 5 mL of 2% Cl_3CCOOH, 0.001 M $NaPP_i$, removed from the filter apparatus, and soaked for 1 h at 4 °C in 1.0 M KCl, 5% Cl_3CCOOH, 0.005 M $NaPP_i$. They were returned to the filter apparatus, rinsed two times with 10 mL of 2% Cl_3CCOOH plus 0.001 M $NaPP_i$, and then with 5 mL of 95% ethanol, and then they were counted in 5 mL of Scintosol (Isolab, Inc.).

Incorporation of ^{32}P into 5′-terminal oligonucleotides was quantitated as follows: RNA was synthesized as described above and the reaction stopped by adding rifampicin to 20 μg/mL and incubating for 5 min. The RNA was isolated by DNase treatment, phenol extraction, Sephadex G-50 chromatography, and ethanol precipitation as described by Miller & Burgess (1978a). The precipitated RNA was resuspended in about 50 μL of double-distilled water and the volume accurately determined by weighing the dissolved sample. Precisely one-fifth of the sample was then digested with RNase T1, fractionated and quantitated as described by Miller & Burgess (1978a).

We have shown that short oligonucleotides less than 15 bases long are retarded on the Sephadex G-50 column and eluted in the front of the substrate peak. In an attempt to quantitate all initiations in these experiments, including those giving rise to short oligonucleotides, the void peak and the first two fractions of the substrate peak were pooled (corresponding to fractions 4–10 in Figure 1, Miller & Burgess, 1978a).

Measurement of Complex Stability in the Presence of Heparin. The stabilities of the RNA polymerase–DNA complexes were determined as follows: A standard reaction containing 10 mM $MgCl_2$ was preincubated for 10 min at 37 °C in the absence of nucleotides. A zero-time point was taken by adding a 200-μL portion of the preincubated reaction to heparin (50 μg/mL) and four nucleoside triphosphates containing [γ-^{32}P]ATP and [γ-^{32}P]GTP. Heparin was added to the large reaction mix to a final concentration of 50 μg/mL and at various times after the addition of heparin 200-μL portions were added to 50 μL of a mixture of four nucleoside triphosphates. [γ-^{32}P]ATP and [γ-^{32}P]GTP concentrations were 0.12 mM with a specific activity of 15 000 dpm/pmol and UTP and CTP concentrations were 0.24 mM. The mixtures were incubated 3 min at 37 °C to allow transcription and quickly chilled to 0 °C. The RNA synthesized in the reactions was isolated and digested, and the 5′-terminal oligonucleotides were subjected to chromatography, autoradiography, and quantitation as described in Miller & Burgess (1978a).

Results and Discussion

Fingerprints of the 5′-triphosphate terminal sequences of RNAs made in vitro using T7 and λcb2 DNA as template and digested with T1 RNase show different patterns of spots. It has been demonstrated that many of these spots represent the 5′-triphosphate oligonucleotides of RNAs initiated at specific promoters (Miller & Burgess, 1978b). Schematic diagrams of fingerprint patterns of RNAs from λcb2 and T7 labeled with [γ-^{32}P]ATP and digested with T1 RNase are given in Figures 1A and 3A of the preceding paper (Miller & Burgess, 1978b). In the following discussion the 5′ termini will be presented as follows: for oligonucleotides from RNAs transcribed from T7 DNA, spot 1 represents the 5′-terminal oligonucleotide from RNA initiated at the A3 promoter, spot 2 is from the A1 promoter, and spots 3, 4, and 5 may represent minor initiations or aborted initiations (Miller & Burgess, 1978b). For oligonucleotides of RNAs transcribed from λcb2 DNA, spot 1 is from RNA initiated at the P_L promoter. Spot 2 represents the 5′ terminus of RNA initiated at the P_R promoter. Spot 5 is from the P_R′ promoter. Spots 4, 7, and 8 are thought to arise from abortive initiation, and the origins of spots 3 and 4 are unknown. In this study RNA is labeled with both [γ-^{32}P]ATP and [γ-^{32}P]GTP and digested with T1 RNase. All GTP-initiated oligonucleotides appear as pppGp which migrates midway between the origin and a GTP marker.

