Ohshima, Y., & Suzuki, Y. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74,* 5363–5367.
Pearson, W. R., Wu, J.-R., & Bonner, J. (1978) *Biochemistry 17,* 51–59.
Rudkin, G. T. (1977) *J. Supramol. Struct., Suppl. 1,* 106.
Rudkin, G. T., & Stollar, B. D. (1977) *Nature (London) 265,* 472–473.
Stollar, B. D. (1970) *Science 169,* 609–611.
Taylor, W. C., Cockburn, A. F., Frankel, G. A., Newkirk, M. J., & Firtel, R. A. (1977) *ICN-UCLA Symp. Mol. Cell. Biol. 8,* 309–313.
Thomas, M., White, R. L., & Davis, R. W. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73,* 2294–2298.
Tiemeier, D. C., Tilghman, S. M., & Leder, P. (1977) *Gene 2,* 173–191.
Trilling, D. M., McNicol, L. A., Stollar, B. D., & Goldberg, E. B. (1970) *Biochim. Biophys. Acta 217,* 365–371.
Vogt, V. M., & Braun, R. (1976) *J. Mol. Biol. 106,* 567–587.
Wagner, A. F., Bugianesi, R. L., & Shen, T. Y. (1971) *Biochem. Biophys. Res. Commun. 45,* 184–189.
Warner, R. C., Samuels, H. H., Abbott, M. T., & Krakow, J. S. (1963) *Proc. Natl. Acad. Sci. U.S.A. 49,* 533–538.
Wellauer, P. K., & Dawid, I. B. (1977) *Cell 10,* 193–212.
Woo, S. L. C., Monahan, J. J., & O'Malley, B. W. (1977) *J. Biol. Chem. 252,* 5789–5797.

Escherichia coli RNA Polymerase–Rifampicin Complexes Bound at Promoter Sites Block RNA Chain Elongation by *Escherichia coli* RNA Polymerase and T7-Specific RNA Polymerase†

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ABSTRACT: *Escherichia coli* RNA polymerase that has been complexed with the drug rifampicin is not able to initiate a long RNA chain but still retains its ability to bind at promoter sites on T7 DNA. When conditions are such that the inactive enzyme forms "open" promoter complexes on T7 DNA, that enzyme serves as a barrier to transcription through the promoter site by RNA polymerase molecules which have initiated transcription on the same DNA strand from distal upstream promoters. Both *E. coli* RNA polymerase and the T7 phage-specific RNA polymerase are sensitive to blockage by these *E. coli* RNA polymerase–rifampicin open complexes. When inert bacterial RNA polymerase is not present on T7 DNA, there is no detectable termination by RNA polymerase molecules which traverse a promoter site in the course of transcription. The ability of *E. coli* RNA polymerase–rifampicin complexes to block chain elongation by RNA polymerase transcribing from distal promoters probably accounts for the coincidence of minor promoter and "terminator" sites on T7 DNA in the studies of Minkley and Pribnow [Minkley, E. G., and Pribnow, D. (1973), *J. Mol. Biol. 77,* 255–277] and can lead to some difficulty in the interpretation of in vitro transcription patterns where overlapping transcription units are present. Since bacteriophage T7 induces inhibitors which bind to and inactivate the bacterial RNA polymerase, it is possible that a similar kind of blocking phenomenon involving binding of inert *E. coli* RNA polymerase at T7 minor promoters plays a regulatory role in the turn off of class I and II T7 RNA synthesis in vivo.

The antibiotic rifampicin is an inhibitor of *E. coli* RNA polymerase which prevents the productive initiation of RNA synthesis but not the elongation of RNA chains (Sippel and Hartmann, 1968). The inhibition of *E. coli* RNA polymerase by rifampicin results from the binding of the drug to the β subunit (Rabussay and Zillig, 1969). This interaction can occur when the RNA polymerase is either free in solution or bound to DNA (Hinkle et al., 1972) but not when the RNA polymerase is in an actively transcribing ternary complex (Eilen and Krakow, 1973).

