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Cycling of Ribonucleic Acid Polymerase To Produce Oligonucleotides during Initiation in Vitro at the *lac* UV5 Promoter†

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ABSTRACT: High-resolution gel electrophoresis has been used to detect and quantitate promoter-specific oligonucleotides produced during initiation of transcription in vitro at the lactose operon (*lac*) UV5 promoter. The resolved products are RNA species of various lengths which correspond to the initial *lac* mRNA sequence. Quantitation shows that many oligonucleotides can be formed per preinitiation complex, including species as long as hexanucleotide. Synthesis occurs without dissociation of the enzyme, as evidenced by levels of synthesis in the presence of heparin, a selective inhibitor of free RNA polymerase. Thus, RNA polymerase cycles at this promoter in vitro producing oligonucleotides reiteratively. In general, the yield of oligonucleotides decreases when the total concentration of all four substrates is increased or when a missing nucleoside triphosphate substrate is added. Nevertheless, oligonucleotide synthesis persists under all conditions tested. Strikingly, the dinucleotide always represents 50% of the total of all oligonucleotides, even when conditions are manipulated to cause a 100-fold variation in this total. This shows that, after formation of the first phosphodiester bond at the *lac* UV5 promoter, dissociation of the dinucleotide is as likely as formation of the second phosphodiester bond. As discussed above, after release of a small RNA, RNA polymerase may then begin another RNA chain, which is again subject to premature release. These considerations lead to a model in which RNA polymerase cycles to produce oligonucleotides during initiation of transcription at the *lac* UV5 promoter in vitro. Production of a long RNA transcript is then essentially an escape from this cycling reaction. The drug rifampicin, which drastically inhibits escape to produce RNA, limits, but does not prevent, the cycling reaction.

Production of RNA by the DNA-dependent RNA polymerase is accomplished in a series of steps. These include formation of a promoter-specific preinitiation complex between enzyme and DNA, conversion to an elongation complex, and termination of transcription (for a review, see Chamberlin, 1976a). The rate and mechanism of each of these steps must be determined in order to understand the factors that may contribute the overall yield of RNA over a wide range of conditions.

The conversion of preinitiation complexes to elongation complexes, often termed "initiation", has been studied using a variety of assays which attempt to isolate this step from others along the pathway. Mangel & Chamberlin (1974) used an assay that relies on the properties of the inhibitor rifampicin. This assay relied on an assumed relationship between the mechanism of rifampicin inhibition and the mechanism of initiation. This assumption has now been shown to be at the least an oversimplification (McClure et al., 1978; McClure & Cech, 1978).

An alternative to this method involves limiting the reaction to the formation of a single phosphodiester bond by exclusion of appropriate nucleoside triphosphates (Johnson & McClure, 1976). This "abortive initiation" reaction leads to reiterative synthesis of dinucleoside tetraphosphate. However, the initiation rate and mechanism determined may not accurately reflect the productive initiation of transcript, since artificial steady-state conditions have been imposed by the absence of substrates required for conversion to elongation complexes.

A third assay involves observation of the rate of acid-precipitable RNA production after addition of nucleoside triphosphates to preinitiation complexes (Stefano & Gralla, 1979; Nierman and Chamberlin, 1979). Such rates, however, can be very rapid, so the utility of this assay is restricted to in vitro systems or conditions where initiation is a relatively slow process.

Thus, in part because of these uncertainties and limitations, the mechanism of the initiation process remains unsettled. An important complication is the recent observation that formation of the first phosphodiester bond to produce dinucleoside tetraphosphate is not always followed by extension to complete RNA transcript. Specifically, McClure & Cech, (1978) have shown that under continuous transcription conditions (all four nucleoside triphosphates present, and no inhibitors added) approximately 2 mol of the promoter-specific oligonucleotide pppApApC accumulates per mol of long-chain RNA from the $\lambda P_{R'}$ promoter. Thus, for this promoter the mechanism of conversion of preinitiation complexes to elongation complexes is more complicated than anticipated.

A systematic and quantitative investigation of such potential oligonucleotide products of transcription has not been reported.

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In part, this may be due to the low resolving power of the detection techniques used, paper chromatography (McClure et al., 1978) and thin-layer chromatography (Nierman and Chamberlin, 1979). Oligonucleotide products may have escaped detection for this reason or because they were eliminated prior to detection by the protocol used. This latter reason applies most often to studies in which the intent was to study long-chain RNA; small products are usually eliminated from in vitro transcription reactions by precipitating the long RNA with alcohol prior to detection and quantitation.

We have attempted to obviate these difficulties by loading reaction mixtures directly on a high-resolution gel electrophoresis system to allow detection of any oligonucleotides produced. This technique reveals that initiation of transcription at the *lac*[1] UV5 promoter is indeed accompanied by the formation of oligonucleotides. Such experiments lead to a model for initiation at this promoter, which includes a pathway involving cycling of RNA polymerase to produce oligonucleotide products.

Materials and Methods

Nucleoside triphosphates, heparin, and rifampicin were purchased from Sigma. $H_3{}^{32}PO_4$, HCl free, was from ICN. All other chemicals were reagent grade or better. *Escherichia coli* RNA polymerase was prepared and assayed according to the method of Burgess & Jendrisak (1975). This preparation catalyzes promoter-specific transcription, as judged by criteria described previously (Stefano & Gralla, 1979).

Isolation of the lac UV5 DNA. The 203-base-pair *lac* UV5 DNA was isolated from a derivative of pBR322 (Bolivar et al., 1977) that contained two copies of the *lac* DNA per plasmid (A. J. Carpousis and J. D. Gralla, unpublished experiments). A 3.0-L culture of cells was grown to late log phase, and plasmid replication was amplified by treatment with 150 μg/mL chloramphenicol overnight. Cells were harvested and a cleared lysate was prepared as follows. The cells were resuspended in 100 mL of ice-cold 25% sucrose–50 mM Tris-HCl, pH 8; 15 mL of 10 mg/mL lysozyme (in water) was added, and the mixture was incubated on ice for 5.0 min. NaEDTA, pH 8 (15 mL, 0.5 M), was added, and the mixture was incubated for another 5.0 min on ice. The cells were lysed by addition of 100 mL of ice-cold 50 mM Tris-HCl, pH 8, 100 mM NaEDTA, and 0.2% Triton X-100. The lysates were centrifuged in a Beckman SW 27 rotor at 27 000 rpm and 4 °C for 30 min. The nonviscous portion of the supernatant (cleared lysate) was decanted carefully.