Time Course. Figure 1 shows the incorporation of [^{3}H]UTP and [γ-^{32}P]ATP and [γ-^{32}P]GTP with time at 0.15 M KCl using T7 DNA as the template. Total incorporation of [γ-^{32}P]ATP and GTP plateaus after 10 min, whereas [^{3}H]UTP is still being incorporated at 20 min (see Figure 1A).

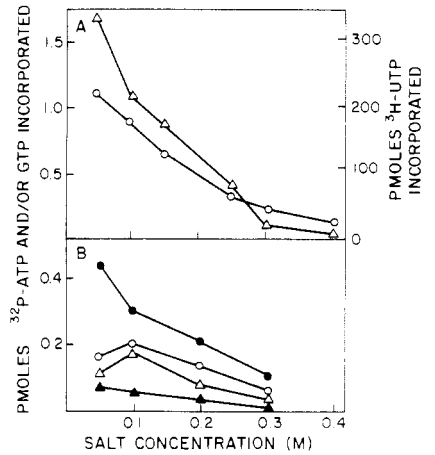

FIGURE 2: Effect of salt concentration on transcription on λcb2 DNA. (A) Effect of salt on initiation and total synthesis. Six standard reactions were carried out as described in Experimental Procedures but at the indicated salt concentrations. Specific activities were 330 dpm/pmol for [^{3}H]UTP and 15 000 dpm/pmol for [γ-^{32}P]ATP. (Δ—Δ) Picomoles of [^{3}H]UTP incorporated; (O—O) pmol of [γ-^{32}P]ATP incorporated. (B) Effect of salt on initiations at individual promoters. Reactions were run at four salt concentrations and 5′-terminal oligonucleotides were analyzed as described in Experimental Procedures. The specific activity of [γ-^{32}P]ATP was 15 000 dpm/pmol. (●—●) Spot 1, P_L promoter; (O—O) spot 4, unidentified start; (Δ—Δ) spot 5, P_R' promoter; (▲—▲) spot 6, minor spot.

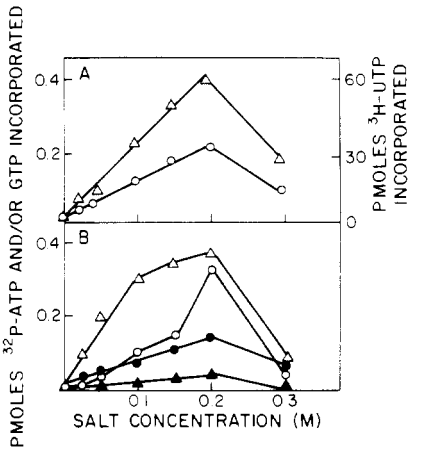

FIGURE 3: Effect of salt concentration on transcription of T7 DNA. (A) Effect of salt on initiation and total synthesis. Specific activities were 330 dpm/pmol for [^{3}H]UTP and 12 000 dpm/pmol each for [γ-^{32}P]ATP and GTP. (Δ—Δ) Picomoles of [^{3}H]UTP incorporated; (O—O) pmol of [γ-^{32}P]ATP amd GTP incorporated. (B) Effect of salt on initiations at individual promoters. (●—●) Spot 1, A3 promoter; (O—O) spot 2, A1 promoter; (Δ—Δ) pppGp, A2 promoter; (▲—▲) spot 5, minor spot.

