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Interaction of RNA Polymerase with *lac*UV5 Promoter DNA during mRNA Initiation and Elongation

Footprinting, Methylation, and Rifampicin-sensitivity Changes Accompanying Transcription Initiation

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We have used enzymatic and chemical probes to follow the movement of *Escherichia coli* RNA polymerase along *lac*UV5 promoter DNA during transcription initiation. The RNA polymerase does not escape from the promoter but remains tightly bound during the synthesis of the initial bases of the transcript. This initial phase of RNA synthesis involves the reiterative synthesis and release of RNA chains up to ten bases long *via* the RNA polymerase cycling reaction and the enzyme remains sensitive to rifampicin inhibition. When longer chains are made, promoter-specific binding is disrupted and the enzyme forms a rifampicin-resistant elongation complex with downstream DNA sequences. This elongation complex covers less than half as much DNA and lacks the DNase I-hypersensitive sites and the base-specific contacts that characterize promoter-bound RNA polymerase. These results lead us to suggest that *lac*UV5 mRNA synthesis is primed by a promoter-bound enzyme complex that synthesizes the initial nine or ten bases in the mRNA chain. Subsequently, when a chain of ten bases, or slightly longer, is made, contacts with promoter DNA are irreversibly disrupted, sigma subunit is lost, and a "true" elongation complex is formed.

1. Introduction

Transcription initiation requires two quite different types of interactions between RNA polymerase and promoter DNA. First, the enzyme must recognize promoter DNA and bind tightly to form an open promoter complex. Subsequently, the enzyme must choose the precise transcription initiation point on the template, initiate the synthesis of an RNA chain and escape from the stable open promoter complex to elongate the mRNA. Thus, the overall process requires a balancing of two conflicting requirements; promoter binding should be tight in order to establish proper recognition, but not so tight as to prevent the polymerase from escaping the promoter to elongate mRNA.

Under certain conditions *in vitro*, RNA polymerase rapidly forms open complexes at the *lac*UV5 promoter, but then escapes from these complexes slowly (Stefano & Gralla, 1979). That is, *lac*UV5 acts as a "slow start" promoter. Indirect experimental evidence indicates that RNA polymerase is unable to escape from the *lac*UV5 promoter even after forming the first four to six mRNA phosphodiester bonds, but is instead initially stalled at the promoter and engaged in non-productive abortive initiation (Carpousis & Gralla, 1980). We called this process RNA polymerase cycling, since the enzyme was observed to produce short RNA chains reiteratively without apparently leaving the promoter, as evidenced by resistance to heparin, a selective inhibitor of free RNA polymerase. Cycling leads to the synthesis of many short RNA chains for each full-length mRNA produced. Subsequent studies *in vitro* found that abundant short RNA production also accompanies synthesis of the bacterial Tn*5* mRNA (Munson & Reznikoff, 1981), the eukaryotic cytoplasmic polyhedrosis virus and reovirus mRNAs (Furuichi, 1981; Yamakawa *et al.*,

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1981), and the adenovirus major late mRNA (Ackerman *et al.*, 1983). Short RNAs were also proposed to be synthesized and used as primers for polyoma virus early transcription (Cowie *et al.*, 1982). Thus, bacterial, viral and mammalian polymerases appear to have the capability to cycle during initiation.

The process of RNA chain initiation is further complicated by the existence of microheterogeneity in the 5′ ends of some bacterial and mammalian transcripts (see Carpousis *et al.*, 1982). How does RNA polymerase cycle to produce short RNAs, and how can microheterogeneous mRNA 5′ ends be initiated from a small region as opposed to at a precise startpoint? A simple mechanism that can account for both phenomena is that promoter-bound RNA polymerase chooses its initiating nucleotide and synthesizes short RNA chains from a small initiation region without actually breaking the strong contacts that bind it to promoter DNA (Carpousis & Gralla, 1980; Carpousis *et al.*, 1982). Below, we extend our knowledge of the process of transcription initiation and find support for this proposal by probing the interaction of RNA polymerase with template DNA during mRNA initiation and elongation at the *lac*UV5 promoter.

2. Materials and Methods

Escherichia coli RNA polymerase was prepared according to the method of Burgess & Jendrisak (1975) and further purified according to the procedure of Gonzalez *et al.* (1977) to obtain sigma-saturated holoenzyme. The holoenzyme was about 70% active as judged by the amount of polymerase needed to saturate the *lac*UV5 promoter (see Stefano & Gralla, 1982*b*). The 203 base-pair *lac*UV5 promoter DNA fragment was prepared as described (Stefano & Gralla, 1980). Nucleoside triphosphates, rifampicin, and heparin were from Sigma. ATP, UTP and GTP were purified further as described (Carpousis & Gralla, 1980). [^{32}P]phosphate, HCl-free, was from ICN. [α-^{32}P]ATP, [α-^{32}P]dATP and [γ-^{32}P]ATP were prepared essentially as described (Carpousis & Gralla, 1980; Carpousis *et al.*, 1982). DNase I and restriction endonuclease *Pvu*II were from Boehringer. A DNase I stock (5 mg/ml in 10^{-4} M-HCl) was prepared and stored at −20°C. Reagents used in DNA modification and sequencing reactions were from Aldrich or Matheson, Coleman and Bell, and were the highest purity grade available.

(a) *RNA polymerase cycling and transcription*

Reaction conditions were essentially as described (Carpousis *et al.*, 1982). A portion (4·0 μl) of RNA polymerase (0·71 μM, active concn) and 10·0 μl of 203-base-pair *lac*UV5 (0·10 μM) in transcription buffer (30 mM-Tris·HCl (pH 8), 100 mM-KCl, 3 mM-$MgCl_2$, 0·1 mM-EDTA, 0·2 mM-dithiothreitol, 0·1 mg acetylated bovine serum albumin/ml) were mixed and incubated at 37°C for 15 min to form open promoter complexes. Heparin (2·0 μl of 1 mg/ml in transcription buffer) was then added and the reaction was incubated at 37°C for 6 min to inactivate any unbound RNA polymerase. Cycling or transcription was then initiated by addition of 4·0 μl containing 1, 2, 3 or all 4 nucleoside triphosphates in transcription buffer and the reaction was incubated for another 15 min at 37°C. The final concentration of nucleoside triphosphates was 100 μM-ATP, -UTP and -GTP, and 10 μM-CTP. [α-^{32}P]ATP at a specific activity of 5 to 20 Ci/mmol was used in these reactions because it labels all species of RNA made at the *lac*UV5 promoter (see Carpousis & Gralla, 1980). The reactions were stopped and the products analyzed by direct loading on high-resolution polyacrylamide gels as described (Carpousis & Gralla, 1980).

(b) *Rifampicin-chase experiment*

Reactions employing rifampicin were similar to those described above. Open complex formation, addition of heparin, and addition of nucleoside triphosphates was the same. After 15 min incubation in the presence of 1, 2 or 3 nucleoside triphosphates 2·0 μl of rifampicin (1 mM in transcription buffer) were added and the reaction was incubated at 37°C for 6 min to inactivate sensitive complexes. Then, 2·0 μl containing the "missing" nucleoside triphosphates (1 mM-UTP and -GTP, 0·1 mM-CTP) in transcription buffer were added and the reactions were incubated for another 15 min at 37°C to permit mRNA elongation.