The cleared lysate was treated with RNase and Pronase as described previously (Stefano & Gralla, 1979). Protein was removed by two extractions with aqueous phenol followed by two extractions with chloroform–isoamyl alcohol (24:1). The nucleic acids were precipitated by making 1 M with ammonium acetate, adding 2 volumes of ethanol, and storing at −20 °C overnight.

Ribooligonucleotides were separated from plasmid by chromatography on a 400-mL Sepharose-6B column (eluted with 10 mM Tris-HCl, pH 8, and 1 mM NaEDTA). The plasmid, which eluted in the void volume, was made 1 M with ammonium acetate and 10 mM with magnesium acetate, and precipitated with 2 volumes of ethanol. The *lac* UV5 DNA was excised from the plasmid by treatment with *EcoRI* as described previously (Stefano & Gralla, 1979). The DNA was ethanol precipitated as described above and resuspended in 3.0 mL of 10 mM Tris-HCl, pH 8, and 1 mM NaEDTA. The 203-base-pair *lac* UV5 DNA was separated from the 4400-base-pair plasmid DNA by velocity sedimentation on sucrose gradients (J. McGhee and G. Felsenfeld, personal communication). Briefly, 0.5-mL aliquots of the *EcoRI* cut DNA were loaded on 12.0-mL 5–20% sucrose gradients (in 10 mM Tris-HCl, pH 8, and 1 mM NaEDTA) and centrifuged in a Beckman SW41 rotor at 33 000 rpm and 24 °C for 18 h. The gradients were fractioned, and the *lac* UV5 DNA, which sedimented about halfway down the gradient, was located by its absorbance at 260 nm. The resolution of this technique decreases as the amount of DNA loaded on the gradient is increased. In this preparation about 0.3 mg of DNA was loaded on each gradient.

Potential contamination of the *lac* UV5 DNA by traces of ribooligonucleotides or plasmid DNA was eliminated by chromatography on DEAE-cellulose (Whatman DE-52). The peak fractions from the sucrose gradients were applied directly to a 5.0-mL DEAE column equilibrated with 0.1 M Tris-acetate, pH 8.5, 0.1 M ammonium acetate, and 1.0 mM NaEDTA. A 50-mL 0.1 to 2.0 M ammonium acetate gradient (containing 0.1 M Tris-acetate, pH 8.5, and 1.0 mM NaEDTA) was run, and the 203-base-pair *lac* UV5 DNA which eluted at about 1.0 M ammonium acetate was located by absorbance at 260 nm. The peak fractions were pooled and precipitated with ethanol as described above (additional ammonium acetate was omitted), and the DNA was resuspended in 2.0 mL of 10 mM Tris-HCl, pH 8, and 1 mM NaEDTA. The concentration of the purified DNA was measured by the absorbance at 260 nm. The final yield was about 100 μg (730 pmol).

Purification and Synthesis of Nucleotides. For experiments involving quantitation of oligonucleotides, ATP, UTP, and GTP were further purified to remove any traces of cross-contaminating nucleoside triphosphates and diphosphates. Purification was essentially as described by McClure et al. (1978) with the following modifications. A 0.9 × 42 cm AGl-X2 (formate) anion exchange column was used. The column was equilibrated with 0.5 M ammonium formate, pH 4.0, and a 260-mL 0.5 to 2.5 M ammonium formate, pH 4.0, gradient was started immediately after the sample was applied. In the DEAE-Sephadex step, a 0.1 to 1.0 M triethylammonium bicarbonate, pH 8, gradient was used. The nucleotides were converted to the sodium salt as described, and the concentrations were determined from the observed absorbance and known extinction coefficients.

[α-^{32}P]ATP (100–300 Ci/mmol) was synthesized enzymatically as described by Reeve & Huang (1979). About 5 mCi of $H_3{}^{32}PO_4$ was used per synthesis (instead of 25 mCi), and reaction volumes were reduced proportionally. Products at each step in the synthesis were analyzed by thin-layer chromatography on poly(ethylenimine)-cellulose (E. Merck) developed with 0.25 M KH_2PO_4. In later preparations, the synthesis was modified for the sake of simplicity. [γ-^{32}P]ATP was synthesized in the first step instead of [γ-^{32}P]dATP. The steps leading to the synthesis of [α-^{32}P]AMP were performed as described by Reeve & Huang (1979). The [α-^{32}P]AMP was converted to [α-^{32}P]ATP with a mixture of myokinase and pyruvate kinase (Symons, 1974). The reaction mixture, which already contained unlabeled ADP (about 200 μM) from the previous steps, was adjusted to the following concentrations: 150 mM Tris-HCl, pH 8, 40 mM KCl, 7 mM $MgCl_2$, 1 mM NaEDTA, and 4 mM potassium phosphoenolpyruvate. About 2 units of myokinase (Sigma, rabbit muscle, ammonium sulfate

[1] Abbreviations used: *lac*, lactose operon; Tris,, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; RNase, ribonuclease; DEAE, diethylaminoethyl; DTT, dithiothreitol; *gal*, galactose operon; *trp*, tryptophan operon; *ara*, arabinose operon; *bio*, biotin operon.

suspension) and 1 unit of pyruvate kinase (Sigma, rabbit muscle, ammonium sulfate suspension) were added. Incubation at 37 °C gave a rapid (less than 20 min) and quantitative (greater than 99%) conversion of [α-^{32}P]AMP to [α-^{32}P]ATP. The exclusion of dATP from the synthesis made boronated-polyacrylamide purification of the [α-^{32}P]ATP unnecessary. The use of myokinase in the last step facilitated the conversion of the [α-^{32}P]AMP to [α-^{32}P]ATP without leading to a large dilution in specific activity.

The reaction mixture was diluted into 0.5 mL of 20 mM triethylammonium bicarbonate, pH 8, and applied to a 4.0-mL DEAE-Sephadex column equilibrated with 0.1 M triethylammonium bicarbonate, pH 8. The column was eluted with a 20-mL 0.1 to 1.0 M triethylammonium bicarbonate, pH 8, gradient followed by 10 mL of 1.0 M triethylammonium bicarbonate, pH 8. The peak [α-^{32}P]ATP fractions, which eluted at the end of the gradient, were pooled and lyophilized until the triethylammonium bicarbonate was removed completely. The label was resuspended in 1 to 2 mL of 50% ethanol and stored at −20 °C. Thin-layer chromatography (as described above) indicated that greater than 98% of the ^{32}P was in the [α-^{32}P]ATP. ATP purified and stored in this manner remained chemically stable and enzymatically active for several weeks.