RNA polymerase that is complexed with rifampicin retains its ability to recognize promoters and to form a stable "open" complex at these sites (Hinkle et al., 1972; Bordier and Dubochet, 1974). This property of RNA polymerase–rifampicin complexes has been used to explain the dominance of rifampicin-sensitive alleles over rifampicin-resistant alleles in *E. coli* strains that are heterodiploid for the *rpoB* gene (Khesin et al., 1971; Ilyina et al., 1971; Austin et al., 1971). It has been shown that rifampicin-inactivated RNA polymerase can bind to promoters and thereby make them unavailable to rifampicin-resistant RNA polymerase (Bordier, 1974).

Recent studies on the in vitro transcription of ϕX174 DNA (Axelrod, 1976) and T7 DNA (Stahl and Chamberlin, 1977) indicate that, when *E. coli* RNA polymerase and rifampicin are employed in RNA synthesis reactions at elevated molar ratios of enzyme to DNA, termination events occur at sites that normally function as promoters which are located downstream from where transcription was initiated. These termination events are believed to result from blockage of RNA chain elongation by RNA polymerase–rifampicin complexes bound at downstream promoters.

In this paper, we examine more precisely the ability of the *E. coli* RNA polymerase–rifampicin complex to block transcription of RNA polymerases which have initiated RNA chains at distal promoters.

Materials and Methods

RNA Polymerases. E. coli RNA polymerase was purified

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from DG156, an RNase I deficient derivative of *E. coli* B/1, and assayed as previously described (Gonzalez et al., 1977). The specific activity of this enzyme preparation was 22 000 units/mg. About 40% of the enzyme molecules were able to initiate an RNA chain, as determined by γ-^{32}P-labeled nucleotide incorporation under conditions where reinitiation was minimized (W. Nierman, personal communication). In the experiments presented in this paper, the molar ratios of RNA polymerase to DNA cited were based on the total weight of enzyme added to the reaction so that the actual molar ratios of active RNA polymerase to DNA are approximately 2.5-fold lower than indicated. T7-specific RNA polymerase (fraction F; 76 000 units/mg) was purified and assayed as described previously (Chamberlin et al., 1970). In each case, 1 unit of RNA polymerase activity represents a rate of 1 nmol of CMP incorporated into RNA per hour as determined in the reaction conditions referenced above.

Substrates and Nucleic Acids. The T3-T7 recombinant phage R8 was provided by Drs. Hausmann and Beier. The growth procedures and genetic constitution of this phage have been described previously (Beier et al., 1977). The T7 deletion D111 was provided by Dr. F. W. Studier. This deletion lacks the region between 1.3 and 3.7% on the T7 genome and thereby eliminates two of the three strong A promoters (A_2 and A_3) and one minor promoter (B) for *E. coli* RNA polymerase (Studier, 1975; Stahl and Chamberlin, 1977). R8 DNA and D111 DNA were isolated from purified phage by phenol extraction as described by Thomas and Abelson (1966). ^{32}P-labeled inorganic phosphate was purchased from New England Nuclear and was converted to [α^{32}P]CTP by the method of Symons (1968). Unlabeled nucleoside triphosphates were purchased from P-L Biochemicals and Calbiochem. Spermidine and dithiothreitol were purchased from Sigma. Rifampicin (B grade) was purchased from Calbiochem.

Transcription of R8 DNA by T7-Specific RNA Polymerase. R8 DNA was transcribed by T7-specific RNA polymerase in a solution (100 μL) containing 40 mM Tris-HCl[1] (pH 8.0); 20 mM $MgCl_2$; 5 mM dithiothreitol; 4 mM spermidine; 0.4 mM ATP, UTP, and GTP; 0.2 mM [α-^{32}P]CTP (200 cpm/pmol), and 0.3 mM R8 DNA. This differs from our previously published reaction conditions (Chamberlin and Ring, 1973) in that 4 mM spermidine replaces bovine serum albumin and 5 mM dithiothreitol replaces β-mercaptoethanol. This concentration of spermidine was found to give nearly optimal T7-specific RNA polymerase activity and eliminates the problems caused by the contamination of serum albumin by ribonuclease. Approximately 1 μg of T7-specific RNA polymerase was used for each reaction.

