Abortive Initiation and Long Ribonucleic Acid Synthesis†

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ABSTRACT: In vitro transcription assays have been used to study the rate of ribonucleic acid (RNA) synthesis from the *Escherichia coli* lactose promoter mutant *lac*L8UV5 contained on a 203-bp (base pair) restriction fragment. The half-time of long (63-base) RNA production from heparin-resistant RNA polymerase–promoter complexes was found to be related to the amount of oligonucleotides released during the initiation process (abortive initiation). Studies indicate that once a ternary complex between the promoter, RNA polymerase, and a newly synthesized RNA between seven and nine nucleotides long is formed, abortive initiation is reduced and the rate of synthesis of long RNAs is increased. The promoter for the left inverted repeat of the transposable element Tn*5* was also examined. It was observed to have a much slower rate of production of long RNAs, and it released oligonucleotides 4 times as often as the lactose promoter. The correlation between the amount of abortive initiation and the half-time of long RNA production is discussed.

Despite many years of intensive investigation, the mechanism of transcription initiation remains unclear. Incubation of RNA polymerase with DNA (containing a promoter) at 37 °C results in the formation of a stable promoter-specific complex that is resistant to heparin attack [for a review, see Chamberlin (1976)]. Early experiments performed on T7 bacterial RNA polymerase promoters suggested that once this stable complex was formed addition of nucleoside triphosphates would result in rapid initiation of RNA synthesis (Mangle & Chamberlin, 1974). These results were recently confirmed for T7 promoters (Nierman & Chamberlin, 1979), but very different results were obtained from studies on the *lac*L8UV5[1] promoter (Stefano & Gralla, 1979). The rate of production of long RNAs from a prebound heparin-resistant complex was found to proceed with a half-time of 1 min. This is 1–2 orders of magnitude slower than that estimated for T7 promoters. These results have stimulated speculation as to whether promoter sequences may affect gene expression through differential rates of initiation of RNA synthesis.

DNA–RNA polymerase complexes had previously been observed to undergo an abortive initiation reaction involving the release of short oligonucleotides even in the presence of all four nucleoside triphosphates (McClure & Cech, 1978). Further studies on the *lac*L8UV5 promoter indicated that small oligonucleotides are synthesized in large quantities from prebound complexes (Carpousis & Gralla, 1980). A recycling model for initiation was proposed to account for the high yield oligonucleotides compared to long RNA transcripts. This model suggests that after each phosphodiester bond is formed a finite probability of dissociating the product exists (for the first 7–9 steps). If the product is dissociated, RNA polymerase can reinitiate RNA synthesis without leaving the DNA template. If product dissociation does not occur, RNA polymerase proceeds on to the next polymerization step. Productive initiation is defined as those initiation events that lead to the synthesis of full-length transcripts (i.e., transcripts that start at the correct site and terminate at or near the end of the restriction fragment).

In this paper, we present data that suggest that the recycling of RNA polymerase is responsible for the apparent long half-time of full-length RNA production. When RNA polymerase is bound in a ternary complex which does not recycle, addition of labeled nucleoside triphosphates leads to rapid synthesis of long RNA products. In an independent system where recycling was increased approximately 4-fold, a similar increase in the half-time of full-length RNA synthesis was observed. The slow production of long RNAs can then be explained as follows. The majority of polymerases are involved in recycling through the initiation process (defined here as the synthesis of the first few phosphodiester bonds). At each step, a certain percentage of the polymerases do not dissociate their RNA product but proceed on to form the next phosphodiester bond. After seven to nine bonds have been formed, elongation continues without further recycling. The percentage of products dissociated at each step (i.e., the amount of abortive initiation) determines the time it takes to escape this recycling process and synthesize a long RNA. The half-time of long RNA synthesis is thus related to the amount of abortive initiation and is a measure of the rate of escape from the recycling process.

Materials and Methods

Heparin and unlabeled nucleoside triphosphates were obtained from Sigma. [α-^{32}P]UTP was obtained from Amersham ($J > 350$ Ci/mmol). Bis(acrylylcystamine) (BAC) was purchased from Bio-Rad Laboratories. RNA polymerase was a gift from L. Maquat and S. Rothstein. Restriction enzymes were obtained from either New England Biolabs or Bethesda Research Labs Inc.