(c) *Preparation of ^{32}P-end-labeled promoter DNA fragments, DNase footprinting and DNA methylation*

*lac*UV5 203 base-pair promoter DNA was (5′-^{32}P)-end-labeled using [γ-^{32}P]ATP and polynucleotide kinase (Maxam & Gilbert, 1980); or (3′-^{32}P)-end-labeled using [α-^{32}P]dATP and DNA polymerase I by essentially the same procedure, described by Schmitz (1981). Singly end-labeled promoter DNA was prepared by digesting the ^{32}P-end-labeled 203 base-pair fragment with *Pvu*II and isolating a 190 base-pair promoter DNA fragment by polyacrylamide gel electrophoresis (Maxam & Gilbert, 1980).

DNase footprinting was performed using a modification of the procedure of Schmitz & Galas (1979). Transcription reactions were performed as described above, except that ^{32}P-end-labeled 190 base-pair promoter DNA (100,000 to 200,000 cts/min per reaction) was used instead of 203 base-pair fragment and the [α-^{32}P]ATP was omitted. After open complex formation and transcription, 5·0 μl of transcription buffer containing 0·4 μg DNase I/ml and 1·5 mM-$CaCl_2$ were added and the reactions were incubated at 37°C for another 6 min to partially digest the radioactively labeled DNA. $CaCl_2$ was added at this step to activate the DNase I. Reactions were stopped by the addition of 100 μl of 50 mM-Tris·HCl (pH 8), 5 mM-NaEDTA, 0·1% (w/v) sodium dodecyl sulfate, 10 μg carrier DNA/ml. The reactions were extracted twice with 100 μl of redistilled, neutralized phenol; desalted using a 1 ml Sephadex G50 column eluted by centrifugation (see modification of the procedure of Penefsky; Carpousis *et al.*, 1982); and precipitated using 0·1 vol. 3 M-sodium acetate and 2 vol. ethanol. The DNase-digested, ^{32}P-end-labeled DNA was analyzed by electrophoresis on denaturing 8% polyacrylamide gels (Maxam & Gilbert, 1980). Polyacrylamide gels were dried on Whatman 3MM paper and autoradiograms were made using Cronex 4 X-ray film and, in some cases, Quanta II intensifying screens (Du Pont) at −70°C.

The DNase footprinting protocol described above was used for DNA methylation with the following changes: 75 mM-dimethylsulfate was used in place of 0·4 μg DNase I/ml. The dimethylsulfate was diluted into transcription buffer immediately before use. Preliminary experiments showed that the dimethylsulfate did not change the pH

of the transcription buffer during the course of the reaction, and that the extent of DNA methylation under our conditions was comparable to the standard "G only" DNA sequencing reaction (Maxam & Gilbert, 1980). The stop mixture used for these reactions included 1 M-β-mercaptoethanol to inactivate unreacted dimethylsulfate. The methylated DNA was deproteinated, desalted and precipitated with ethanol as described above. Methylated DNA was suspended in 10 μl of water and 15 μl of hydrazine were added to preferentially cleave any methylated cytosine residues (Kierkegaard *et al.*, 1983). The mixture was incubated at room temperature for 5 min and the reaction was then stopped by the addition of 100 μl of 5 M-acetic acid. The reaction was desalted as described above and the DNA was cleaved with 1 M-piperidine following standard DNA sequencing procedures (Maxam & Gilbert, 1980). The piperidine was neutralized by the addition of 0·25 vol. 5 M-acetic acid; the DNA was desalted, precipitated with ethanol and analyzed by gel electrophoresis as described above.

3. Results

The experimental approach involves using rifampicin, DNase I and dimethylsulfate probes to follow the progress of RNA polymerase as it escapes from the *lac*UV5 promoter to form mRNA *in vitro*. Combinations of nucleoside triphosphates will be added to transcription reactions in order to restrict synthesis to short RNA chains of known length. Probes will then be applied to determine how far the enzyme has moved along the DNA and at what point the open promoter complex is replaced by a complex fully committed to chain elongation.

(a) *At what chain length during mRNA initiation at the* lac*UV5 promoter is a rifampicin-resistant complex formed?*

The major transcripts *in vitro* from the *lac*UV5 promoter start from two adjacent purine bases to give an mRNA 5′ end sequence of pppGpApApUpUp . . . or pppApApUpUpGp . . . (Maizels, 1973). Transcription *in vitro* in the presence of all four nucleoside triphosphates yields, in addition to full-length mRNA, an excess of short RNA chains up to ten bases long (Carpousis & Gralla, 1980). The majority of these short RNA chains initiate with ATP and their sequence corresponds to the 5′ end of the ATP-initiated full-length mRNA. The synthesis of these short RNAs can be increased by omitting nucleotides required for chain elongation. The length of the short RNAs synthesized in the presence of various nucleotide combinations can be monitored easily by gel electrophoresis.

Open promoter complexes were formed and heparin was added to inactivate any free RNA polymerase, thereby preventing re-binding to promoters that might become vacant during transcription. Transcription was then initiated by the addition of certain nucleoside triphosphates including [α-^{32}P]ATP. The products were loaded directly onto a high-resolution denaturing polyacrylamide gel, and all RNA species produced from two bases long to full-length transcript were separated (Carpousis & Gralla, 1980). Figure 1, lanes 2 to 5, shows the results of an experiment where one, two, three or all four nucleoside triphosphates were added to preformed open promoter complexes. Lane 2 shows that, in the presence of ATP, the initial dinucleotide (pppApA) of the ATP-initiated *lac* mRNA accounts for virtually all of the RNA synthesized. Lanes 3 to 5 display the products of identical reactions, except that ATP and UTP were added (lane 3), ATP, UTP and GTP were added (lane 4), or all four nucleoside triphosphates were added (lane 5). The inclusion of additional nucleotides leads to RNA species of increasing length. These short RNAs were identified previously (Carpousis & Gralla, 1980), and Munson & Reznikoff (1981) have reported similar results. Reactions are performed here under the conditions to be used in footprinting and methylation experiments. Note that, under all conditions, a large excess of short RNAs relative to full-length mRNA ("T") is produced, as reported previously.

Next, we wished to determine whether the addition of the various nucleotide combinations leads to the formation of a rifampicin-resistant complex. The drug rifampicin inhibits initiation by RNA polymerase, but does not affect mRNA elongation by pre-initiated enzymes (Sippel & Hartman, 1968; So & Downey, 1970). That is, if rifampicin is added to transcription reactions, full-length mRNA can be made only if RNA polymerases have already initiated and are committed to chain elongation. Therefore, if a nucleotide combination leads to the formation of such complexes, then the subsequent addition of rifampicin cannot prevent the appearance of full-length transcripts. This can be tested by adding "missing" nucleotides after drug addition and allowing any rifampicin-resistant complexes to complete runoff transcription.