Transcription of R8 DNA by T7-specific RNA polymerase in the presence of the inactive *E. coli* RNA polymerase-rifampicin complex was carried out as follows: *E. coli* RNA polymerase was complexed with rifampicin by the addition of a concentrated rifampicin solution to a 1.06 mg/mL enzyme solution to give a final concentration of 60 μg/mL rifampicin. The *E. coli* RNA polymerase-rifampicin complex was then added to a transcription reaction solution containing all components except the T7-specific RNA polymerase. The amount of *E. coli* RNA polymerase-rifampicin complex added to the assay was varied to give an *E. coli* RNA polymerase to R8 DNA molar ratio from 0 to 40. The *E. coli* RNA polymerase-rifampicin complex was allowed to bind to promoters on R8 DNA by incubating the assay solution for 10 min at 37 °C. Transcription was initiated by the addition of T7-specific RNA polymerase, and incubation at 37 °C was allowed to continue for an additional 10 min. The reaction was terminated by the addition of 30 μL of a buffered detergent solution (0.1 M Na_2EDTA, 0.1% $NaDodSO_4$, 27 mM boric acid, 0.1% Bromphenol blue, and 30% glycerol), and 10-μL aliquots from each reaction were then assayed for acid-insoluble radioactivity. A sample of each transcription reaction (volume sufficient to contain 30 000–40 000 cpm) was then analyzed by electrophoresis on a 2% acrylamide–0.5% agarose slab gel as previously described (Golomb and Chamberlin, 1974). Control reactions containing the *E. coli* RNA polymerase-rifampicin complex but no T7-specific RNA polymerase showed no significant incorporation into acid-insoluble material.

Transcription of D111 DNA by E. coli RNA Polymerase in the Presence of E. coli RNA Polymerase Complexed with Rifampicin. Transcription of D111 DNA by *E. coli* RNA polymerase holoenzyme was carried out in a solution (100 μL) containing 40 mM Tris-HCl (pH 8.0); 10 mM $MgCl_2$; 10 mM β-mercaptoethanol; 2 mM spermidine; 0.25 mM ATP, UTP, and GTP; 10 μM [α-^{32}P]CTP (700 cpm/pmol), and 0.3 mM D111 DNA. *E. coli* RNA polymerase was bound to promoters on D111 DNA by incubating the enzyme for 10 min at 37 °C in the reaction solution containing all components except the nucleoside triphosphates. A molar ratio of enzyme to DNA of 2 was employed. RNA synthesis was initiated by the addition of a solution (15 μL) containing nucleoside triphosphates and *E. coli* RNA polymerase-rifampicin complex. The concentration of rifampicin in this solution was 80 μg/mL, and the concentration of enzyme was varied from 0 to 425 μg/mL to give a final ratio of RNA polymerase-rifampicin complex to DNA of 0 to 32. RNA synthesis was allowed to proceed for 30 min at 37 °C and was then terminated by the addition of 30 μL of the buffered detergent solution as before. Samples (10 μL) of each reaction were assayed for acid-insoluble radioactivity, and a sample of appropriate volume (120 000 cpm) was analyzed by electrophoresis on a 1.75% acrylamide–0.5% agarose gel.

Results

Binding of the E. coli RNA Polymerase-Rifampicin Complex at a Promoter Site Blocks Chain Elongation by E. coli RNA Polymerase. Under normal assay conditions, *E. coli* RNA polymerase holoenzyme has been shown to transcribe primarily a single size class of RNA of 2.4×10^6 amu (Millette et al., 1970; Dunn and Studier, 1973a) originating from a cluster of three promoter sites (A_1, A_2, and A_3) at the left end of T7 DNA and terminating at a site near 20% on T7 DNA. In the case of D111 DNA (where A_2 and A_3 are deleted), only one transcript of 2.2×10^6 amu is made under normal assay conditions (Figure 1a). However, when elevated molar ratios of *E. coli* RNA polymerase to T7 DNA (E/DNA = 12) are incubated at 37 °C with T7 DNA in order to form the highly stable "open" promoter complex prior to initiation of RNA synthesis by the addition of nucleoside triphosphates ("prebinding" conditions), at least four other promoters are used with efficiencies comparable to that of the A promoters (Stahl and Chamberlin, 1977). These promoters were originally described by Minkley and Pribnow (1973) in studies of dinucleotide-primed transcription.