The 203-base-pair (bp) restriction fragment containing the lactose control region was isolated from pRZ3 (wild-type promoter), pRZ3111 (P^{r}111; Maquat et al., 1980), and pRZ3UV5 (L8UV5) by digestion with *Hae*III [for plasmid constructions, see Hardies et al. (1979) and Maquat & Reznikoff (1978)]. The transposon Tn*5* promoter was isolated by digestion of pRZ102 (Jorgensen et al., 1979) with *Hpa*I and *Eco*RI. Restriction fragments were separated on 5% polyacrylamide tube gels containing 0.2% BAC, 10% glycerol, 89 mM Tris–borate, pH 8.3, and 2.8 mM EDTA. The appropriate band was cut out and dissolved in 2-mercaptoethanol as previously described (Hansen, 1976). The dissolved gel was diluted 10-fold with water and applied to a 0.25-mL DEAE-

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[1] Abbreviations used: EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; DEAE, diethylaminoethyl; NaDodSO$_4$, sodium dodecyl sulfate; BPB, bromophenol blue; XC, xylene cyanol; *lac*, lactose operon.

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cellulose column (Whatman DE52). The DNA was eluted with 0.01 M Tris, pH 7.9, 1.0 mM EDTA, and 1.0 M NaCl after extensive washing with low-salt buffers. A sample of 3.5 volumes of 100% ethanol was used to precipitate the DNA. The pellet was washed with 95% ethanol, redissolved in 0.01 M Tris, pH 7.9, 0.1 mM EDTA, and 0.01 M NaCl, and dialyzed against the same buffer.

Transcription assays were performed in the buffer described by Majors (1975) at 37 °C. A 0.75-pmol aliquot of restriction fragment and 0.75 pmol of RNA polymerase were used per assay, except where noted. Heparin was added to a concentration of 100 μg/mL. When cold nucleoside triphosphates were added in the preincubation experiments, their concentrations were as follows: ATP and GTP, 400 μM; UTP, 20 μM. An additional 200 μM ATP and GTP, 20 μM CTP, 10 μM UTP, and 25 μCi of [α-^{32}P]UTP were added where labeled nucleoside triphosphates were indicated in the preincubation experiments. In assays where there was no preincubation, the UTP concentration in the labeled nucleoside triphosphates was increased to 20 μM.

Reaction volumes were either 240 or 80 μL (containing the above-mentioned picomoles of fragment and polymerase) with 30 or 10 μL withdrawn per time point. In the former case, the reaction was stopped with 100 μL of a solution containing 30 mM EDTA, 50 μg of tRNA, and 0.3 M sodium acetate. This was then ethanol precipitated and resuspended in sample buffer (20% glycerol, 7 M urea, 0.10% $NaDodSO_4$, 89 mM Tris–borate, pH 8.3, BPB, and XC). After being boiled for 1 min the samples were applied to a 20% polyacrylamide–7 M urea gel and electrophoresed. In the latter case, the reactions were stopped by addition of 10 μL of the sample buffer, followed by 1 min at 100 °C. These were then loaded and electrophoresed as described above.

Bands were visualized by autoradiography, excised, and counted by Cerenkov radiation. The relative amount of each oligonucleotide was determined by the number of UMPs it contained and the radioactivity incorporated. A 0.01-μL sample of the reaction mixture was counted by Cerenkov radiation to determine the specific activity of the UTP in the assay. This was used to calculate the picomoles of RNA synthesized.

Results

Rate of Productive Initiation. Restriction fragments containing the 203-bp lactose control region direct the synthesis of a 63-base RNA transcript (Majors, 1975). For measurement of the rate of synthesis of this transcript independent of the binding process, RNA polymerase is first incubated with the DNA for 10 min to allow formation of stable promoter-specific complexes. Heparin is added to inactivate any unbound polymerases, followed by the addition of labeled nucleoside triphosphates. The rate of incorporation of the label into the full-length transcript is then determined (Stefano & Gralla, 1979).

We have measured a half-time of productive initiation of approximately 1 min for the *lac*L8UV5 promoter. In addition, the wild-type *lac* promoter and *lac*P^{r}111 promoter were assayed and also found to have a half-time of 1 min (data not shown). This is in agreement with the half-time obtained by Stefano & Gralla (1979) for the *lac*L8UV5 promoter.

Preincubation with Unlabeled Nucleoside Triphosphates. An assay was developed to determine if the half-time of productive initiation is affected by limited transcription during a preincubation period. RNA polymerase is first incubated for 10 min with the restriction fragment containing the promoter. After a 1-min heparin challenge, either ATP alone, ATP and UTP, or ATP, UTP, and GTP are added. Preincubation with the designated unlabeled nucleoside triphosphates is continued for 5 min, then labeled nucleoside triphosphates are added, and the rate of formation of the full-length transcript is measured.