Transcription reactions identical to those described above when ATP, ATP and UTP, or ATP, UTP and GTP were present were carried out. If rifampicin is added to inactivate uninitiated RNA polymerases followed by the addition of "missing" nucleoside triphosphates, only some nucleotide pre-additions lead to the appearance of full-length mRNA. The results of such a "chase" experiment are also shown in Figure 1, lanes 6 to 8. In lanes 6 and 7, ATP or ATP and UTP were added first, followed by the addition of rifampicin and then the missing nucleoside triphosphate. Note that no runoff transcript is produced under these conditions. Since many RNA chains up to four nucleotides in length were produced before the addition of rifampicin, it is clear that a rifampicin-resistant complex is not formed before the formation of a tetranucleotide at this promoter.

A different result is obtained when longer chains are synthesized in the presence of three nucleoside triphosphates. Figure 1, lane 8, shows the results of transcription reactions where ATP, UTP and GTP were added first, followed by the addition of rifampicin and CTP. Note that a full-length runoff

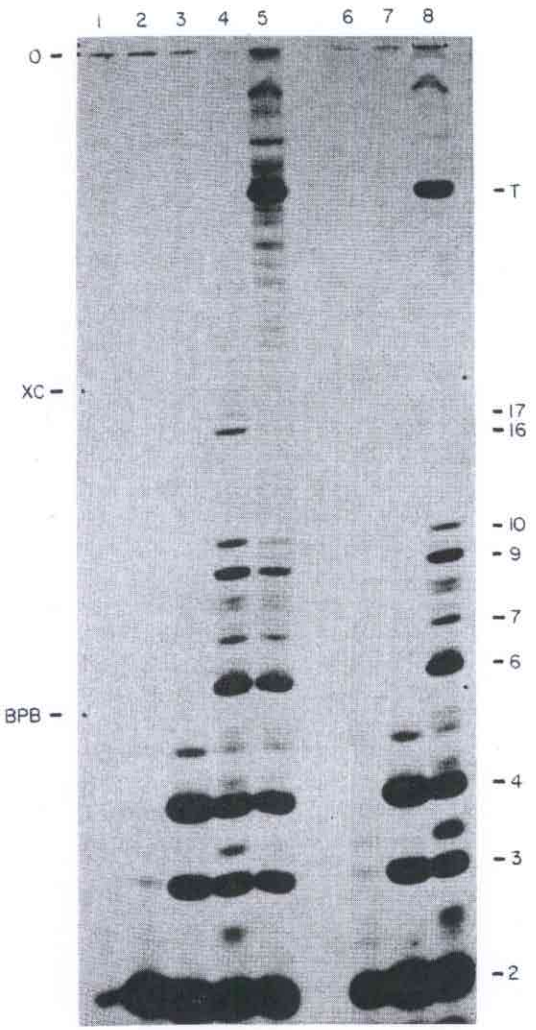

Figure 1. Autoradiogram of RNAs synthesized by the *lac*UV5 promoter. Lane 1, control in which ATP was present but promoter DNA was omitted from the reaction; lane 2, synthesis in the presence of ATP; lane 3, synthesis in the presence of ATP and UTP; lane 4, synthesis in the presence of ATP, UTP and GTP; lane 5, synthesis in the presence of ATP, UTP, GTP and CTP; lane 6, synthesis in the presence of ATP followed by the addition of rifampicin, then UTP, GTP and CTP; lane 7, synthesis in the presence of ATP and UTP followed by the addition of rifampicin, then GTP and CTP; lane 8, synthesis in the presence of ATP, UTP and GTP followed by the addition of rifampicin, then CTP. The position of products 2, 3, 4, 6, 7, 9, 10, 16 and 17 bases long and the full-length runoff transcript (T) is indicated on the right. Quantitation of the incorporated radioactivity in lane 5 indicates that, under the conditions used here, about 50 short RNAs are made for each full-length transcript synthesized, and the yield of full-length mRNA is about 35% relative to the input template DNA (data not shown). Heparin was added to these reactions after open promoter complex formation (see Materials and Methods). If heparin is added before polymerase addition, synthesis is abolished totally (see Carpousis & Gralla, 1980). The faint bands in the no promoter DNA control (lane 1) are due to minor contaminants in the [α-^{32}P]ATP. The origin (O) and the positions of the marker dyes xylene cyanol (XC) and bromophenol blue (BPB) are indicated on the left. The [α-^{32}P]ATP used in these reactions is not visible because it ran off the bottom of the gel.

transcript is synthesized in this reaction (compare lanes 4 and 8). That is, the addition of these three nucleotides leads to the formation of a rifampicin-resistant complex that can be chased into mRNA in the presence of the drug.

This "rifampicin-chase" experiment also reveals the approximate length of nascent RNA that must be synthesized in order to form the rifampicin-resistant complex. At the time of drug addition, all DNA-bound RNA polymerases will have just completed the synthesis of an RNA chain of some length. If a particular enzyme is committed to mRNA elongation, it will be unaffected by drug addition; the addition of the missing nucleotides will then allow that enzyme to continue beyond the site of pausing and complete runoff transcription. This will be revealed by the disappearance of the short RNAs that are waiting at the sites of paused synthesis. A comparison of lanes 4 and 8 in Figure 1 shows only two short RNAs, which are completely chased into full-length runoff transcript. These RNAs are nascent chains 16 and 17 bases long, corresponding to ATP and GTP-initiated species paused at the same point (see Fig. 2). Clearly, all RNA polymerases paused at this position are in rifampicin-resistant complexes.

The major RNA bands (2, 3, 4, 6, 7, 9, 10, 16, 17 and T) in lanes 4 and 8 were excised and the radioactivity counted to learn if any shorter length RNAs might be at least partially chased into runoff transcript in the presence of rifampicin. There was no detectable chase of any product up to nine bases long. Therefore, no detectable paused rifampicin-resistant complexes are formed at this promoter before formation of nascent RNA at least ten bases long. The RNA band of length ten was partially chased into runoff RNA. Quantitation revealed that approximately one-third of this species disappeared upon addition of rifampicin and CTP. The amount of ten-base-long RNA chased into full-length mRNA accounted for about 10% of the total mRNA synthesized in the chase, with the other 90% coming from the 16 and 17-base-long products. From this result, we conclude that a fraction of the rifampicin-resistant RNA polymerases are paused at position +10. The majority of complexes, however, are paused at position +16.

Figure 2 summarizes these data by showing the major RNA chains detected in these experiments and indicating whether their synthesis results in a rifampicin-resistant complex. The products 16 and 17 bases long correspond to ATP and GTP-initiated RNA chains paused at the site of addition of the second CTP to the *lac* mRNA chain. The omission of CTP should have restricted synthesis to chains ten bases long or less. However, there is apparently significant "readthrough" upon addition of ATP, UTP and GTP. This may be due to either a trace contamination of CTP in the reaction or occasional misincorporation of a different nucleoside triphosphate for CTP. Since the nucleoside triphosphates employed in these reactions were highly purified (see Carpousis & Gralla, 1980), the contribution due