In their original studies, Minkley and Pribnow (1973) and Minkley (1974) found that these "minor" promoter sites appeared to serve as sites for RNA chain termination, as well as sites where initiation could occur. Stahl and Chamberlin (1977) found that when a "prebinding" reaction program is used with D111 DNA, three transcripts are made from the

[1] Abbreviations used: Tris-HCl, 2-amino-2-hydroxymethyl-1,3-propanediol; EDTA, (ethylenedinitrilo)tetraacetic acid; $NaDodSO_4$, sodium dodecyl sulfate.

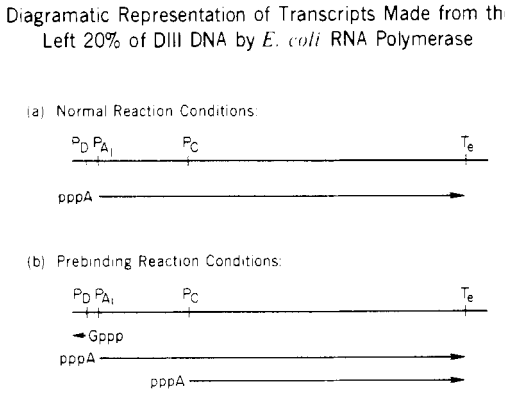

FIGURE 1: Diagrammatic representation of transcripts made from the left 20% of D111 DNA by *E. coli* RNA polymerase under normal reaction conditions and prebinding reaction conditions.

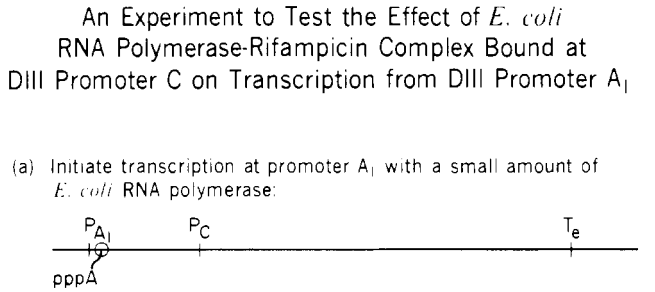

FIGURE 2: An experiment to test the effect of *E. coli* RNA polymerase-rifampicin complex bound at D111 promoter C on transcription from D111 promoter A_1.

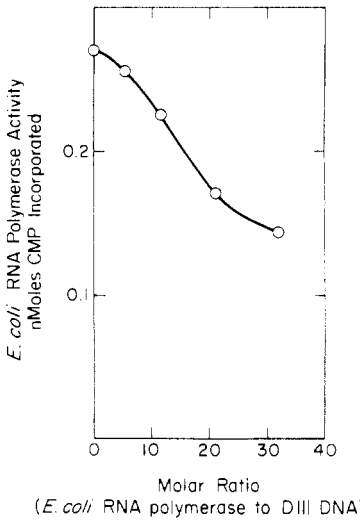

FIGURE 3: Effect of *E. coli* RNA polymerase-rifampicin complex on the transcription of D111 DNA by active *E. coli* RNA polymerase which has initiated from the A_1 promoter. Reaction conditions are described under Materials and Methods.

early region, originating from the major promoter A_1 and the two minor promoters C and D (Figure 1b). However, in their studies no termination of the A_1 transcript was observed to occur in the vicinity of promoter C. Hence, the C promoter does not act per se to terminate RNA chains that are elongated through it at any appreciable rate. Since the work of Minkley and Pribnow utilized both high molar ratios of *E. coli* polymerase and rifampicin, we posed the question of whether or not an inactive *E. coli* RNA polymerase molecule bound at promoter C would block RNA chain elongation by an *E. coli* RNA polymerase molecule initiated at the A_1 promoter.