Initiations at specific promoters were measured by isolating the RNA from the reaction mixture, digesting it with T1 RNase, and fractionating it by PEI-cellulose thin-layer chromatography. Figure 1B shows the incorporation of [γ-^{32}P]ATP or GTP into 5′-terminal oligonucleotides as a function of time of incorporation. Under the standard conditions of synthesis between one-third and one-half of the total initiation observed at 20 min occurs within the first minute. All spots on the chromatogram were present in RNA synthesized in the first few minutes and all spots became more labeled with longer incorporation times. Thus there was no evidence for any late starting promoters or promoters which initiated only once. A 10-min time point was chosen for subsequent experiments on the basis of this experiment.

Effects of Salt. Variations in the concentration of KCl or NaCl have a striking effect on transcription at the level of binding (Hinkle & Chamberlin, 1972; Seeburg & Schaller, 1975; Seeburg et al., 1977), initiation (So et al., 1967; Schäfer & Zillig, 1973; Mangel & Chamberlin, 1974b), elongation (Bremer, 1970), and termination and reinitiation (Millette et al., 1970; Maitra et al., 1970).

To determine the effect of salt on total RNA synthesis and initiation, the incorporation of [^{3}H]UTP and [γ-^{32}P]NTP into Cl_3CCOOH-precipitable counts was measured at different salt concentrations. To determine the effect of salt on initiation of RNA synthesis at individual promoters, RNA synthesized at different KCl concentrations was labeled with [γ-^{32}P]ATP and [γ-^{32}P]GTP, isolated, digested, and subjected to chromatography, autoradiography, and quantitation.

(a) λcb2. As the KCl concentration increases the amount of total RNA synthesis and initiation decreases (Figure 2A). Different promoters appear to vary somewhat in their sensitivity to salt (Figure 2B). Spot 1 decreases dramatically with increasing salt whereas spot 5 shows a peak at 0.1 M KCl. Spot 6 is representative of the minor spots, decreasing with increasing salt but not so dramatically as the decrease observed in spot 1. GTP-initiated starts were not determined in this experiment. The fingerprints of 5′-terminal oligonucleotides from RNA transcribed from λcb2 DNA at high and low salt were similar, with no new spots appearing at low salt. Therefore, it does not appear that low salt enhances nonselective initiation.

(b) T7. Unlike λcb2 DNA, T7 DNA is transcribed maximally at a salt concentration of 0.2 M KCl (Figure 3A). Initiations at individual promoters are stimulated at 0.2 M salt although to different extents (Figure 3B). Spot 2 is greatly stimulated at high salt and becomes the main ATP-initiated transcript. This is in agreement with Iida & Matsuge (1974) who observed a preference for this starting sequence when T7 was transcribed at 0.2 M KCl.

Chamberlin & Ring (1972) observed that a decrease in the number of ATP- and GTP-initiated chains occurred at NaCl concentrations greater than 0.1 M in a 2-min reaction at 27 °C. However, when reinitiation was allowed to occur, a stimulation of synthesis of ATP-initiated chains was observed at high salt, suggesting efficient recycling of polymerase to promoters initiating with ATP. No corresponding increase in GTP-initiated chains was observed. Therefore, the stimulation of synthesis at 0.2 M KCl observed here may be due to reinitiation which occurs at high salt and not at low salt. The differences observed between the results presented here with GTP-initiated RNA synthesis in response to increases in salt concentration and those obtained by Chamberlin & Ring may be due to the fact that these experiments were carried out at 37 °C and theirs at 27 °C.

Nonselective initiation, manifest as the appearance of new 5′-terminal oligonucleotide spots, was not observed at low salt when T7 DNA was used as a template.

Dausse et al. (1976) have investigated the effect of salt on the utilization of the three early T7 promoters by fractionation on polyacrylamide gels of the three 5′-terminal fragments generated by cleavage of T7 RNA with RNase III. Their findings suggested that the A1, A2, and A3 promoters were dramatically different in their response to salt. However, problems with incomplete cleavage of RNA at high salt and the analysis of data as percentage of total recovery make those data difficult to interpret and compare with ours.