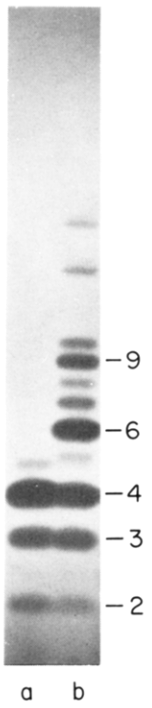

FIGURE 1: Autoradiogram of species produced upon incubation with specific nucleoside triphosphates. The restriction fragment containing the *lac*L8UV5 promoter was used as template. Sizes of RNA products were determined by eluting RNA from the gel and analyzing by homochromatography. Markers were a gift from W. McClain. Less than 4% of the RNA in lane b is more than 10 nucleotides long. A small percentage of the transcription starts at the G residue located at −1. This presumably gives rise to the pentanucleotide in lane a and the decanucleotide in lane b. Reactions contained the following: 400 μM ATP, 20 μM [α-^{32}P]UTP (lane a); 400 μM ATP, GTP, and 20 μM [α-^{32}P]UTP (lane b).

The 5′ sequence of *lac* mRNA is pppApApUpUpGpUpGpApGpC (Maizels, 1973). The addition of only ATP to the transcription assay should allow formation of the dinucleotide pppApA. ATP and UTP should allow RNA polymerase to synthesize the tetranucleotide pppApApUpU, and when ATP, UTP, and GTP are preincubated with the polymerase–DNA complex, RNA products up to nine nucleotides should be formed. Figure 1 shows that these are the major products synthesized during the preincubations. The low level of long transcripts indicates minimal contamination by the omitted nucleoside triphosphates in these experiments.

Table I shows the half-times of productive initiation measured after preincubation with the indicated unlabeled nucleoside triphosphates as described above. Preincubation with ATP has no effect on the rate of productive initiation. Preincubation with ATP and UTP has only a minor effect on the half-time, but preincubation with ATP, UTP, and GTP reduced the half-time from 60 s to less than 20 s.

Quantitation of Oligonucleotides. To further investigate this, we examined all small transcripts synthesized during these experiments. Figure 2 shows an autoradiogram of an assay performed without preincubation with nucleoside triphosphates, as described in the first experiment above. Quantitation of the three major bands (tetramer, hexamer, and full length) yielded an average of 10–12 oligonucleotides for each full-length transcript produced. Figure 3A shows the

Table I: Half-times of Productive Initiation[a]

experiment	nucleoside triphosphates present during preincubation ATP	UTP	GTP	half-time (s)
1	–	–	–	60
2	+	–	–	60
3	+	+	–	45
4	+	+	+	<20

[a] Half-times of productive initiation were determined by using *lac*L8UV5 as template. The assay is described in the text; the reaction conditions are described under Materials and Methods. The half-time is the time at which 50% of all full-length transcripts have been made.

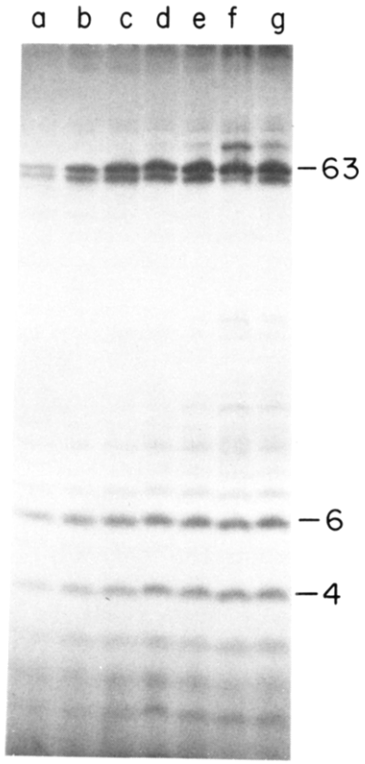

FIGURE 2: Time course of RNA products synthesized from *lac*L8UV5 promoter. RNA polymerase was incubated with the *Hae*III restriction fragment containing the *lac* control region at 37 °C for 10 min. Labeled nucleoside triphosphates were added after a 1-min heparin challenge (see Materials and Methods for details). The band migrating below the full-length transcript was identified by Majors (1975) as a paused product, possibly due to RNA polymerase stopping prior to running off the restriction fragment. Both bands were excised and Cerenkov radiation counted to determine the amount of full-length transcript. Transcription was stopped at the following times: 40 s (a); 1 min (b); 1.5 min (c); 2 min (d); 3 min (e); 5 min (f); 10 min (g). The amount of RNA produced after 10 min was determined for the three major bands: tetramer, 0.11 pmol; hexamer, 0.13 pmol; full length, 0.072 pmol.

labeled products synthesized after a 5-min preincubation with unlabeled ATP and UTP. There are approximately 11 oligonucleotides made per full-length transcript under these conditions. The radioactive products made after preincubation with ATP, UTP, and GTP are shown in Figure 3B. In this assay, there were only two oligonucleotides made per full-length transcript.