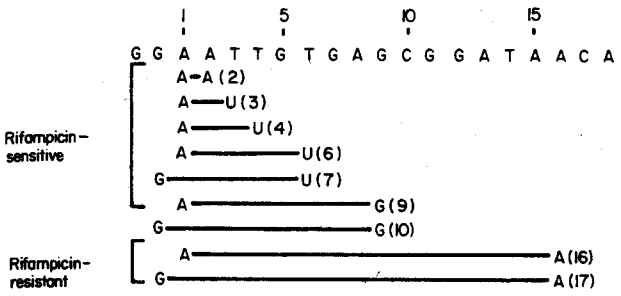

Figure 2. Sequence of the "top strand" of the *lac*UV5 initiation region and short RNA products synthesized from this region (hyphens omitted for clarity). The sequence is numbered relative to the predominant start point (+1). The base at the 5′ and 3′ termini of each short RNA is shown and the number of nucleotides in each RNA is indicated to the right in parentheses. Products 2 to 9 bases long are made reiteratively by a rifampicin-sensitive initiation complex. Products 16 and 17 nucleotides long are associated with a paused rifampicin-resistant elongation complex. A minor fraction of the products 10 bases long are apparently associated with a rifampicin-resistant complex. However, the majority of products 10 bases long cannot be elongated to full-length in RNA in the presence of rifampicin (see the text for discussion).

to contaminating CTP should be very small. Misincorporation seems more probable, since the experimental protocol requires RNA polymerase to pause for a long time (up to 15 min) at the site where the first CTP is added to the chain, allowing ample opportunity for the addition of a non-complementary nucleotide for CTP. Longer exposures of the gel in Figure 1 show very few RNA chains greater than 17 bases long synthesized in the presence of ATP, UTP and GTP (lane 4). The site of addition of the second CTP in the *lac*UV5 mRNA is known to be a very strong pause site when the CTP concentration is low (Maizels, 1973). Therefore, although the majority of the polymerases read-through the first CTP in the *lac* mRNA under these conditions, they are efficiently paused at the site of addition of the second CTP.

At this point, we can make the following conclusions relevant to the nature of complexes that will be subjected to DNase I footprinting and methylation. When either ATP, or ATP and UTP are added, no rifampicin-resistant complexes are formed, despite the observation that many RNAs up to four bases long are synthesized. In the presence of ATP, UTP and GTP, rifampicin-resistant complexes are formed with about 90% of the RNA polymerase paused at position +16 in the sequence shown in Figure 2.

(b) *Interaction of RNA polymerase with* lac *DNA during mRNA initiation and elongation probed by DNase I footprinting*

The aim of the experiments presented below is to learn the extent and position of the RNA polymerase interaction with the promoter-containing DNA as RNA of increasing length is synthesized. We used the DNase footprinting method (Schmitz & Galas, 1979) to determine the precise location of RNA polymerase on the sequenced promoter DNA. The basic transcription experiments described above were repeated, then DNase I was added to probe the interaction of the RNA polymerase with promoter DNA. The polymerase interaction sites on both strands can be identified, since either the "top" or the "bottom" strand can be ^{32}P-end-labeled independently. Lane 1 in Figure 3 shows the partial DNase I cleavage patterns of the (a) top and (b) bottom strands without RNA polymerase. Lanes 7 and 8 in Figure 3(a) and (b) show parallel DNA sequencing reactions of these same DNAs to provide precise reference points for DNase I cleavage. The sequence is numbered relative to the start site of the ATP-initiated *lac*UV5 mRNA (+1).

The partial DNase I cleavages were repeated on polymerase–promoter complexes after RNAs of various lengths were synthesized. In the absence of RNA synthesis (lane 2, Fig. 3(a) and (b)), the footprint is that of the stable open promoter complex. The protection due to bound polymerase extends from approximately −50 to +20, and there are striking DNase I-hypersensitive sites on both strands in the region from −20 to −50. This pattern is very similar if not identical to that observed by Schmitz & Galas (1979). In other experiments, nucleotides were added to the complexes allowing formation of RNA chains of up to the following lengths (Fig. 3(a) and (b)): two (ATP added; lane 3), four (ATP and UTP added; lane 4), 16 or 17 (ATP, UTP, and GTP added; lane 5), and full-length mRNA (ATP, UTP, GTP, and CTP added; lane 6).

Qualitatively, the results of these experiments are simple to interpret. The patterns of protection in Figure 3 appear identical whether the complex synthesizes no RNA (lane 2), two base long RNA (lane 3), or chains up to four bases long (lane 4). One can compare lanes 2, 3, and 4 (Fig. 3(a) and (b)) band by band and observe no changes in intensity that would indicate movement of the polymerase. This is also true near the right (downstream) border of the footprint, where polymerase might be expected to move and protect additional sequences in the presence of ATP and UTP. There is no detectable change in the extent or position or binding nor is there any change in the characteristic internal DNase-hypersensitive sites. These autoradiograms were also scanned with a densitometer in an attempt to detect any movement of RNA polymerase further into the transcribed region, but none was detected. It is clear from these results that the polymerase remains bound at the promoter during the reiterative synthesis of RNA chains up to four bases long. Recall that, by the criterion of rifampicin sensitivity, these RNA polymerases are still not initiated.

Figure 3. DNase footprints before, during and after mRNA initiation at the *lac*UV5 promoter. The footprints in this Figure show the interaction of RNA polymerase with (a) the top strand and (b) the bottom strand of the promoter DNA. In each panel, lane 1 is a control showing the digestion of promoter DNA when heparin is added before RNA polymerase. This lane is identical to controls in which RNA polymerase is omitted (not shown) and it shows that the heparin efficiently prevents binding to promoter DNA but does not interfere with DNase digestion. Lanes 2 to 6 show RNA polymerase footprints. No nucleotides were added (lane 2); ATP was added (lane 3); ATP and UTP were added (lane 4); ATP, UTP and GTP were added (lane 5); or ATP, UTP, GTP and CTP were added (lane 6). Lanes 7 and 8 are DNA sequencing reactions showing G + A or C + T cleavages (reaction performed according to Maxam & Gilbert, 1980). The sequencing patterns were used to align the known sequence of the promoter DNA with the DNase digestion pattern, and the DNase cleavage positions are numbered relative to the start of transcriptions (+1 being the *lac* A_1 start point). In (b), the DNA sequencing and DNase ladders do not exactly line up. This is particularly noticeable with the shorter products and is due to the fact that the DNase products have 3′ hydroxyl groups, whereas the sequencing products contain 3′ phosphate groups. This additional phosphate makes the short sequencing products migrate noticeably faster than their DNase I counterparts.

By contrast, when ATP, UTP and GTP are added to form a rifampicin-resistant complex with about 90% of the polymerases paused at position +16, there is a significant change in the footprint pattern. Nearly all of the protection from approximately −50 to −5 is lost, and the DNase I-hypersensitive sites characteristic of the open promoter complex are greatly diminished (lane 5, Fig. 3(a) and (b)). The polymerase now protects a much smaller region extending from about −5 to +25 and no new DNase I hypersensitive sites appear in this region. Note that there is clearly some new protection on the top strand (lane 5, Fig. 3(a)) at positions +24 and +26. These data demonstrate that the RNA polymerase finally leaves the promoter upon synthesis of nascent mRNA chains paused predominantly at position +16 to form an elongation complex. Note that the elongation complex is formed under the same conditions that lead to rifampicin-resistance (see above).