In order to study the influence of the inactive *E. coli* RNA polymerase-rifampicin complex bound at promoter C on transcription through that region by *E. coli* RNA polymerase involved in chain elongation, the experimental approach diagrammed in Figure 2a–d was used. *E. coli* RNA polymerase was first bound to D111 DNA at a molar ratio of enzyme to DNA of 2. Under these conditions, transcription is primarily from the A_1 promoter as shown in track b of Figure 4. RNA synthesis was initiated by the addition of nucleoside triphosphates, rifampicin, and increasing concentrations of RNA polymerase-rifampicin complex. Upon the addition of nucleoside triphosphates and rifampicin, most of the RNA polymerase bound to the A_1 promoter will initiate an RNA chain and thereby become resistant to the drug within less than 1 s (Figure 2a). The RNA polymerase-rifampicin complex added to the reaction along with the nucleoside triphosphates and rifampicin, though unable to initiate an RNA chain, can still bind tightly to promoters (Hinkle et al., 1972)(Figure 2b). After allowing sufficient time for all RNA chains to grow to maximum length, gel electrophoretic analysis of the size of the resulting RNA transcripts will reveal directly whether D111 RNA chains initiated at the A_1 promoter have terminated at promoter C (Figure 2c) or have read through promoter C to reach the 20% terminator (Figure 2d).

The addition of increasing amounts of *E. coli* RNA polymerase-rifampicin complex to the *E. coli* RNA polymerase assay described under Materials and Methods leads to a significant decrease in the amount of RNA synthesis by the RNA polymerase bound to the A_1 promoter (Figure 3), suggesting immediately that blocking is occurring. This decrease in RNA synthesis is directly dependent upon the amount of *E. coli* RNA polymerase-rifampicin complex added to the reaction.

The transcripts formed in each reaction shown in Figure 3 was subsequently analyzed by gel electrophoresis (Figure 4). In the absence of added rifampicin-inactivated *E. coli* RNA polymerase, one major transcript of 2.2×10^6 amu (track b) is observed. This transcript is the product of the RNA polymerase which initiated an RNA chain at the A_1 promoter and elongated to the terminator at 19.3% on the T7 genome (Millette et al., 1970). Two minor transcripts of 1.7×10^6 and 1.2×10^6 amu are also observed, and these correspond to transcripts initiated at the minor promoters C and E, respectively (Minkley and Pribnow, 1973; Stahl and Chamberlin, 1977). However, there is no detectable amount of an RNA species of the size expected for a transcript initiated at A_1 and terminated at C. In tracks c–f increasing amounts of *E. coli*

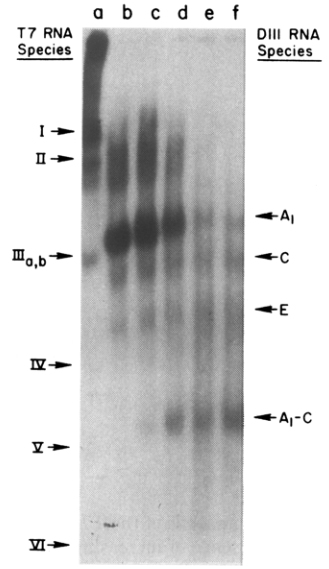

FIGURE 4: Electrophoretic analysis of the transcripts read from the A_1 promoter of D111 DNA by *E. coli* RNA polymerase in the presence of increasing amounts of *E. coli* RNA polymerase–rifampicin complex. *E. coli* RNA polymerase (0.4 μg) was bound to D111 DNA (10 μg) at 37 °C to form open promoter complexes as described under Materials and Methods. RNA synthesis was initiated by adding a mixture of nucleoside triphosphates and rifampicin (track b) together with 1.06 (track c), 2.12 (track d), 4.24 (track e), and 6.36 μg of *E. coli* RNA polymerase (track f). Equal amounts of labeled RNA (120 000 cpm) were loaded on tracks b–f. T7-specific RNA polymerase transcripts of T7 DNA (Golomb and Chamberlin, 1974) were run as molecular weight standards (track a).