The above experiments suggest that RNA polymerase can recycle through the initiation process after synthesizing oligonucleotides four bases long but that this process is greatly reduced after the polymerase has synthesized a transcript nine bases long. It is important to verify that the oligonucleotides are synthesized by polymerases that are recycling rather than

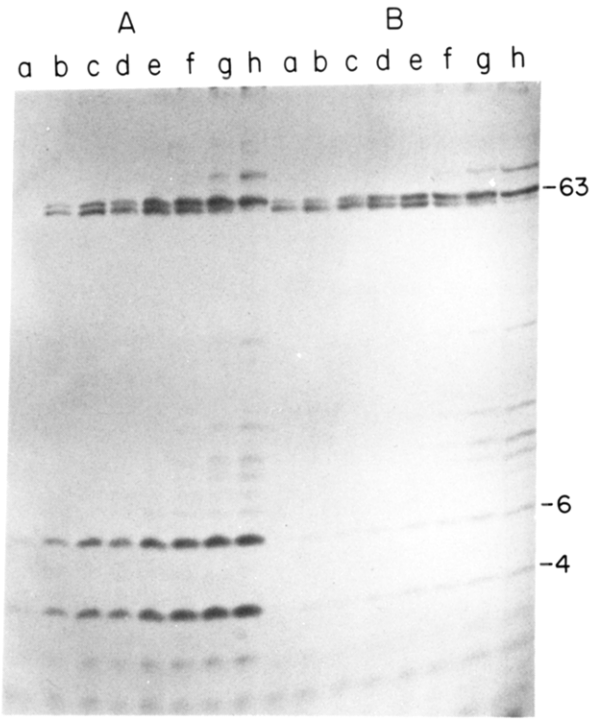

FIGURE 3: Time course of RNA products synthesized after preincubation with unlabeled nucleoside triphosphates. The *lac*L8UV5 promoter was used as template. Assays were as described in the text. At zero time, labeled nucleoside triphosphates were added. Transcription was stopped at the following times: 20 s (a); 40 s (b); 1 min (c); 1.5 min (d); 2 min (e); 3 min (f); 5 min (g); 10 min (h). (A) Preincubation with ATP and UTP. The amount of RNA produced after 5 min for the three major bands was approximately the following: tetramer, 0.15 pmol; hexamer, 0.090 pmol; full length, 0.021 pmol. (B) Preincubation with ATP, UTP, and GTP. Picomoles of RNA quantitated after 5 min were approximately the following: tetramer, 0.027; hexamer, 0.024; full length, 0.018.

by polymerases that terminate prematurely. To do this, we determined the amount of RNA products made per promoter in a standard assay in which there was no preincubation with unlabeled ribonucleoside triphosphates. To ensure that the majority of promoters were complexed with an active polymerase, a 30:1 molar excess of polymerase to promoter was used in this experiment. The *lac*L8UV5 promoter was used as template, and the three major products (see above) were quantitated. On the average, one transcript was produced from every promoter in 20 s. After 5 min 2.5 transcripts were synthesized per promoter. As will be described under the Discussion, these are underestimations of the true values. (Again, there were approximately ten oligonucleotides per full-length transcript.) These results indicate a recycling mechanism, not an early termination mechanism.

Transposon Tn5 Promoter. Preliminary studies were performed on the promotor located in the left inverted repeat of the transposable element Tn5. A 250-bp *Hpa*I–*Eco*RI restriction fragment containing the promoter directs the synthesis of a 93-base transcript (Rothstein et al., 1980). We measured the rate of production of this transcript from a prebound heparin-resistant complex and found the half-time to be approximately 5 min.

The 5′-mRNA sequence is pppApApCpUpUpCpUpGpCpU, (R. Johnson, unpublished experiments), so any RNA less than four nucleotides long is not labeled with the [α-^{32}P]UTP used in these assays. Figure 4 shows the products synthesized under these conditions. Bands corresponding to four to eight nucleotides in length, as well as the full-length transcript, were excised and Cerenkov radiation counted.