Finally, lane 6 in Figure 3(a) and (b) shows the footprint that results when all four nucleoside triphosphates are added. Most of the protection and hypersensitivity is lost and the pattern is essentially the same as that in which RNA polymerase was omitted (Fig. 3(a) and (b), lane 1). This loss of footprint is a consequence of polymerase movement away from the promoter during full-length mRNA synthesis. Since these experiments were done in the presence of heparin, which selectively inactivates free RNA polymerase, new open promoter complex formation and re-initiation is inhibited.

The patterns resulting from the addition of three or four nucleoside triphosphates (lanes 5 and 6, Fig. 3(a) and (b)) show some residual DNase I-hypersensitivity, characteristic of the open promoter complex. This is likely due to the failure of a minor fraction of the RNA polymerases to initiate (move) under the conditions used in these experiments. Visual inspection of the autoradiograms can be somewhat misleading, because the bands on the autoradiograms corresponding to DNase-hypersensitive sites are heavily exposed. From the loss of protection in the −50 to −5 region, it is clear that nearly all of the RNA polymerases have initiated upon addition of three or four nucleoside triphosphates. Failure of a minor fraction of the polymerase to move could be due to insufficient time to allow all the polymerases to initiate, or possibly a small fraction of the bound RNA polymerase is incapable of initiating mRNA synthesis.

(c) *Interaction of RNA polymerase with* lac *DNA during mRNA initiation and elongation probed by DNA methylation*

The DNase I footprint experiments presented above give information about the location of the RNA polymerase on the template DNA. The chemical modification of DNA with dimethylsulfate in the presence of bound RNA polymerase probes specific contact points in the nucleoprotein complex and therefore can detect more subtle changes in the interaction (for a review, see Siebenlist *et al.*, 1980). Kierkegaard *et al.* (1983) described a related method using dimethylsulfate that can detect cytosine methylation in single-stranded DNA and thus identify such residues within the melted region of an open promoter complex. Combining both methods, melted G·C base-pairs and some specific contacts with G residues can be detected in the same reaction. We performed experiments under the same conditions as the DNase I footprinting described above but using dimethylsulfate modification instead of nuclease digestion to probe promoter–polymerase complexes. The aim of these experiments was to identify specific contacts to the promoter DNA and also to locate the "melted" sequences in complexes involved in the *lac* mRNA initiation.

Figure 4 shows autoradiograms of methylation experiments using templates where the (a) top or (b) bottom strand of the DNA was end-labeled. In Figure 4(a) and (b), lane 1, methylation was performed in the absence of added RNA polymerase. The subsequent cleavage protocol leads to a ladder with strong bands that correspond to cleavage at G residues and faint bands that correspond to the cleavage at pyrimidine residues. These assignments were confirmed by the partial sequence analysis shown in lanes 7 and 8. Lane 2 shows the same methylation experiment repeated on the open promoter complex in the absence of added nucleotides. Certain bands are either diminished or enhanced in the presence of enzyme, indicating either decreased or increased reactivity of those residues to dimethylsulfate attack. This pattern is characteristic of the specific binding of RNA polymerase to *lac*UV5 DNA in an open promoter complex. As reported previously (Siebenlist *et al.*, 1980), the principal protected G residues flank the −10 region on the top strand (−6 and −13) and lie within the −35 region on the bottom strand (−32). As also reported previously, single-stranded C residues are detected at positions −1, −2, −4 and −6 on the bottom strand (Fig. 4(b)), as evidenced by their methylation at the normal base-pairing position and appearance as strong bands only in the presence of RNA polymerase (Kierkegaard *et al.*, 1983). This whole collection of bands provides the best markers for the molecular contacts near −10, −35 and the melted region, which characterize the stable open promoter complex.

Next, the basic experiment was repeated under transcription conditions where short RNAs were being synthesized but a rifampicin-resistant elongation complex had not yet formed. Figure 4 shows the patterns that result when the methylation probe was applied while short RNA chains 2 bases long (lane 3, Fig. 4(a) and (b)) or up to four bases long (lane 4) were being synthesized as described above. Careful inspection of the autoradiogram