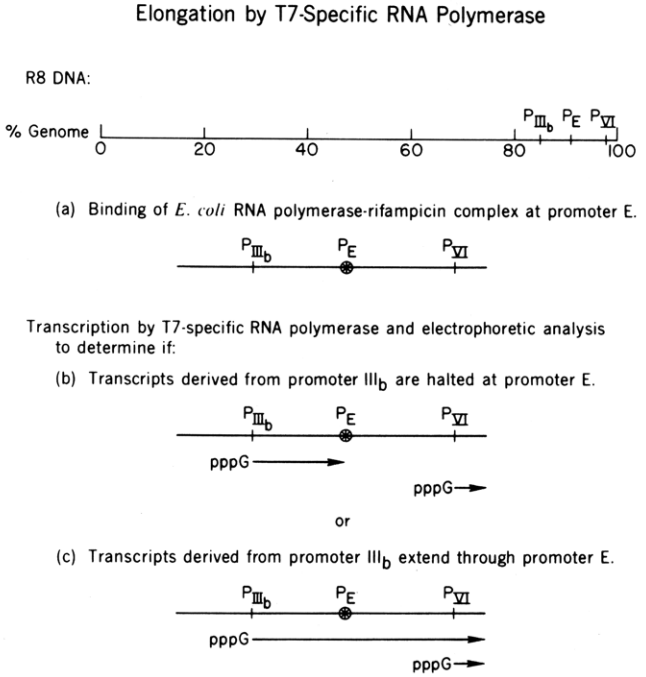

FIGURE 5: A test of the ability of *E. coli* RNA polymerase–rifampicin complex bound at a promoter site to block RNA chain elongation by T7-specific RNA polymerase.

RNA polymerase–rifampicin complex were added to the RNA reaction along with rifampicin and nucleoside triphosphates to give molar ratios of RNA polymerase to DNA of 5.3, 10.6, 21.2, and 31.8, respectively. The addition of increasing amounts of RNA polymerase–rifampicin complex results in decreasing amounts of the 2.2×10^6 amu transcript. When the RNA polymerase to DNA molar ratio reaches 32, there is less of this A_1 transcript than of the minor transcripts C and E which are relatively unaffected by the addition of RNA polymerase–rifampicin complex. Concurrent with the disappearance in the A_1 transcript is the appearance of increasing amounts of a new transcript with an apparent molecular weight of $0.48–0.5 \times 10^6$ amu. We believe that this transcript results from the binding of *E. coli* RNA polymerase–rifampicin complex to the C promoter. The molecular weight of this transcript conforms very closely to the length of a transcript initiated from promoter A_1 at 1.1% on the standard T7 map (Hsieh and Wang, 1976; Williams and Chamberlin, 1977) and terminated at promoter C near 8% on the standard T7 map (Minkley and Pribnow, 1973; Stahl and Chamberlin, 1977). Blocking of actively elongating RNA polymerase at the C promoter has also been observed previously in this laboratory when moderately high RNA polymerase to DNA ratios were used in a prebinding reaction protocol, and nucleoside triphosphates and rifampicin were then added together to initiate RNA synthesis under conditions where a substantial amount of the RNA polymerase would become inactivated prior to chain initiation (Stahl and Chamberlin, 1977).

In the experiments described above, low nucleotide concentrations were used to slow the elongation rate so that the RNA polymerase–rifampicin complex would have sufficient time to form an "open" promoter complex at promoter C before the RNA polymerase which initiated at promoter A_1 elongated to that region of the DNA. In an analogous experiment to that shown in Figures 3 and 4, the RNA polymerase bound at promoter A_1 was allowed to initiate an RNA chain at 37 °C and was immediately shifted to 10 °C prior to the addition of RNA polymerase–rifampicin complex. The reaction was then continued for 80 min at 10 °C so that enzyme molecules which had initiated at the A_1 promoter would have enough time to reach the terminator at 19.3%. Under these conditions, no decrease of activity was observed upon addition of increasing amounts of RNA polymerase–rifampicin complex, and analysis of the reaction products by gel electrophoresis did not show any significant amount of the A to C transcript (data not shown). Since 10 °C is well below the transition temperature of promoter C (Stahl and Chamberlin, 1977), we conclude that *E. coli* RNA polymerase–rifampicin complex must be in an "open" promoter complex to block actively elongating RNA polymerase.