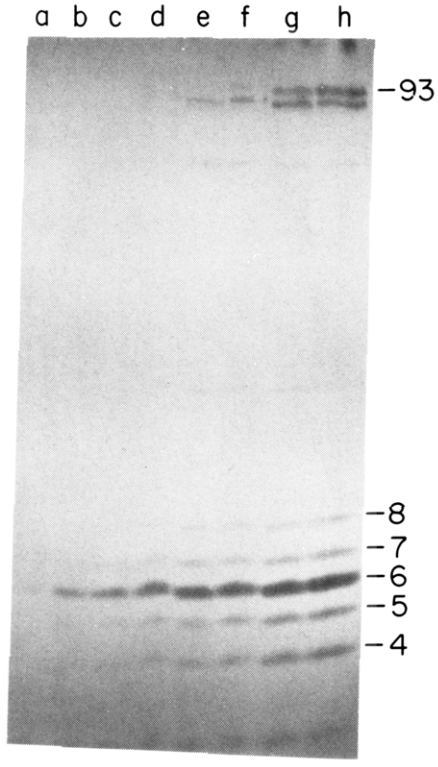

FIGURE 4: Transcripts synthesized from the Tn*5* promoter. RNA polymerase was incubated with the restriction fragment at 37 °C for 10 min. After a 1-min heparin challenge, labeled nucleoside triphosphates were added. Transcription was stopped at the following times: 20 s (a); 40 s (b); 1 min (c); 1.5 min (d); 2 min (e); 3 min (f); 5 min (g); 10 min (h). The band migrating below the 93-base transcript is believed to be a paused product, as in the *lac* system. Both bands were counted as full-length transcripts. Picomoles of RNA produced after 10 min were as follows: tetramer, 0.023; pentamer, 0.008; hexamer, 0.032; heptamer, 0.005; octamer, 0.003; full length, 0.002.

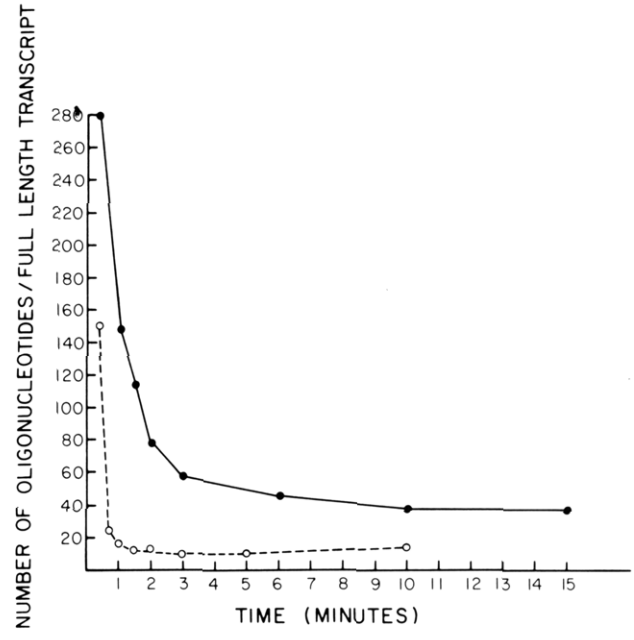

FIGURE 5: Ratio of oligonucleotides produced per full-length transcript. The RNA bands that were quantitated were described in the text for Tn*5*. For the *lac* promoter, the oligonucleotides quantitated were the trinucleotide, tetranucleotide, and the hexanucleotide. The full-length transcripts were determined as described in the legend to Figure 3. Tn*5* (●), *lac* (○).

Figure 5 shows how the ratio of abortive initiation products to full-length transcript changes with time. The ratio decreases from 281 at 20 s to 40 at 6 min. One explanation for the initial high ratio is that there is a certain amount of pausing occurring as the polymerase makes the first few phosphodiester bonds. Some of these early oligonucleotides are paused products that are eventually chased into full-length transcripts. After several minutes, the ratio becomes steady at approximately 40 oligonucleotides per full-length transcript. As can be seen from Figure 5, the *lac* promoter also has an initial high ratio that rapidly becomes steady at approximately 11 oligonucleotides per full-length transcript.