Effects of Temperature. Temperature appears to have a major effect in determining whether initiation will occur at a particular promoter complex. A cooperative transition from

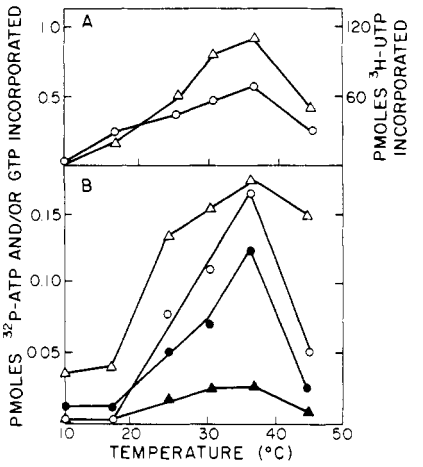

FIGURE 4: Effect of temperature on transcription of T7 DNA. Transcription was carried out under standard conditions except that 0.17 M KCl was used and the temperature of the preincubation and transcription was varied as indicated. Specific activities of [γ-^{32}P]ATP and GTP were 20 000 dpm/pmol. The results are the average of two experiments. (A) Effect of temperature on initiation and total synthesis. (Δ—Δ) Picomoles of [^{3}H]UTP incorporated; (O—O) pmol of [γ-^{32}P]ATP and GTP incorporated. (B) Effect of temperature on initiations at individual promoters. (●—●) Spot 1, A3 promoter; (O—O) spot 2, A1 promoter; (Δ—Δ) pppGp, A2 promoter; (▲—▲) spot 4, minor spot.

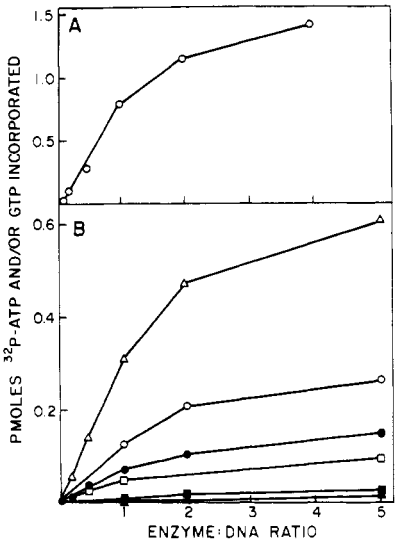

FIGURE 5: Effect of enzyme to DNA weight ratio on transcription of T7 DNA. Specific activities of [γ-^{32}P]ATP and GTP were 15 000 and 20 000 dpm/pmol, respectively. The results are the average of two experiments. (A) Effect of enzyme to DNA ratio on initiation as measured by [γ-^{32}P]ATP plus GTP incorporation (O—O). (B) Effect of enzyme to DNA ratio on initiations at individual promoters. (●—●) Spot 1, A3 promoter; (O—O) spot 2, A1 promoter; (Δ—Δ) pppGp, A2 promoter; (▲—▲) spot 3, minor spot; (□—□) spot 4, minor spot; (■—■) spot 5, minor spot.

a closed promoter complex to an open promoter complex occurs at 18 °C with T7 promoters at low salt. At temperatures above this, an equilibrium in favor of open complex formation is established. When the temperature is lowered the closed complex may be favored (Hinkle & Chamberlin, 1972; Mangel & Chamberlin, 1974c; Seeburg & Schaller, 1975). The transition temperature of a particular promoter is a measure of the ability of that promoter to melt out and initiate RNA synthesis. The transition temperature of a promoter is affected by many parameters including the configuration of the DNA (Richardson, 1975), the concentration of ions (Nakanishi et al., 1975), organic solvents (Nakanishi et al., 1974), and the source of the RNA polymerase (Baralle & Travers, 1976). Therefore, the effects of temperature cannot be viewed independently of the other conditions of transcription.