Binding of the E. coli RNA Polymerase–Rifampicin Complex at a Promoter Site Blocks Chain Elongation by the T7-Specific RNA Polymerase. The conclusions we have discussed above raise the question of whether the *E. coli* RNA polymerase–rifampicin complex bound at a promoter can also block RNA chain elongation by T7-specific RNA polymerase. This was tested employing DNA from the T3–T7 recombinant phage R8 as DNA template (Beier et al., 1977). The salient features of this template are outlined in Figure 5. R8 DNA contains only the rightmost 32% of T7 and the rest of the template is derived from T3. This template contains only promoters III_b and VI for the T7-specific RNA polymerase, which are located at 85% and 98.5% on the standard T7 genome, respectively. R8 DNA also contains the *E. coli* RNA polymerase minor promoter E located at 92% on the standard T7 genome (Stahl and Chamberlin, 1977). An outline of the experiment used to demonstrate blocking of the T7-specific RNA polymerase by *E. coli* RNA polymerase–rifampicin complex bound at promoter E is shown in Figure 5.

The T7-specific RNA polymerase is resistant to rifampicin (Chamberlin et al., 1970), and, hence, normally, the addition

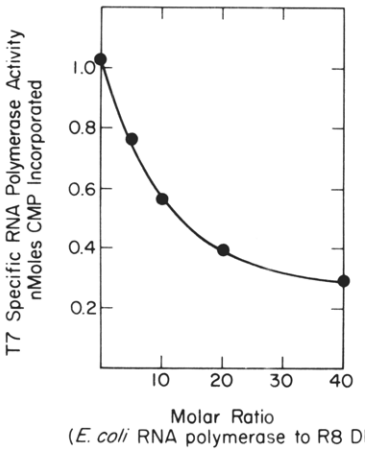

FIGURE 6: Effect of *E. coli* RNA polymerase–rifampicin complex on the transcription of R8 DNA by T7-specific RNA polymerase. Reaction conditions are described under Materials and Methods.

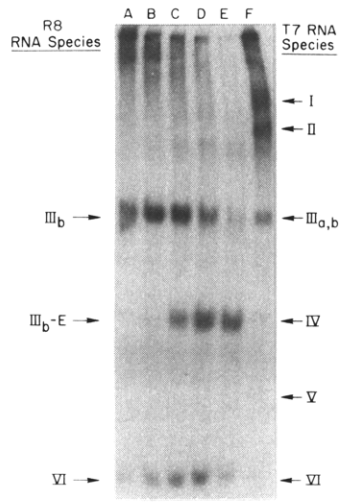

FIGURE 7: Electrophoretic analysis of the T7-specific RNA polymerase transcripts made in the presence of increasing amounts of *E. coli* RNA polymerase–rifampicin complex. Reactions were carried out as described under Materials and Methods. Increasing amounts of *E. coli* RNA polymerase–rifampicin complex as noted were bound to 10 μg of R8 DNA at 37 °C to form open complexes prior to the addition of 1.4 μg of T7-specific RNA polymerase: track A, none; track B, 1.0 μg; track C, 2.0 μg; track D, 4.0 μg; track E, 8.0 μg. Equal amounts of labeled RNA (40 000 cpm) were loaded on tracks A–D. Track E contained only 70% that amount. T7-specific RNA polymerase transcripts of T7 DNA were run as molecular weight standards in track F.

of even high concentrations of this drug has no effect on the transcription by this enzyme. However, when rifampicin together with *E. coli* RNA polymerase is added to a reaction in which T7-specific RNA polymerase transcribes R8 DNA, there is a substantial decrease in the amount of RNA synthesis by the T7-specific RNA polymerase as the amount of bacterial enzyme is increased (Figure 6). This immediately suggests that the *E. coli* RNA polymerase–rifampicin complex is able to block transcription by the phage enzyme.