Transcription, Electrophoresis, and Counting. All the reactions in this paper used the following standard salts: 30 mM Tris-HCl, pH 8, 100 mM KCl, 3 mM $MgCl_2$, 0.1 mM EDTA, and 0.2 mM DTT. ATP was added as an equimolar mixture of $MgCl_2$ and ATP, raising the final $MgCl_2$ concentration. RNA polymerase–DNA complexes were formed by a 10.0-min incubation at 37 °C with 1.6 pmol of *lac* UV5 DNA and 4.0 pmol of RNA polymerase in 6.0 μL of standard salts plus 0.1 mg/mL bovine serum albumin acetylated according to the method of Gonzalez et al (1977). At this time 2.0 μL of standard salts (control), standard salts plus 1.2 mg/mL heparin, or standard salts plus 300 mM rifampicin were added, and the mixture was incubated another 5.0 min. The reaction was started by addition of the nucleoside triphosphates ([α-^{32}P]ATP diluted approximately 300-fold to 0.1 to 1.0 Ci/mmol) in 4.0 μL of standard salts. After 10.0 min at 37 °C, the reaction was stopped by the addition of 3.0 μL of 0.25 M NaEDTA, 0.25% xylene cyanol, and 0.25% bromophenol blue.

Products were analyzed by electrophoresis on 25% acrylamide (including 1:29 methylenebis(acrylamide)), 7 M urea, 50 mM Tris borate, pH 8, and 1 mM NaEDTA gels (0.05 × 20 × 40 cm). Water saturated with urea (5 μL) was added to the reaction mixture, and 10.0 μL was loaded directly onto the gel. Electrophoresis was at 1000 V for 8 to 10 h. By this time, the fast moving dye moved approximately 20 cm from the sample well. The gels were autoradiographed on Cronex 4 X-ray film (Du Pont). The bands of interest were excised and counted by Cerenkov radiation. The Cerenkov countng efficiency was determined as follows. A standard radioactive gel slice was first counted Cerenkov and then soaked in buffer until greater than 95% of the radioactivity was eluted. The eluted ^{32}P was determined by liquid scintillation counting assuming 100% efficiency for ^{32}P. Cerenkov counting efficiency of the original gel slice was then calculated to be 35%. The number of picomoles of [^{32}P]ATP incorporated was calculated using this 35% efficiency and the known specific activity of ATP.

Results

The 5′ sequence of the predominant in vitro mRNA from the *lac* UV5 promoter is pppApApUpUpGpUpGpApGpC (Maizels, 1973). Promoter-specific transcription from a DNA restriction fragment containing only this promoter has been demonstrated in vitro (Majors, 1975; Stefano & Gralla, 1979; Maquat & Reznikoff, 1978). The UV5 mutation allows transcription to proceed in the absence of active catabolite activator protein in vivo (Silverstone et al., 1970) and in vitro (see above references). A great deal of information concerning the molecular topography of *E. coli* RNA polymerase–promoter interactions derives from study of this promoter (Johnsrud, 1978; Simpson, 1979; Schmitz & Galas, 1979). The rate of productive initiation of transcript has also been studied (Stefano & Gralla, 1979). Therefore, we searched for potential oligonucleotide products of transcription initiation using this very well-characterized template of purified *lac* UV5 DNA fragment.

Detection of Complementary Oligonucleotides. We adapted the recent application of high-resolution gel electrophoresis for nuclei acid sequencing (Maxam & Gilbert, 1977; Sanger & Coulson, 1978; Donis-Keller et al., 1977) to the detection of oligonucleotides formed during the initiation phase of RNA synthesis. Figure 1A shows that when ATP alone is added to a preinitiation complex, a predominant radioactive RNA species is resolved on the gel autoradiogram (lane b). The band has the mobility appropriate for the expected product pppApA (see above 5′-RNA sequence), slightly slower moving than the radioactive ATP. Addition of ATP and UTP together stimulates the production of two longer prominent radioactive species (Figure 1, lane c). These slower moving bands have mobilities appropriate for the expected products pppApApU and pppApApUpU. These identifications were confirmed by repeating the experiments using both [α-^{32}P]ATP and [^{3}H]-UTP as substrates (Figure 1, lane d). The ^{3}H/^{32}P ratio was approximately 2.2 times as high for the product identified as pppApApUpU as for the product identified as pppApApU, nearly the expected ratio of 2. Negligible tritium was incorporated into the band identified as pppApA. Thus, we conclude that the electrophoresis system is capable of resolving the detecting oligonucleotides produced at the *lac* UV5 promoter.

Quantitation of Oligonucleotides. Next, we used this method to quantitate any oligonucleotides formed during a 10-min reaction in the presence of various concentrations and combinations of ATP, UTP, GTP, and CTP. Various reaction mixtures were loaded directly onto the acrylamide gel and subjected to electrophoresis, and an autoradiogram was developed (Figure 1B). Notice that inclusion of nucleoside triphosphates excluded above stimulates the appearance of longer oligonucleotides (lanes i–l). Radioactive bands were excised from the gel and counted directly to determine the abundance of various oligonucleotides. Table I shows the amount of ATP incorporated into each species under varying conditions.

Table I shows that many species are produced in vast molar abundance compared to the amount of DNA present, which is only 1.6 pmol per reaction. It is possible that this overproduction is due in part to a reaction pathway involving premature termination, dissociation of RNA polymerase from the DNA template, and reassociation followed by reinitiation. We eliminated any possible contribution from such a pathway by repeating the experiment shown in Figure 1 in the presence of heparin, an inhibitor of free, but not DNA-bound, RNA polymerase (for a discussion, see Chamberlin, 1976b). Table I shows that even in the presence of heparin (data shown in parentheses), oligonucleotides are produced in great molar excess over the amount of DNA template present. Thus, RNA polymerase can produce complementary oligonucleotides reiteratively *without* dissociating from the DNA, as evidenced

Table I: Picomoles of AMP Incorporated into Promoter-Specific Ribooligonucleotides[a]

	(1) 800 μM A[b]	(2) 800 μM A, 40 μM U	(3) 800 μM A, 800 μM U	(4) 800 μM A, 40 μM UG	(5) 800 μM A, 800 μM UG	(6) 800 μM A, 40 μM UGC	(7) 800 μM A, 800 μM UGC
2-mer	1583 (1510)	660 (632)	883 (800)	175 (136)	28 (18)	111 (49)	37 (8)
3-mer	96 (83)	198 (196)	254 (226)	37 (27)	7 (4)	24 (10)	8 (2)
4-mer	14 (11)	398 (380)	575 (528)	112 (90)	8 (6)	75 (34)	6 (3)
6-mer	-[c]	2 (2)	16 (13)	32 (25)	5 (3)	18 (10)	3 (1)
9-mer	-	-	-	8 (6)	5 (3)	-	-

[a] These results are the average of two experiments similar to the one described in Figure 1. The results in parentheses are the average of two experiments carried out in the presence of heparin (see Materials and Methods). Incorporation was quantitated as described under Materials and Methods. Backgrounds from a reaction in which DNA was omitted (see Figure 1, lane e) were subtracted. The values that were averaged agreed with each other to within 10% of the average value, except for very low incorporation (less than 10 pmol of AMP). [b] A = ATP, U = UTP, G = GTP, and C = CTP. [c] A dash indicates that incorporation was below the level of detection of the methods employed.