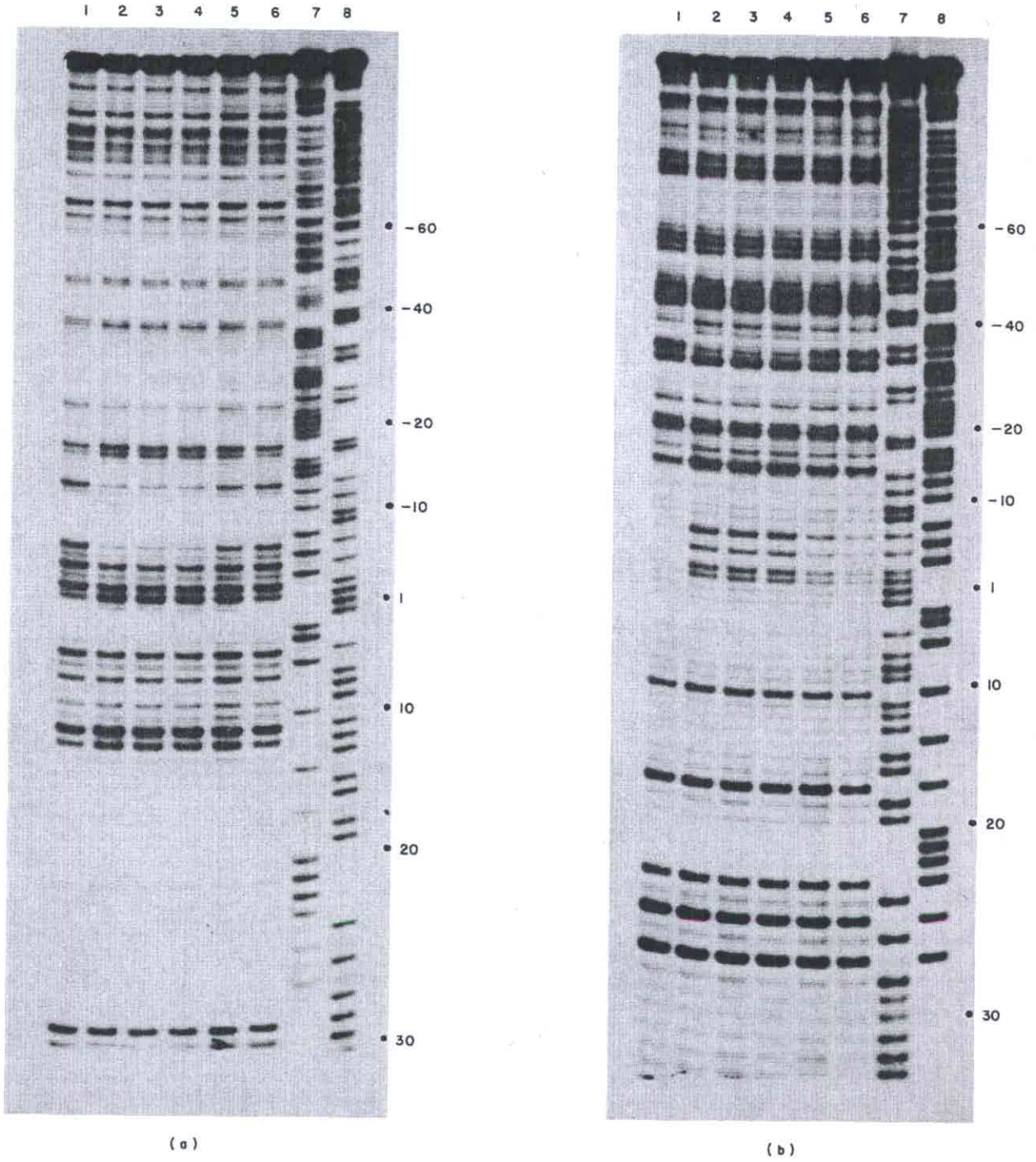

Figure 4. DNA methylation before, during and after mRNA initiation at the *lac*UV5 promoter. The experiment presented in this Figure is identical to that presented in Fig. 3, except that dimethylsulfate was used instead of DNase I. See the legend to Fig. 3 for a description of panels and lanes. Lane 1 ((a) and (b)) is a control in which heparin was added before RNA polymerase. This lane is identical to controls in which polymerase was omitted (not shown), and it shows that the heparin efficiently prevents binding to the promoter DNA but does not interfere with the methylation reactions. The heavy bands in the pattern represent methylations of guanine residues in the major groove of the helix and the very light bands are cleavages at pyrimidine residues that occur during the brief treatment with hydrazine after the DNA is methylated (see Materials and Methods). Binding of polymerase to double helical DNA can give either protection and enhancement of guanine methylation, and formation of melted regions leads to methylation and cleavage of single-stranded cytosine residues. In this Figure, lane 7 ((a) and (b)) shows C+T DNA sequencing cleavages and lane 8 ((a) and (b)) shows G+A sequencing cleavages.

reveals no change in the methylation pattern after the reiterative synthesis and release of short RNAs up to four bases long. The specific contacts between the polymerase and the DNA near the −10 and −35 region are not disrupted by the synthesis of a short RNA chain, nor is there a detectable change in the position or extent of the melted DNA region. These results demonstrate that the specific contacts that bind the polymerase to the promoter are not yet broken, despite abundant short RNA synthesis. Furthermore, they complement the DNase I footprinting results that showed no detectable movement of the polymerase under these conditions. Taken together, these results show that the detailed interaction of the RNA polymerase with the promoter DNA is not altered under these conditions.

By contrast, a different result is obtained in the presence of ATP, UTP and GTP, when a rifampicin-resistant elongation complex is formed. Figure 4(a) and (b), lane 5, shows the methylation pattern when about 90% of the polymerases are in an elongation complex with transcription paused at position +16. Note that most of the distinctive methylation pattern induced by contacts characteristic of the open promoter complex is lost. Enhancement or suppression of G residue methylation in the double helical portion of the promoter DNA disappears, as does most of the methylation of melted C residues at positions −1, −2, −4 and −6 on the bottom strand (Fig. 4(b), lane 5). This result demonstrates that the specific contacts in the promoter DNA have now been disrupted and the melted region about the mRNA start site has renatured. This is in agreement with the DNase footprinting, which showed that the RNA polymerase moves from the promoter under these conditions to form an elongation complex.

It is somewhat surprising that the methylation probe does not reveal any specific contacts characteristic of an elongation complex. Figure 4(a) and (b), lane 5, shows no significant enhancement or protection of G residues from dimethylsulfate attack in the region from −5 to +25 where DNase I footprinting shows protection by the elongation complex (see above). As discussed below, this may be due to the non-specific nature of the interaction between RNA polymerase and template DNA during elongation. Furthermore, we fail to detect clearly any melted G·C base-pairs in this complex. On the bottom strand there are C residues from −5 to +25 at −4, −2, −1, +5, +11 and +12. These residues are likely to be base-paired either to the top strand of the template DNA or the nascent mRNA, and are therefore protected from attack by dimethylsulfate. On the top strand from −5 to +25 there are C residues only at +10, +17 and +23. The residues at +10 and +17 should be melted. However, Figure 5(a), lane 5, shows that there may be some weak methylation at +10, but the residue at +17 is clearly not attacked by dimethylsulfate. Possible explanations of this result are discussed below.

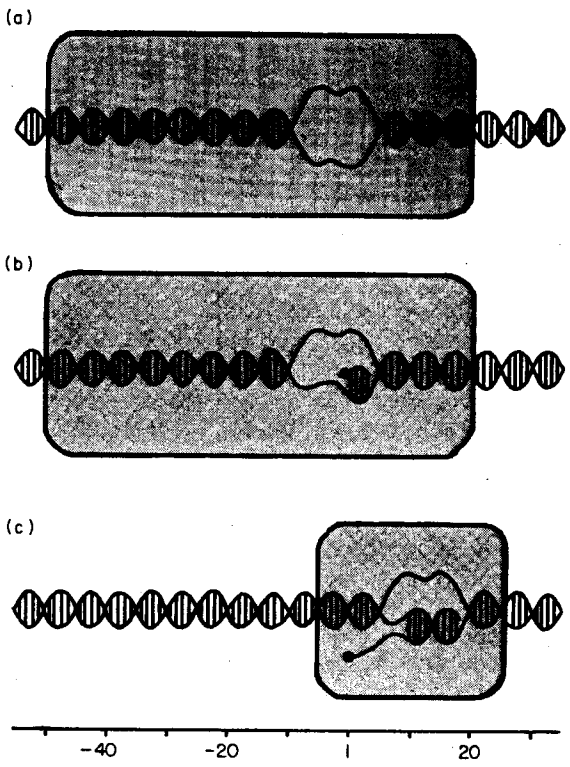

Figure 5. Schematic diagrams of polymerase–DNA complexes involved in *lac* mRNA initiation and elongation. (a) Stable open promoter complex formed before initiation of mRNA synthesis; (b) transient promoter-bound complex synthesizing short RNA chain; and (c) stable ternary elongation complex paused at position +16. The boundaries of the polymerase are drawn to correspond to the results from the footprinting data and the scale at the bottom of the Figure shows the position along the helical DNA (in base-pairs) numbered relative to the site of initiation of the major *lac*UV5 mRNA (at +1). In (b) and (c), the dot at about +1 marks the 5′ end of the nascent RNA chain. The exact boundaries of the melted regions and mRNA–DNA hybrids are not known and this aspect of the diagram is therefore an approximation (see Discussion).

Finally, Figure 4(a) and (b), lane 6, shows the methylation pattern when all four nucleoside triphosphates are added and full-length mRNA is synthesized. The pattern is very similar to that obtained in the absence of added RNA polymerase, indicating that most of the RNA polymerase has moved from the promoter to synthesize full-length mRNA. As was the case in the DNase footprinting experiments, a small fraction of the polymerases appear to have failed to initiate (move) under these conditions. This is most evident in Figure 4(b), lanes 5 and 6, where a small fraction of the C residues at positions −1, −2, −4 and −6 appear to remain melted. However, the loss of G residue protection at −6 and −13 on the top strand and −32 on the bottom clearly shows that most of the polymerases have moved.