Assay Conditions. The possibility that changes in the in vitro assay conditions might alter the half-time of productive initiation was explored. Variations in the polymerase to DNA ratio, heparin concentration (from 10 to 300 μg/mL), salt concentration, and UTP concentration (from 10 to 200 μM) did not affect the half-time. Initiating transcription by addition of magnesium instead of nucleoside triphosphates also had no effect on the half-time.

Assays were performed by utilizing supercoiled and linear plasmids to test whether the high yield of oligonucleotides was a result of using a restriction fragment as a template. A substantial amount of oligonucleotides was seen, and although these were not quantitated, it is clear that abortive initiation also occurs with these templates.

Discussion

Two lines of evidence have been presented that suggest that the half-time of productive initiation is directly related to the amount of abortive initiation or recycling that can occur. When RNA polymerase is allowed to undergo limited transcription in the presence of ATP, UTP, and GTP, a complex is formed that has a much lower capacity for recycling through the initiation process and a much higher rate of synthesis of the full-length transcript. This complex is not formed when only ATP and UTP are added to the incubation mixture. These results indicate that the formation of a ternary complex composed of the polymerase, DNA, and a newly synthesized RNA between four and nine bases in length decreases the ability of the polymerase to recycle through the initiation process. This may be due to either the stability of the complex or the inability of the polymerase to start over after moving away from the promoter. Since oligonucleotides up to six bases in length can be made reiteratively from the *lac* promoter, we further suggest that the complex with a lower capacity for recycling is formed after synthesis of an RNA between seven and nine nucleotides long. In fact, when challenged with radioactive nucleotides, ternary complexes containing nine-base-long RNA synthesize a full-length transcript in less than a third of the time it normally takes.

The second correlation between abortive initiation and the rate of productive initiation stems from comparative studies between the *lac* system and the Tn*5* system. The Tn*5* promoter was seen to have approximately 4 times as many abortive initiation products relative to the full-length transcripts as the *lac* promoter, and the half-time was found to be more than 5 times as long. Consistent with this model is the extremely rapid half-time and relatively little abortive initiation found in the T7 system (Nierman & Chamberlin, 1979).

The amount of oligonucleotides measured is an underestimation of the true quantities produced under these reaction conditions. This is due to the fact that the dinucleoside tetraphosphate pppApA and the trinucleoside pentaphosphate pppApApU were not quantitated. The former was not labeled by the [α-^{32}P]UTP, and the latter frequently comigrated with a radioactive contaminant found in the commercial [α-^{32}P]-UTP. It has recently been shown that 50% of all RNA made

from the *lac* promoter is the dinucleoside tetraphosphate and that a substantial amount of the trinucleotide is also aborted (Carpousis & Gralla, 1980). This would mean our oligonucleotide yields should at least be doubled. Even with these limitations, our results clearly indicate that small RNAs are aborted a high percentage of the time and that more than one transcript is made per promoter, confirming observations made by Carpousis and Gralla.

It is clear from analysis of Tn*5*, *lac*PL8UV5, *lac*P^{r}115 (R. Johnson, unpublished experiments), and λP_L (G. T. Horn and R. D. Wells, unpublished experiments) that these promoters each produce abortive initiation products in characteristic sizes and quantities. Whether the amount of abortive initiation is determined by the promoter sequence or by the mRNA sequence cannot as yet be determined. The wild-type *lac* promoter and the *lac*L8UV5 promoter differ by two base pairs within the promoter sequence (T/A → A/T at −8, G/C → A/T at −9) but behave similarly in these assays. The *lac*P^{r}111 (C/G → A/T at +10) promoter changes both the promoter activity and the mRNA sequence, as compared to either the wild-type or the *lac*L8UV5 promoter. It also has the same half-time of productive initiation. These results indicate that the sequences changed by these mutations do not affect the amount of abortive initiation. More promoter mutants should be studied before any conclusions as to the role the promoter sequence plays in abortive initiation can be drawn. The Tn*5* promoter has both a different promoter sequence and a different mRNA sequence, so one cannot distinguish which is important.

Recent studies on poly(dA-dT) have yielded very interesting results with regard to stable complex formation and RNA sequence (Sylvester & Cashel, 1980). It was found that ApUpA is made in stoichiometric amounts from ApU and ATP and forms a stable ternary complex with the polymerase and DNA. On the other hand, UpApU is made in catalytic amounts from UpA and UTP and does not form a stable ternary complex. These results suggest a relationship between the mRNA sequence and the amount of abortive initiation or stable complex formation. Studies on natural promoters with different mRNA sequences may provide further insight into the role mRNA sequences play in abortive initiation.

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