At 0.17 KCl, initiation and total RNA synthesis on T7 DNA occur maximally at 37 °C (Figure 4A). The data shown in Figure 4B suggest that initiation at individual promoters also occurs maximally at 37 °C. When the transition temperatures are determined from Figure 4B, promoters A1 and A3 show transition temperatures of about 27 and 28 °C, respectively. The chains initiating with GTP show a transition temperature of 22 °C. A rather unexpected result was the dramatic decrease in initiation, especially ATP initiations, observed at temperatures greater than the temperature of maximum initiation. This decrease in synthesis may reflect a change in the polymerase induced by the temperature, but it is difficult to separate effects of temperature on the DNA from effects on the polymerase. However, it is clear that GTP initiations are much less affected by high temperature (45 °C) and thus that the decrease in ATP initiations is not just due to decreased enzyme activity.

The results described here suggest that A1 and A3 promoters behave similarly in response to temperature and differ from A2. Dausse et al. (1976) found the transition temperatures of three sites to be markedly different, A3 being used predominantly at low temperature with A1 and A2 being activated as the temperature increased. In addition they also found that the temperature effects were dependent on the salt concentration. The data in that work are difficult to interpret as described above. Our preliminary evidence (data not shown) confirms that the transition temperatures of individual promoters are affected by salt concentration. In order to understand more fully the effect of salt and temperature on promoter site selectivity and initiation, a series of experiments should be carried out in which temperature curves are determined at several salts and salt curves determined at several temperatures.

Effect of Enzyme to DNA Ratio. Promoters may differ in their affinity for RNA polymerase. If polymerase were limiting, only promoters with a high affinity for the enzyme would be able to initiate. Increasing the polymerase concentration would then enable promoters with a lesser affinity to initiate transcription (see Stahl & Chamberlin, 1977). If enzyme were available in great excess over the number of promoters on the template, nonselective initiations might occur; however, no new species of RNA have been detected with increasing concentrations of enzyme (Takanami et al., 1970; Brody et al., 1970; Chamberlin & Ring, 1972).

When T7 DNA is transcribed with increasing amounts of RNA polymerase holoenzyme, both the total initiations (Figure 5A) and the initiations at individual promoters (Figure 5B) increase. No new spots appeared at high enzyme levels, suggesting that no detectable nonselective initiations were occurring. Initiation increases somewhat even after enough polymerase has been added to saturate all known promoters, since under these conditions repeated initiation can occur at a single promoter during the 10-min reaction. It is also clear that not all of the polymerase is able to initiate since at a weight ratio of enzyme to DNA of 1, which corresponds to a molar ratio of about 60, only 4–5 initiations are occurring per DNA molecule.

Stability of Complexes as Measured by Decay in the Presence of Heparin. Heparin is a polyanion which binds to the β' subunit of polymerase and competes with the DNA for a template binding site on the polymerase before the open complex has been formed (Zillig et al., 1970; Walter et al., 1967). While heparin will bind and inactivate free enzyme and by displacement will dissociate polymerase which is weakly