4. Discussion

(a) *Polymerase–DNA interactions during mRNA initiation and elongation*

These experiments show that RNA polymerase remains specifically bound to the *lac*UV5 promoter even after initial RNA synthesis has begun. This result was predicted previously based on kinetic experiments (Carpousis & Gralla, 1980; Carpousis *et al.*, 1982) and is consistent with experiments by others. Achberger *et al.* (1982) and LeGrice & Sonenshein (personal communication) have footprinted *Bacillus subtilis* RNA polymerase complexes to which initiating nucleotides were added. Their results are similar to those presented here in that polymerase apparently does not escape from the promoter during the earliest stages of RNA synthesis, although subtle changes in the protection pattern were observed. Spassky & Buc (personal communication) have used dinucleotide primers to initiate short RNA chains at the *lac*UV5 promoter and have made similar observations.

Previously, it was suggested that RNA polymerase could choose its initiating nucleotide from a small region and synthesize short RNA chains without actually leaving the promoter and moving along the DNA. In order to explain the reiterative synthesis of short RNAs and 5′ nucleotide heterogeneity at the *lac* promoters, it was postulated that open promoter complexes contained a "flexible" template strand (Carpousis & Gralla, 1980; Carpousis *et al.*, 1982). Movement of this strand (or the melted DNA bubble) within an otherwise fixed open promoter complex could allow the accommodation of short RNA chains with various 5′ and 3′ termini without actually breaking contacts to the promoter DNA. In this model of the open promoter complex, one can easily imagine some movement of the melted template DNA occurring during initial RNA synthesis without actually breaking the strong contacts to the promoter. This is illustrated in Figure 5. Panel (a) is a diagram of the open promoter complex. In Figure 5(b), a short RNA chain is synthesized forming an mRNA–DNA hybrid. The complex in (b) is essentially the same as the complex in (a), except for the short mRNA–DNA hybrid. If the short nascent mRNA is released instead of elongated further, then re-initiation can occur by simply relaxing the template DNA strand to its initial position. Furthermore, flexibility in the template strand can accommodate initiation from several different start sites.

Strictly speaking, we cannot rule out the possibility that the polymerase transiently breaks promoter-specific contacts during short RNA synthesis and then rapidly reforms these contacts when a short RNA is released. A short-lived transient complex would not be detected by the methods employed here. However, it seems unlikely that the promoter-specific contacts could be reformed rapidly if completely broken. Short RNA synthesis at the *lac*UV5 promoter can approach a rate of 100 chains per minute in the presence of high concentrations of nucleoside triphosphates (Gralla *et al.*, 1980). Even if such a transient complex exists, the key point is that the overall process is fully reversible at this stage. The polymerase remains promoter-bound after short RNA synthesis and release.

We have shown that, when the enzyme has synthesized nascent mRNA paused predominantly at position +16, the contacts to the promoter are broken and a rifampicin-resistant elongation complex is formed. Figure 5(c) shows a diagram of this paused elongation complex. Taken together with data from others, the results presented here lead to a crude picture of the elongation complex. The DNase footprinting shows that RNA polymerase covers, at most, 30 base-pairs of DNA, consistent with a previous estimate of the size of nuclease-protected phage T7 DNA or poly(dA·T) fragments from random elongation complexes (Rohrer & Zillig, 1977). The 30 base-pairs include approximately nine base-pairs downstream and 20 base-pairs upstream from the point at which the next base is to be added (recall that the polymerase is paused at position +16 and the footprint extends from about −5 to +25). The 21 upstream base-pairs include an RNA–DNA hybrid whose length has been estimated at 12 base-pairs (for a review, see von Hippel *et al.*, 1984), leaving slightly less than one turn of helical DNA at the upstream end of the complex. Downstream from the transcription point there can be, at most, a few melted base-pairs, since the total amount of DNA unwinding in an elongation complex is estimated to be 17 bases long (Gamper & Hearst, 1982; von Hippel *et al.*, 1984), leaving a short region of helical DNA at the downstream end of the complex. Less than half of the DNA protected by the paused elongation complex is double helical with the remainder being unwound or part of the mRNA–DNA hybrid.

One aspect of the data concerning the elongation complex is unexpected. Although the DNA is protected from DNase attack as efficiently as DNA in the open complex, methylation probe experiments reveal no protected G residues and only one weakly methylated single-stranded C residue. The lack of base-specific contacts is the less surprising of the two, since elongation is a relatively sequence-independent process. In fact, the situation is the same as the interaction between polymerase and the transcribed regions of open promoter complex that is also DNase-protected but not specifically contacted (Figs 3 and 4; Schmitz & Galas, 1979; Siebenlist *et al.*, 1980). The inability to detect melted cytosine residues is more surprising. The presumptive melted cytosine residues on the bottom strand of the elongation complex all lie between −4 and +12 and are likely protected by base-pairing either to nascent mRNA or the top strand of DNA. On the top strand, there are cytosine residues at positions +10 and +17; we can surmise only that their base-pairing residues are protected by contacts to the core RNA polymerase and thus are inaccessible to attack by dimethyl-

sulfate. Failure to detect cytosine residues that are presumed to be melted is not unprecedented. Kierkegaard *et al.* (1983) were unable to detect melted cytosine residues in the *trp* open promoter complex, even though DNase footprinting clearly revealed a complex. They postulated that the *trp* complex was only transiently melted. However, it is also plausible that the melted cytosine residues in the *trp* complex are protected by the RNA polymerase. Furthermore, there is precedent for protection of a melted residue in the *lac*UV5 open promoter complex. The melted G residue at −6 on the top strand is strongly protected from methylation, yet the corresponding C at −6 on the bottom strand is methylated, confirming that this base-pair is indeed melted (see Fig. 4).

(b) *When does RNA polymerase escape from promoter DNA to form an elongation complex?*

Early work on *E. coli* chain initiation focused mainly on the enzymology of initial phosphodiester bond formation. In part, these studies were made possible by assays that uncoupled the formation of the initial phosphodiester bond from the subsequent elongation of the chain. Mangel & Chamberlin (1974) developed a kinetic assay for chain initiation based on a competition between binding of initial nucleotides and rifampicin. Johnston & McClure (1976) developed an "abortive initiation" assay based on the steady-state synthesis of the initial dinucleotide in the mRNA chain. An implicit assumption in the interpretation of some of these early experiments was that chain initiation was simply the binding of the initial nucleotides and the formation of the initial phosphodiester bond. While this can apply to some promoters, the chain initiation process is clearly more complex at promoters that exhibit extensive cycling, such as *lac*UV5. The results presented above show that RNA polymerase does not form a rifampicin-resistant complex or escape from the *lac*UV5 promoter until a nascent mRNA chain about ten bases long is made. This conclusion is consistent with the observations reported by Munson & Reznikoff (1981), who showed that preincubation with ATP, UTP and GTP converted *lac*UV5 open complexes to a form that exhibited less cycling and faster productive chain initiation.

This point of conversion to an elongation complex coincides approximately with the point of release of the sigma subunit from RNA polymerase holoenzyme. Sigma is released after an RNA chain of eight residues is formed on a poly(dA·T) template (Hansen & McClure, 1980), and is suggested to be released at a similar point on the *lac*UV5 promoter (unpublished results cited by Hansen & McClure, 1980). Therefore, the simplest view appears to be that sigma subunit anchors the polymerase to the promoter during the formation of the first full helical turn of the mRNA–DNA hybrid. Escape from the promoter then involves the breakage of strong polymerase–promoter DNA contacts in the non-transcribed portion of the promoter (Fig. 5) and release of the sigma subunit. The elongation complex thus formed has an entirely new character; it binds the nascent mRNA chain tightly, thus preventing RNA chain release, lacks sigma subunit, covers a rather short DNA segment, contains less than 50% helical DNA, and has escaped from sequence-specific contacts with the promoter DNA (see Fig. 5).