Table II: Moles of Promoter-Specific Ribooligonucleotides per Mole of *lac* UV5 DNA, in the Presence of Heparin[a]

	(1) 800 μM A[b]	(2) 800 μM A, 40 μM U	(3) 800 μM A, 800 μM U	(4) 800 μM A, 40 μM UG	(5) 800 μM A, 800 μM UG	(6) 800 μM A, 40 μM UGC	(7) 800 μM A, 800 μM UGC
2-mer	481	201	255	43	6	16	~3
3-mer	26	62	72	9	~1	~3	~1
4-mer	4	121	168	29	~2	11	~2
6-mer	-[c]	~1[d]	4	8	~1	~3	-
9-mer	-	-	-	~1	~1	-	-
total	511	385	499	90	11	33	~6

[a] This table was derived from Table I. Picomoles of complementary ribooligonucleotide were calculated from the picomoles of AMP incorporated divided by the number of AMP residues in the oligonucleotide. This number was then divided by the 1.6 pmol of *lac* UV5 DNA that was in the reaction (see Materials and Methods). [b] A = ATP, U = UTP, G = GTP, and C = CTP. [c] A dash indicates less than 1 mol of complementary ribooligonucleotide per mol of *lac* UV5 DNA. [d] Given as an approximate value to indicate that the value shown should be considered an estimate because of the low level of product synthesized (see Table I for statement about accuracy of data).

Table III: pppApA as a Percent of the Total Promoter-Specific Ribooligonucleotides[a]

	(1) 800 μM A,[b] 40 μM U	(2) 800 μM A, 800 μM U	(3) 800 μM A, 40 μM UG	(4) 800 μM A, 800 μM UG	(5) 800 μM A, 40 μM UGC	(6) 800 μM A, 800 μM UGC
no heparin	53	51	48	54	48	~70[c]
+heparin	52	51	48	54	48	~60

[a] This table was derived from Table I. Picomoles of complementary ribooligonucleotides were calculated as described in Table II. Picomoles of pppApA were divided by total picomoles of complementary ribooligonucleotides and multiplied by 100. [b] A = ATP, U = UTP, G = GTP, and C = CTP. [c] Given as an approximate value to indicate that the value shown should be considered an estimate because of the low level of product synthesized (see Table I for statement about accuracy of data).

by the heparin resistance of the synthesis.

Table II quantitates the yield of each oligonucleotide produced per DNA template, in the presence of heparin. These quantitites should be considered minimum estimates. Although the individual nucleoside triphosphates were highly purified (see Materials and Methods), trace amounts of contaminants may be present. Note, for example, that in the presence of ATP alone 5% of the products are longer than pppApA, implying a slight contamination with UTP. This probably leads to a slight underestimation of the amount of pppApA formed. Table II shows that oligonucleotides up to at least 6-mers (pppApApUpUpGpU) are formed reiteratively at the promoter under a wide variety of conditions. This result has been obtained in four separate experiments. This demonstrates that RNA polymerase is capable of synthesizing a long complementary oligonucleotide, releasing the oligomer, and then reinitiating synthesis without dissociating from the template in a manner in which would allow inactivation by heparin.

Table II (bottom row) shows that the total yield of oligonucleotides is decreased when a missing substrate is added to the reaction. Specifically, either the addition of missing GTP to ATP + UTP reactions (compare totals, columns 4 and 5 with 2 and 3), or the inclusion of CTP as the fourth substrate (compare totals, columns 6 and 7 with 4 and 5), leads to a reduction in total oligonucleotide yields. This simply suggests that, as the RNA chain is extended past the 4-mer, the probability of reiterative synthesis is reduced. There is a further reduction in yield (but oligomers persist) when UTP, GTP, and CTP are raised from 40 to 800 μM (compare totals; column 6 with 7, column 4 with 5). These data demonstrate a correlation between those conditions which favor extension of the growing chain beyond the 4-mer and a reduction in the number of reiterative initiation events by RNA polymerase.

This correlation is supported by the experiment shown in Figure 2. In this experiment the concentrations of UTP, GTP, and CTP were varied from 25 to 800 μM, while ATP was fixed at 800 μM. Although oligonucleotide synthesis persists over a wide range of concentrations, the yields as evidenced by the intensity of bands on the autoradiograph are lowered as the substrate concentration increases. A similar result has been obtained by J. Stefano (unpublished results).

A Constant Fraction of pppApA Is Always Produced. In spite of the wide variation in total oligonucleotide yield, there is a striking conservation of the fraction of total products represented by pppApA (Table III). Notice that pppApA remains approximately half of all oligonucleotides either in the presence or absence of heparin under conditions where the total yield of all species varies from as high as 499 to as low as 6 pmol (see Table II for total yields). This implies that,