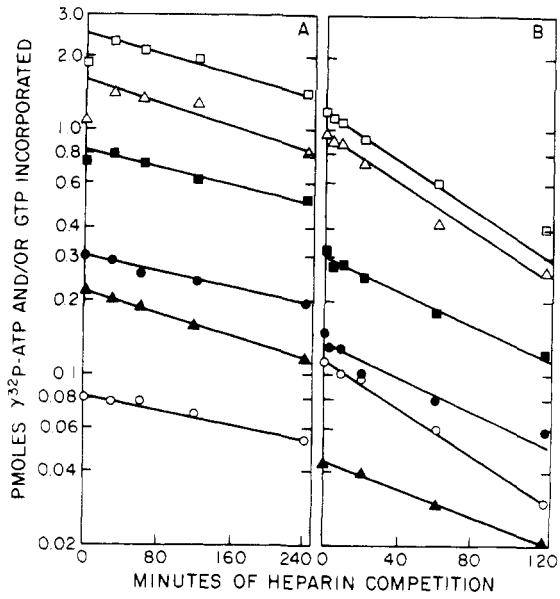

FIGURE 6: Time course of inactivation of RNA polymerase by heparin. RNA synthesis at various times after additon of heparin was carried out as described in Experimental Procedures. (A) λcb2 5′-triphosphate oligonucleotides. (□—□) Total starts (ATP + GTP, $t_{1/2}$ = 4.9 h; (△—△) [γ-^{32}P]GTP starts, $t_{1/2}$ = 4.2 h; (■—■) total [γ-^{32}P]ATP starts, $t_{1/2}$ = 5.6 h; (●—●) spot 1 (P_L), $t_{1/2}$ = 6.3 h; (▲—▲) spot 5 (P_R', $t_{1/2}$ = 4.4 h; (○—○) spot 4 (minor start), $t_{1/2}$ = 6.4 h. (B) T7 5′-triphosphate oligonucleotides. (□—□) Total starts (ATP + GTP), $t_{1/2}$ = 1.0 h; (△—△) [γ-^{32}P]GTP starts, $t_{1/2}$ = 1.0 h; (■—■) total [γ-^{32}P]ATP starts, $t_{1/2}$ = 1.5 h; (●—●) spot 1 (A3), $t_{1/2}$ = 1.4 h; (○—○) spot 2 (A1), $t_{1/2}$ = 1.0 h; (▲—▲) spot 4, $t_{1/2}$ = 1.7.

bound to the DNA (Zillig et al., 1970; Schäfer & Zillig, 1973), heparin will also attack and dissociate polymerase bound in open complexes. The rate of attack at different promoters varies (Pfeffer et al., 1977). Heparin was added to a standard reaction mixture in the absence of nucleoside triphosphates as described in Experimental Procedures. As the complex is attacked by heparin it is unable to initiate when the nucleotides are added. These experiments measure the sensitivity of each promoter complex to heparin attack rather than the stability of the open complex.

Figure 6A shows the rate of heparin attack on λcb2 promoter complexes initiating with ATP and GTP. Figure 6B shows the rate of T7 heparin attack on promoter complexes initiating with ATP. The T7 A3 promoter (spot 1) appears to be more stable than the A1 promoter (spot 2). The individual promoter complexes formed on λcb2 DNA also show some variation in their stability. P_L appear to be more stable than P_R'. Considerable variation in the stability of promoter complexes to heparin attack has also been shown by Seeburg et al. (1977) and by Stahl & Chamberlin (1977). λcb2 promoters appear to be about four times more stable than T7 promoters under these conditions. It is not clear how promoter stability relates to the ability to start. In terms of total number of initiations and total incorporation of labeled nucleotides, T7 DNA can be considered a better template for in vitro RNA transcription with *E. coli* RNA polymerase than λcb2 DNA. If the polymerase is bound very tightly, it may not move away from the promoter region as readily as a polymerase bound less tightly and therefore less stably. Although the intrinsic rate of initiation does not appear to vary with salt and temperature (Mangel & Chamberlin, 1974a-c), it may vary between promoters. P_R' is a very strong promoter in vitro (Dalberg & Blattner, 1973; Rosenberg et al., 1975) and yet it appears to be less stable to heparin challenge than P_L.

Conclusions

Experiments which involve altering the conditions of transcription and examining the effects of these alterations on transcription allow one to define conditions which affect the selectivity of initiation and to determine the variables which influence promoter strength. Previous studies on gross transcription and initiation have demonstrated that protein factors, salt, divalent cations, temperature, and DNA configuration are important in determining whether RNA synthesis can occur. But to come to any precise understanding, individual promoters must be examined systematically under a wide variety of conditions. The experiments described in this paper illustrate the use of our newly developed method of 5′-terminal oligonucleotide analysis for the determination of the effects of reaction conditions on initiation of transcription at individual promoters.