These results have shown that the initial bases in the *lac*UV5 mRNA are synthesized by a promoter-bound holoenzyme complex. *E. coli* RNA polymerase holoenzyme differs from many other nucleic acid polymerases in that it can initiate chains *de novo*. That is, it can "prime" RNA chain synthesis as well as elongate nascent chains. Therefore, the promoter-bound holoenzyme complex can be viewed as a "primase" that is quite distinct from the elongation complex. The entire initiation phase can be viewed as the production of a tightly bound primer. After the formation of about one helical turn of mRNA–DNA hybrid by the mRNA primase complex, a "true" ternary elongation complex is finally formed.

The rifampicin-sensitivity results suggest that the promoter-bound holoenzyme complex remains sensitive to rifampicin inhibition even after the synthesis and release of short RNA chains up to ten bases long. McClure & Cech (1978) proposed a mechanism of rifampicin inhibition that involved the steric blockage of the initial translocation step in mRNA initiation. That is, if rifampicin binds to the polymerase before the initial translocation step, then translocation is blocked and subsequent elongation is prevented. However, once the initial translocation occurs, the drug can no longer bind to the polymerase and block elongation. The above rifampicin-sensitivity results are consistent with this scheme if one assumes that rifampicin can block "re-initiation" at any time after a short chain is released during the RNA polymerase cycling reaction. In this view, the conversion from a rifampicin-sensitive complex to one that is resistant depends on the stability of the nascent mRNA chain. Once a tightly bound nascent chain is formed, the complex will be "truly" resistant to rifampicin. It is worth pointing out that rifampicin resistance may not coincide with the point at which the sigma subunit is released and the polymerase escapes from promoter DNA. If a short RNA, two or three bases long, remains tightly bound, then rifampicin resistance is likely to be achieved before sigma release and promoter escape. It also seems likely that such promoters would form stable, salt-resistant ternary complexes upon addition of the initial nucleoside triphosphates. For intance, this is probably true of the T7 early promoters, since they efficiently initiate mRNA chains at very low concentrations of nucleoside triphosphate and rarely release short RNAs during the initiation phase (Nierman & Chamberlin, 1979,1980).

(c) *Can the process of RNA polymerase escape from promoter DNA have regulatory consequences?*

Taken together with previous observations, these results provide an over-view of how RNA polymerase interacts with *lac* DNA to initiate mRNA. Stable recognition of the promoter involves specific melting of the DNA (see Siebenlist *et al.*, 1980), which is probably preceded by a torsional untwisting of the helix as the enzyme attempts to bind to both the −10 and −35 regions (Stefano & Gralla, 1982*a*). Open promoter complex formation does not depend on the presence of nucleoside triphosphates and, once formed, complexes are stable for hours (see Carpousis *et al.*, 1982). The enzymatic and chemical probe results reported here demonstrate that the very tight specific contacts that stabilize the open complex are not immediately broken upon addition of nucleoside triphosphates and the formation of a short RNA chain. Instead, the enzyme is held at the promoter as it repeatedly fails to complete the initiated chain and cycles non-productively to produce short RNAs (Carpousis & Gralla, 1980; Munson & Reznikoff, 1981). Finally, the enzyme breaks free of these strong contacts and escapes from the promoter to form a stable elongation complex containing tightly bound mRNA.

If the process of RNA polymerase escape is to have important regulatory consequences, it must be capable of being the rate-limiting step in mRNA production for some promoters. Previous results showed that promoters have differing escape or chain initiation rates. *lac*UV5 and Tn*5* are "slow start" promoters, *trp* and *lac* p^s intermediate, and certain phage T7 and λ promoters are "rapid start" (Stefano & Gralla, 1979; Gralla *et al.*, 1980; Nierman & Chamberlin, 1979,1980; Munson & Reznikoff, 1981; Horowitz & Platt, 1982). *lac*UV5 slow start promoters and T7 fast start promoters differ in rate by two orders of magnitude. The introduction of two mutations into the *lac*UV5 promoter to create the *lac* L305xp^s promoter accelerates the rate of escape by an order of magnitude (Carpousis *et al.*, 1982). Moreover, under certain conditions *in vitro*, the rate of escape at the *lac*UV5 promoter can be slower than the rate of open complex formation, a prerequisite for regulation at this step (Stefano & Gralla, 1979). Therefore, it is clear that promoters vary widely in their intrinsic chain initiation rates, and it is conceivable that some promoters are rate-limited at this step.

Evidence supporting the difficulty of escape from some promoters also comes from studies that reveal an excess of short over full-length mRNA chains during transcription *in vitro*. In the case of the slow start *lac*UV5 (Carpousis & Gralla, 1980) and Tn*5* promoters (Munson & Reznikoff, 1981), such observations were in fact interpreted in terms of promoter-bound cycling, but similar observations have been made at the *gal* and λ P'_R promoters (Di Lauro *et al.*, 1979; McClure & Cech, 1978). Related observations have also been made for eukaryotic RNA-dependent RNA polymerases (Furuichi, 1981; Yamakawa *et al.*, 1981), and for RNA polymerases from mouse transcribing polyoma promoters (Cowie *et al.*, 1982) and from human HeLa cells transcribing adenovirus DNA (Ackerman *et al.*, 1983). Their observations suggest that aspects of the escape mechanism described for *lac*UV5 transcription initiation may apply to other systems.

On the other hand, cycling does not appear to be an important phenomenon at other promoters (see Nierman & Chamberlin, 1979,1980); and it is not known if a significant amount of cycling occurs *in vivo*. The rate of mRNA chain initiation increases and the extent of RNA polymerase cycling decreases with increasing concentrations of nucleoside triphosphates (see Mangel & Chamberlin, 1974; McClure *et al.*, 1978; Gralla *et al.*, 1980; Munson & Reznikoff, 1981). Furthermore, the effect of such *in vivo* factors as DNA supercoiling, nucleoside mono- and diphosphates, which may be competitors during chain initiation, and other metabolites that may be regulatory effectors are not known. In the very few cases where promoter strength comparisons have been made, the expression levels *in vivo* correlate qualitatively with the rate of open complex formation (Maquat & Reznikoff, 1978; Stefano *et al.*, 1980; Ackerson & Gralla, 1982; McClure *et al.*, 1982). One interesting observation concerns a family of closely related mutant *lac* promoters in which the rate of escape ("chain initiation rate") in this series of promoters was found to decrease as the rate of open complex formation increased (Carpousis *et al.*, 1982). This raised the interesting possibility that attempts to create very strong natural or synthetic promoters that bind RNA polymerase rapidly may encounter difficulties; some promoters that bind RNA polymerase rapidly (and tightly) may become "stuck" and initiate full-length mRNA slowly (see, for example, Rossi *et al.*, 1983). Thus, a delicate balance may exist between the promoter sequence requirements for rapid binding and the requirements for rapid escape from the promoter to produce full-length mRNA.

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