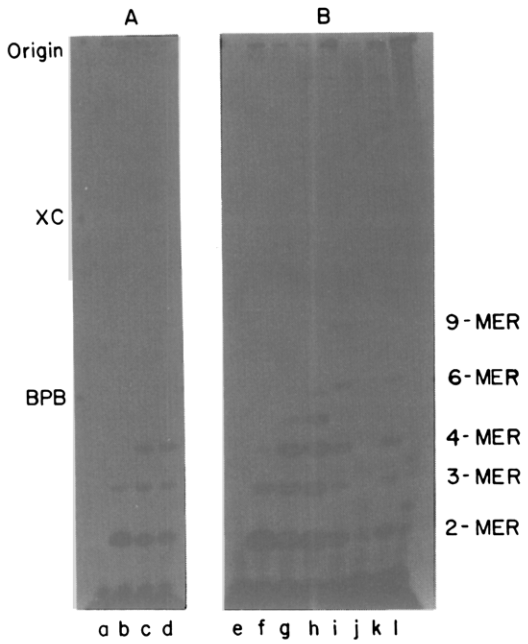

FIGURE 1: (A) Autoradiogram of denaturing polyacrylamide gel electrophoretic separation of complementary oligonucleotides. All reactions contained 800 μM [α-^{32}P]ATP: (lane a) control in which DNA was omitted from the reaction; (lane b) synthesis in the presence of ATP alone; the predominant band (2-mer) is pppApA; (lane c) synthesis in the presence of ATP and 40 μM UTP; the 3-mer and 4-mer are pppApApU and pppApApUpU, respectively; (lane d) same as c, except that the reaction contained [^{3}H]UTP. The ^{3}H/^{32}P ratio in lane d (see text) was measured by excising appropriate bands, eluting the radioactive RNA, and measuring incorporated ^{3}H and ^{32}P by liquid scintillation counting. XC (xylene cyanol) and BPB (bromophenol blue) are electrophoresis marker dyes. (B) Autoradiogram of species produced using various concentrations of nucleoside triphosphates. All reactions contained 800 μM [α-^{32}P]ATP: (lane e) control in which DNA was omitted from the reaction. Reactions contained: ATP alone (lane f); ATP with 40 μM UTP (lane g); ATP with 800 μM UTP (lane h); ATP with 40 μM UTP and GTP (lane i); ATP with 800 μM UTP and GTP (lane j); ATP with 40 μM UTP, GTP, and CTP (lane k); ATP with 800 μM UTP, GTP, and CTP (lane l). Lanes f, g, and h are greatly overexposed to allow simultaneous visualization of the products in lanes i through l. The aberrant 3-mer products in overexposed lane f and 5-mer and 6-mer products in overexposed lanes g and h represent less than 5% of the total [α-^{32}P]AMP incorporated into oligonucleotides. Note that in lanes i through l little or no 5-mer is detectable, even though the 4-mer and 6-mer are easily visualized. We have never observed significant quantities of 5-, 7-, or 8-mers. In lanes j and l, the products (2- and 3-mers) of intermediate mobility are probably oligonucleotides with pppG at the 5′ terminus. This is a consequence of the very high GTP concentrations present in these reactions (see text discussion of Figure 3).

on the average, each time an RNA polymerase molecule "initiates" by forming pppApA it is approximately equally probable that the chain will be extended or that the dinucleotide tetraphosphate will dissociate leading to another round of initiation.

Strictly, the supposition that pppApA dissociation is as likely as condensation with UTP to form pppApApU assumes that there is not a burst of pppApA synthesis at the beginning of the reaction which is eventually equalled by the synthesis of longer oligomers later in the reaction. This is not the case, as the fraction of the total products which is pppApA remains relatively constant near 50% during the entire course of the reaction (data not shown).

Action of Rifampicin. As discussed previously, rifampicin inhibits long-chain RNA synthesis. Its mechanism of action, however, remains uncertain, though it is commonly assumed to act at the "initiation" step. Since initiation at this promoter is clearly more complex than assumed, we wished to investigate

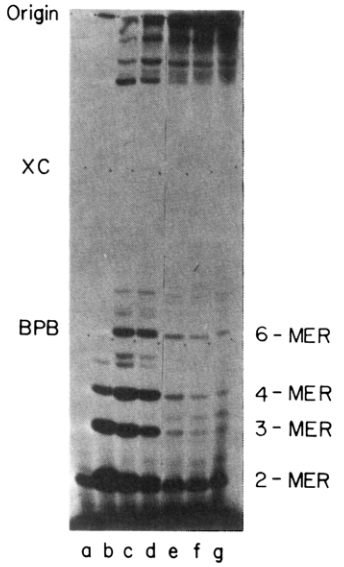

FIGURE 2: Synthesis of complementary oligonucleotides in the presence of all four nucleotide triphosphates. Electrophoresis was as described in Figure 1. This experiment was done in the presence of heparin (200 μg/mL final concentration). All reactions contained 800 μM [α-^{32}P]ATP: (lane a) control in which DNA was omitted from the reaction; (lane b) synthesis in the presence of ATP alone; (lane c) ATP with 25 μM UTP, GTP, and CTP; (lane d) ATP with 50 μM UTP, GTP, and CTP; (lane e) ATP with 200 μM UTP, GTP, and CTP; (lane f) ATP with 400 μM UTP, GTP, and CTP; (lane g) ATP with 800 μM UTP, GTP, and CTP. The species that migrates as 2-mer in the no DNA control (lane a) is a very minor contaminant that occasionally appears in the [α-^{32}P]ATP preparations. Lanes b, c, and d are greatly overexposed to allow visualization of oligomers in lanes e, f, and g.

the influence of the drug. The experiment is simply to allow initiation as shown in Figure 1, except that rifampicin is added to the reaction mixture during the 5-min incubation preceding addition of nucleoside triphosphates. Synthesis then proceeds for 10 min, and gel electrophoresis is used as before to detect any oligonucleotide products.

The resulting autoradiogram (Figure 3) shows that the primary effect of rifampicin is to prevent extension of the growing RNA chain after two phosphodiester bonds have formed. That is, 2-mers and 3-mers continue to be produced reiteratively in the presence of this inhibitor. Comparison of the amounts of 2-mers and 3-mers formed (Table IV) with identical experiments performed in the absence of rifampicin (Table I) allows the inhibitor's effects to be summarized simply. At a fixed concentration of nucleoside triphosphate, rifampicin slows formation of the first phosphodiester, inhibits dramatically formation of the second phosphodiester, and makes formation of the third so unfavorable as to prevent detection of the product. As a consequence, RNA polymerase rarely escapes from cycling to produce oligonucleotides and synthesis of long RNA is inhibited.

The autoradiogram shown in Figure 3A reveals two minor bands resulting from addition of 800 μM ATP, UTP, and GTP to preinitiation complexes containing rifampicin (lane f). Recall that the UV5 *lac* promoter can initiate with the sequence GAAUUG, although AAUUG is the favored sequence (Maizels, 1973). These minor species are very likely to be pppGpA and pppGpApA for the following reasons. These two minor bands are undetectable in the absence of GTP (lanes b–d), barely detectable with added 40 μM GTP (lane e), and detectable with added 800 μM GTP (lane f). Thus, their appearance is clearly GTP dependent. Both species have mobilities slightly slower than their GTP-independent 2-mer and 3-mer counterparts, pppApA and pppApApU. This is the

Table IV: Picomoles of AMP Incorporated into Promoter-Specific Ribooligonucleotides in the Presence of Rifampicin[a]

	(1) 800 μM A[b]	(2) 800 μM A, 40 μM U	(3) 800 μM A, 800 μM U	(4) 800 μM A, 40 μM UG	(5) 800 μM A, 800 μM UG	(6) 800 μM A, 40 μM G	(8) 800 μM A, 800 μM G
2-mer	312	336	599	298	433	319	218
2-mer*[c]	-[d]	-	-	-	19	-	21
3-mer	3	18	31	16	25	-	-
3-mer*[c]	-	-	-	-	12	-	15

[a] The experiment described in Figure 3 was quantitated (see Materials and Methods for details). [b] A = ATP, U = UTP, G = GTP, and C = CTP. [c] 2-mer* and 3-mer* are products with slightly slower mobilities than 2-mer and 3-mer. These products have pppG at their 5′ termini (see text for discussion). [d] Indicates that incorporation was below the level of detection of the methods employed.