When this work was started it was assumed that each initiation at a promoter would lead to the synthesis of a particular 5′-terminal end and that quantitation of the corresponding 5′-terminal oligonucletide would allow accurate quantitation of initiation at that promoter. It is now known and has been discussed in the preceding paper that abortive initiation can occur, leading to the termination after formation of the first phosphodiester bond, and that ambiguous starting can occur one base before the normal start site at some promoters. It is presently not known whether these reactions can occur at all promoters or precisely how they are affected by variations in reaction conditions, but it is clear that each reaction would cause underestimation of the number of initiations we measure at a particular promoter. Until the generality and characteristics of these reactions become known, the method we describe for 5′-terminal oligonucleotide analysis will probably be more useful for qualitative than for precise quantitative studies of initiation. We can, nevertheless, conclude from the studies presented here that while individual promoters may respond differently to changes in salt concentration, temperature, enzyme to DNA ratio, and to the presence of heparin, no dramatic changes in selectivity of initiation are observed as a result of these changes.

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Enzymatic Oligoribonucleotide Synthesis with T4 RNA Ligase†

Thomas E. England and Olke C. Uhlenbeck*

ABSTRACT: The substrate specificity of T4 RNA ligase has been examined to determine whether the intermolecular reaction is sufficiently general to realize its potential in the enzymatic synthesis of oligoribonucleotides of defined sequence. Reactions between a variety of acceptor molecules with 3′- and 5′-hydroxyl groups and donor molecules with 3′- and 5′-phosphates indicate that the minimal substrates are a trinucleoside diphosphate acceptor and a nucleoside 3′,5′-bisphosphate donor. Increasing the chain length of either the acceptor or donor has little effect on the rate or extent of reaction. Although the base composition of the donor has only a small effect on the reaction rate, the presence of uridine in the acceptor greatly reduces the amount of product formed. The presence of a phosphate on the 3′ terminus of the donor molecule permits a unique intermolecular product with a 5′-hydroxyl and a 3′-phosphate. By enzymatically either adding a 5′-phosphate or removing the 3′-phosphate, a new donor or acceptor is prepared so synthesis of an oligomer chain can proceed in either direction. With the simplicity of this enzymatic pathway and the rather broad substrate specificity of T4 RNA ligase, a convenient method for the synthesis of oligoribonucleotides is established.

RNA ligase isolated from T4-infected *E. coli* catalyzes the ATP-dependent formation of a phosphodiester bond between terminal 5′-phosphates and 3′-hydroxyls of oligoribonucleotides (Silber et al., 1972; Walker et al., 1975; Kaufmann & Kallenbach, 1975). Although intramolecular cyclization can occur, intermolecular joining of an acceptor molecule with a 3′-hydroxyl and a donor molecule with a 5′-phosphate is achieved either by using a donor too short to cyclize (Walker et al., 1975) or by protecting the 3′-hydroxyl of the donor molecule (Sninsky et al., 1976; Uhlenbeck & Cameron, 1977). The intermolecular reaction proceeds through the formation of a covalent enzyme-AMP complex (Cranston et al., 1974) and the transfer of the AMP to the 5′-phosphate of the donor to form an adenylylated oligonucleotide (Ohtsuka et al., 1976; Sninsky et al., 1976). Under favorable conditions a majority of the adenylylated donor is tranferred to the acceptor to form the intermolecular product. In one case where the reaction was studied in detail, relatively high concentrations of two oligomers could be joined in excellent yield with modest amounts of enzyme (Uhlenbeck & Cameron, 1977). Thus, the intermolecular reaction of T4 RNA ligase would appear to be of great importance for the enzymatic synthesis of oligoribonucleotides of defined sequence.

In this work we will examine the substrate specificity of

† From the Department of Biochemistry, University of Illinois, Urbana, Illinois 61801. *Received December 5, 1977.* This work was supported by a grant from the National Institutes of Health (GM 19059).

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