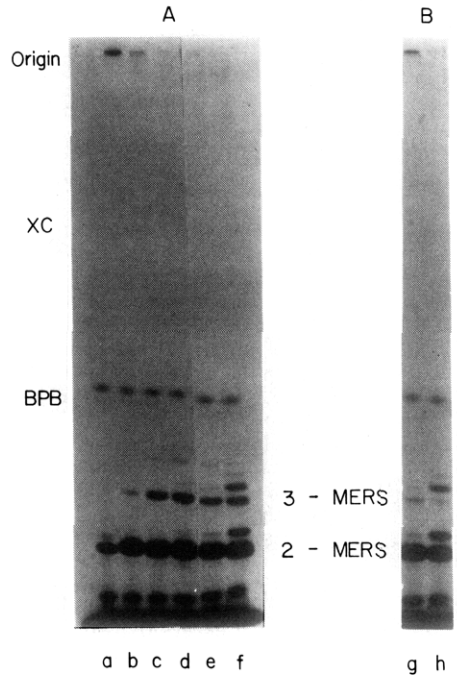

FIGURE 3: Synthesis of oligonucleotides in the presence of rifampicin. Electrophoresis was as described in Figure 1. Rifampicin was added to a final concentration of 50 μM and reactions contained 800 μM [α-^{32}P]ATP: (lane a) control in which DNA was omitted from the reaction. Reactions contained: ATP alone (lane b); ATP with 40 μM UTP (lane c); ATP with 800 μM UTP (lane d); ATP with 40 μM UTP and GTP (lane e); ATP with 800 μM UTP and GTP (lane f); ATP with 40 μM GTP (lane g); ATP with 800 μM GTP (lane h). In lanes f and h, the products (2- and 3-mers) of intermediate mobility are probably oligonucleotides with pppG at the 5′ terminus. This is a consequence of the very high GTP concentrations present in these reactions (see discussion in text). The particular preparation of [α-^{32}P]ATP used for these experiments contains minor contaminants which appear in all lanes including the control in which DNA was omitted (lane a).

mobility shift expected for G-containing oligonucleotides of this length (Frank & Köster, 1979). Supporting evidence comes from the experiment shown in Figure 3B, in which synthesis occurs in the absence of UTP. ATP (800 μM) and GTP (40 μM) produce barely detectable minor bands (lane g), but 800 μM ATP and 800 μM GTP (lane h) lead to increased levels of both minor species. Thus, the minor bands may be produced in the absence of UTP, are dependent on added GTP, and have the mobilities consistent with those of the expected products pppGpA and pppGpApA. These data (see Table IV, columns 5 and 6) confirm that G starts are allowed but disfavored, and are consistent with rifampicin allowing formation of the first two but not the third phosphodiester bond.

Discussion

Initiation Pathway. These experiments show that the initiation phase of RNA synthesis at the *lac* UV5 promoter is accompanied by the reiterative formation of small promoter-specific oligonucleotides. These small products may be formed in amounts greater than the amount of DNA template even in the presence of heparin, which inactivates free but not DNA-bound RNA polymerase. Thus, multiple rounds of oligonucleotide synthesis occur while a single RNA polymerase–DNA complex is "initiating" RNA synthesis.

The dinucleoside tetraphosphate pppApA is always the most abundant product formed (recall that the RNA sequence is pppApApUpUpGpUpGpApGpC...). One striking observation is that although the nucleoside triphosphate substrate concentration can be manipulated to cause the *total* oligonucleotide yield to vary by two orders of magnitude, pppApA always comprises about half of the total products. This is true even when the UTP concentration is 800 μM. Therefore, even at this very high concentration of UTP, it is equally probable that pppApA will dissociate or condense with UTP to form pppApApU. Moreover, 3-mers, 4-mers, and 6-mers continue to be produced over a wide range of concentrations of nucleoside triphosphates, indicating that these species also have reasonable probabilities of premature dissociation. Our data are not sufficiently precise to allow calculation of these probabilities under various conditions. We can only estimate crudely that the 3-mer has a one in four chance of dissociation and the 4-mer a three in four chance of dissociation when the concentrations of UTP and GTP are 40 μM. It is likely that these probabilities fall with increasing concentration. However, at all concentrations *total* yields of oligonucleotides (either in the presence or absence of heparin) continue to exceed the molar amount of DNA template present.

These considerations lead to a cycling model for initiation of transcription at this promoter. Conversion of preinitiation complexes to elongation complexes is proposed to take place along the following pathway (see Figure 4). Binding of two ATPs leads to formation of pppApA, which dissociates in about half the complexes. Those RNA polymerase molecules which are free of pppApA bind two more ATPs to produce another molecule of pppApA, which again has a 50:50 probability of dissociation. Thus, approximately 1 mol of pppApA accumulates per mol of RNA polymerase which escapes by addition of UTP to form pppApApU or longer species. Each extension of the RNA chain leads to formation of a product which has its own probability of dissociation. The cumulative effect of the finite probabilities of oligonucleotide dissociation leads to accumulation of oligonucleotides. Each dissociation, of course, requires RNA polymerase to begin the process over again, including the initial steps with the accompanying high probability of pppApA dissociation. In this view, productive initiation (of long transcript) can be considered an escape by RNA polymerase from cycling to produce oligonucleotides.

At what point is this escape accomplished, that is, at what chain length is a stable elongation complex achieved? McClure & Cech (1978) raised the possibility that stability

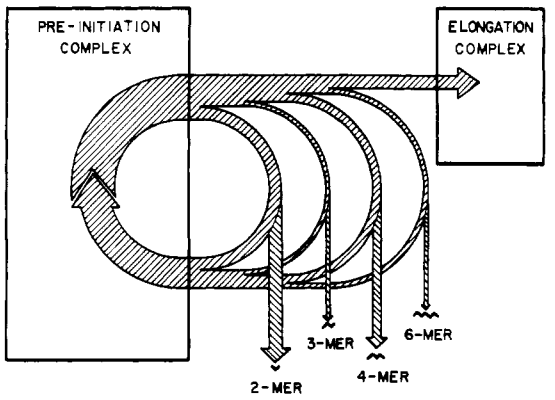

FIGURE 4: Proposed cycling pathway for initiation at the *lac* UV5 promoter (see Discussion in text). Initiation (formation of elongation complex) is an escape from cycling to produce oligonucleotides (2-mers, 3-mers, etc.). The pathways that lead to the preinitiation complex after the release of oligomer do not involve free RNA polymerase. Preinitiation complex is the RNA polymerase–DNA complex that does not contain RNA or oligomer. Elongation complex is a ternary complex of RNA polymerase, DNA, and RNA that does not release its RNA product until the full-length chain is synthesized. The binding of the individual nucleoside triphosphates is not shown. See text for full description.

may not be achieved until a variable number of nucleotides had been incorporated into the nascent RNA chain. Our data demonstrate that stability is not achieved on the *lac* UV5 promoter prior to formation of a 4-mer and very likely not prior to formation of a 6-mer. Recall that the 4-mer and the 6-mer can be formed in the presence of heparin in amounts greater than the amount of DNA template. This indicates that RNA polymerase can reinitiate after forming these species without dissociating from the DNA template, and, therefore, the complexes involved in oligonucleotide synthesis retain some of the important characteristics of preinitiation complexes. Moreover, the dissociation of oligonucleotides on this time scale is inconsistent with the behavior of elongation complexes, which have a reported half-life of 1 week at 4 °C on one template (Rhodes & Chamberlin, 1974). The latter half-life is of course very long compared to the fraction of a second it normally takes to elongate a chain by one nucleotide. The observation that, for the 4-mer and 6-mer, dissociation can actually compete with addition suggests that oligomer dissociation is extremely rapid, or that addition of a nucleotide is relatively slow; neither is a property of elongation complexes. Taken together, these considerations imply that RNA polymerase at the *lac* UV5 promoter cannot form a stable elongation complex and thereby escape irreversibly from cycling, until at least three–five phosphodiester bonds have formed.

How is it possible, mechanistically, for an RNA polymerase to synthesize a long complementary oligonucleotide, release this product, and reinitiate synthesis without the enzyme dissociating from the DNA? If the whole enzyme had actually moved along the DNA during synthesis, the return process is difficult to imagine. However, there is one possible mechanism that does not require the enzyme to move and contact a DNA region different from that contacted in the preinitiation complex. This speculative mechanism relies on the single strandedness of one helical turn of DNA surrounding the initiation sequence (Siebenlist, 1979). Since single-stranded DNA has an average phosphate–phosphate distance twice that of helical DNA, one might imagine a "slack" in the DNA within the preinitiation complex. During initial synthesis, this "slack" might be taken up as the copied single-stranded DNA "threads" through the enzyme active site and becomes base paired to RNA. Premature release of a base-paired complementary RNA would simply restore the slack, allowing reinitiation to occur. We raise this possible mechanism for initiation simply to demonstrate that reiterative oligomer synthesis could occur without significantly altering the primary contacts characteristic of a preinitiation complex.

Is This pathway Utilized at Other Promoters? There is evidence available from previous studies of two other promoters that indicates that cycling to produce oligonucleotides without polymerase dissociation from DNA is not unique to the *lac* UV5 promoter. Our analysis of in vitro transcription data from the *gal* promoter (DiLauro et al., 1979) indicates that initiation proceeds through a pathway involving cycling of RNA polymerase to produce oligonucleotides reiteratively. High-resolution gel electrophoresis was used to follow *gal* mRNA production; autoradiograms show oligonucleotides accumulating up to at least the 7-mer (Figure 7 of DiLauro et al., 1979). Since these species include many fewer [^{32}P]UMP residues than the long transcript, yet are of comparable exposure on the autoradiogram, we infer that they are produced in great molar excess over long RNA. This phenomenon occurs in the presence of heparin and 100 μM of each nucleoside triphosphate. Moreover, the RNA mixture was precipitated with ethanol prior to electrophoresis, which probably leads to only a fraction of oligonucleotide represented on the gel autoradiogram. In another experiment the amounts of 3-mers and 9-mers synthesized were determined (Table 2 of DiLauro et al., 1979). From those latter data we estimate that these species were produced, in the presence of heparin, in molar amounts greater than the DNA template. Taken together, these data indicate that reiterative formation of oligonucleotides also accompanies initiation of transcription in vitro at the *gal* promoter. It cannot be determined whether this pathway is followed on other bacterial promoters that have been transcribed in vitro, since the amount of oligonucleotide is normally reduced by ethanol solubilization prior to detection of transcript.

Analysis of transcription data from the $\lambda P_{R'}$ promoter is also suggestive of a cycling pathway. Recall that continuous transcription from this promoter leads to the accumulation of 2 mol of pppApApC per mol of long RNA (McClure & Cech, 1978). This experiment, however, was done in the absence of heparin, so contributions from the dissociation and reassociation of RNA polymerase cannot be estimated. Therefore, there are two possible interpretations of this result. One possibility is that, out of three RNA polymerase molecules that initiate, two subsequently terminate prematurely to produce pppApApC. Alternatively, a single RNA polymerase may cycle during initiation and produce on the average two molecules of pppApApC before escaping to produce long RNA. This latter possibility only is similar to the mechanism described for the *lac* UV5 promoter. This alternative is supported, but not proven, by indirect experiments indicating that pppApApC can be formed in the presence of heparin and can be formed very rapidly (in the presence of rifampicin) (McClure & Cech, 1978). Whether or not the two promoters share a common mechanism, the suggestion that there may be a delicate balance between dissociation and elongation of transcript (McClure et al., 1978) is true for both $\lambda P_{R'}$ and *lac* UV5.

The ability of RNA polymerase to form more than one dinucleoside tetraphosphate without dissociating from the DNA template was first described for λb2 DNA (Johnston & McClure, 1976). This reaction was termed "abortive initiation", presumably since productive initiation was aborted by the exclusion of the nucleoside triphosphate required to

extend the RNA chain. Subsequent studies of abortive initiation (Smagowicz & Scheit, 1977; McClure et al., 1978; McClure & Cech, 1978; Nierman & Chamberlin, 1979) have focused on kinetic analyses. However, these kinetic analyses did not require the use of heparin, which prevents dissociation and reassociation of RNA polymerase during the time course of reaction. By contrast, in this study we require the presence of heparin to demonstrate the reiterative production of oligonucleotides central to the concept of a cycling pathway.

Certain features of our results have counterparts in some of the experiments in studies which focused primarily on abortive initiation. For example, addition of missing nucleoside triphosphates always led to reduction in the yield of dinucleoside tetraphosphate (Johnston & McClure, 1976; McClure et al., 1978), as is usually the case with the *lac* UV5 promoter. However, on the T7 A_1 promoter, no species longer than dinucleoside tetraphosphate were detected (Nierman & Chamberlin, 1979). The method of detection was thin-layer chromatography, which is less sensitive than the gel electrophoresis method used in the present study. Thus, it is conceivable that low levels of such products may have escaped detection. Alternatively, the cycling pathway described for the *lac* UV5 (and probably *gal*) promoter may not be utilized at this promoter.

Effects of Rifampicin. The primary effect of the inhibitor rifampicin at the *lac* UV5 promoter is to inhibit the formation of the first few phosphodiester bonds. No species longer than 3-mers are detectable in the presence of the drug. Yet 2-mers and 3-mers continue to be produced reiteratively, indicating that RNA polymerase, which has not yet formed a stable elongation complex, can still cycle in the presence of this inhibitor. Thus, the consequence of rifampicin addition is that escape from cycling is inhibited and long-chain RNA does not accumulate.

The effect of rifampicin reported here is very similar to the effects on the $\lambda P_{R'}$ promoter but somewhat different from that reported for λP_L and λP_R (McClure & Cech, 1978). In the latter case, formation of the third phosphodiester was blocked, as is also true for the *lac* UV5 promoter. These authors proposed a simple model of steric interference to account for the inhibition observed at P_L and P_R and consider $P_{R'}$ a probable exception to this model. They further raised the possibility that promoters like $P_{R'}$ and *lac* might be excepted from this model, since they are capable of initiating at either of two adjacent positions. Our data do not address the molecular basis for the observed effects. Although *lac* UV5 and $\lambda P_{R'}$ do fall into the same class based on simple observation of rifampicin effect, we do not consider the existence of adjacent start sites as necessarily the most likely cause of this classification (see section below on possible classes of promoters).

Rifampicin has been used in a challenge assay of initiation rate (Mangel & Chamberlin, 1974; Rhodes & Chamberlin, 1975). This assay involves a simple kinetic competition for preinitiation complexes between rifampicin, which inactivates the complex, and nucleoside triphosphates which leads to rifampicin resistance. The interpretation of this assay if applied to the *lac* UV5 promoter would be difficult, since one or two phosphodiester bonds can form in the presence of rifampicin, and since initiation proceeds along a complex cycling pathway. It is not clear in general what types of conclusions based on the challenge assay are affected by such considerations. These considerations emphasize the desirability of using direct assays for initiation (Stefano & Gralla, 1979; Nierman & Chamberlin, 1979).

Implications for Productive Initiation. The rate of appearance of *lac* UV5 transcript upon addition of a fixed concentration of nucleoside triphosphates (Stefano & Gralla, 1979) is one to two orders of magnitude slower than that estimated on a T7 DNA template (Mangel & Chamberlin, 1974; Rhodes & Chamberlin, 1975; Nierman & Chamberlin, 1979). This raises the possibility (Stefano & Gralla, 1979) that there may be classes of promoters which differ in overall initiation rate. The results presented here may provide at least a partial explanation for the observed difference in initiation rate.

The deduced pathway for production of RNA at the *lac* UV5 promoter is shown in Figure 4. After addition of each nucleotide to the growing short RNA there is a finite probability that the oligonucleotide product will dissociate and that RNA polymerase will begin the initiation process over again. Thus, only a fraction of "initiation" events (formation of the first phosphodiester) actually result in escape from cycling (to form oligonucleotides) and produce long RNA. A single RNA polymerase will have actually "initiated" a number of times before it escapes to produce RNA. Therefore, the rate of appearance of RNA depends both on the rate of formation of the first phosphodiester and on the number of times this must be formed prior to appearance of RNA. Since *lac* UV5 complexes cycle frequently, the rate of productive initiation (leading to long RNA) would be slowed proportionally. Moreover, we have found, using an abortive initiation assay, that the rate of pppApA formation is rather slow at moderate concentrations of ATP, probably due to a disfavored binding of the penultimate ATP to the preinitiation complex (unpublished experiments). Thus, the *lac* UV5 promoter may be a slow initiator for two reasons: formation of the first phosphodiester bond is slow, and cycling requires that this slow step be repeated a variable number of times prior to escape to produce long RNA.

There are two other potential ways that cycling to produce oligonucleotides could affect the rate of production of transcript. First, there is the possibility that oligonucleotide dissociation does not always lead to reinitiation. Thus, the appearance of small RNAs may actually lead to a reduction in the number of long RNAs produced and thereby alter the efficiency of productive initiation (McClure et al., 1978). Our data are not sufficiently precise to determine what fraction, if any, of oligonucleotide dissociations do not allow subsequent reinitiation. A second possible effect would be that the cycling pathway, which is sensitive to the concentration of nucleoside triphosphates, might also be sensitive to compounds present in vivo that might resemble these nucleosides. There are, of course, many such compounds that might act as inhibitors; for example, nucleoside mono- and diphosphates can be incorporated into 5′ termini in vitro (McClure et al., 1978), and the role of magic spot guanine nucleotides in transcriptional control is still unsettled.

Are There Classes of Promoter That Differ with Respect to Initiation? As discussed above, the cycling pathway deduced for the *lac* UV5 promoter may also be used at the *gal* and $\lambda P_{R'}$ promoters and is probably not prominent at the T7 A_1 promoter (Nierman & Chamberlin, 1979). Are there features of these promoters that determine the extent of utilization of the cycling pathway?

Both the *gal* and $\lambda P_{R'}$ promoters, although they do not resemble each other, have a feature that resembles the *lac* promoter. That is, the *gal* and *lac* promoters resemble each other in that they have evolved to allow the assistance of catabolite activator protein in transcription. It is possible that

the cycling pathway is prominent at promoters that have evolved in this way. Alternatively, the *lac* and $\lambda P_{R'}$ promoters resemble each other in that both initiate RNA with the sequence pppApA. We might expect $\lambda P_{R'}$ to be a slower initiator at moderate concentrations of ATP, as is the *lac* UV5 promoter, as a consequence of this sequence. It is possible in these cases that the cycling pathway is utilized to allow further reduction of initiation rate. We note parenthetically that the "rule" that transcription initiates with a purine–pyrimidine pair is violated very often. Examples of purine–purine starts include many of the most intensively studied promoters, including the *lac* Z, *trp*, $\lambda P_{R'}$, *araC*, and *lac*·i promoters and several promoters from phages fd and ϕX [see Rosenberg & Court (1979) for references]. Moreover, the *bio* and several ribosomal promoters (Otsuka & Abelson, 1978; Gilbert et al., 1979) initiate with a pyrimidine and, therefore, may also be very slow initiators. These speculative ideas concerning the rate and pathway of initiation are largely untested. Whether classes of promoters exist which exhibit important differences at the level of initiation remains to be established.

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