To determine the nature and specificity of this inhibition, the RNA products from the reactions shown in Figure 6 were analyzed by gel electrophoresis (Figure 7). As expected, T7-specific RNA polymerase produces two major transcripts with R8 DNA which have molecular weights of 2×10^6 and 0.22×10^6 amu (track A). These correspond to the T7 RNA species III_b and VI, respectively (Golomb and Chamberlin, 1974). In tracks B–E, increasing amounts of *E. coli* RNA polymerase–rifampicin complex were allowed to bind to R8 DNA at 37 °C prior to the addition of the T7-specific enzyme to give final molar ratios of *E. coli* RNA polymerase to DNA of 5, 10, 20, and 40, respectively. As the amount of *E. coli* RNA polymerase is increased, there is a corresponding decrease in the amount of RNA species III_b observed on the gel, and a new transcript of molecular weight 0.82×10^6 amu appears. The molecular weight of this new transcript agrees well with the molecular weight predicted for a transcript initiated at promoter III_b, near 85% on the standard T7 map and terminated at the *E. coli* RNA polymerase minor promoter E at 92%. A binding site for *E. coli* RNA polymerase corresponding to promoter E has recently been mapped by the electron-microscopic technique of Williams (1977) at 92.2 ± 0.1% on the standard T7 map (Kadesch and Chamberlin, personal communication). Addition of *E. coli* RNA polymerase–rifampicin complex has no effect on the amount of T7 RNA species VI, confirming our expectation that the truncation of the T7 RNA species III_b is specifically due to the presence of a bacterial promoter in this transcription unit and not to some direct inhibitory effect of *E. coli* RNA polymerase–rifampicin complex on the T7-specific RNA polymerase molecule itself.

The elongation of T7 RNA species III_b by T7-specific RNA polymerase is blocked by the *E. coli* RNA polymerase–rifampicin complex of promoter E only when conditions permit the formation of an "open promoter complex" by the latter enzyme. When the experiment shown in Figures 6 and 7 was repeated at 10 °C instead of 37 °C, no decrease in activity was observed upon the addition of *E. coli* RNA polymerase–rifampicin complex, and analysis of the reaction products on gels showed only T7 RNA species III_b and VI (data not shown).

In these experiments, we have observed complete blocking of transcription only when the amount of *E. coli* RNA polymerase–rifampicin complex was present in approximately fourfold excess over the total number of known promoters. The incomplete blocking of transcription at lower RNA polymerase to DNA molar ratios is probably not due to the low efficiency of stopping transcription by a single RNA polymerase molecule. Only one RNA polymerase molecule binds to each promoter (Stahl and Chamberlin, 1977; Williams and Chamberlin, 1977), and adding more RNA polymerase should not affect its ability to block transcription. The more likely explanation for this observation is that 60% of the *E. coli* RNA polymerase molecules in the preparation used were unable to initiate an RNA chain to begin with and, therefore, may have also been unable to form a stable open promoter complex. In addition, it may well be that the stability of active RNA polymerase in the rifampicin complex is reduced and that somewhat less enzyme is available to actually bind to promoter sites.

Discussion

Rifampicin has been commonly used as an inhibitor of transcription in both in vitro and in vivo experiments. The interpretation of the results in these experiments has assumed that rifampicin inhibits only the initiation of transcription by *E. coli* RNA polymerase. We have shown in this paper that *E. coli* RNA polymerase which has been complexed with rifampicin and bound at downstream promoters can block the elongation of both *E. coli* RNA polymerase and T7-specific RNA polymerase.

In the experiment where we show the blocking of actively elongating *E. coli* RNA polymerase at promoter C (Figure 4), the amount of blocking observed for any given *E. coli* RNA polymerase–rifampicin complex concentration is dependent

on both the rate of open promoter complex formation by the *E. coli* RNA polymerase–rifampicin complex and on the time it takes the actively elongating *E. coli* RNA polymerase to reach that downstream promoter. Under our normal reaction conditions, *E. coli* RNA polymerase has an elongation rate of 30 nucleotides per second on T7 DNA (Neff and Chamberlin, 1978). An RNA polymerase which has initiated a chain at the A_1 promoter would, therefore, reach promoter C in about 45 s on D111 DNA using normal reaction conditions. Since the $t_{1/2}$ of open complex formation for promoter C is approximately 40 s (Stahl and Chamberlin, 1977), we maximized the amount of *E. coli* RNA polymerase–rifampicin complex that would be bound at promoter C by lowering the CTP concentration to give about a twofold decrease in the rate of elongation of the active *E. coli* RNA polymerase. Using these conditions, we can observe the A–C blocked transcript at even moderately low *E. coli* RNA polymerase–rifampicin complex to DNA molar ratios (Figure 4, track C). When the rate of elongation was reduced further by lowering all the triphosphate concentrations to 100 μM, not only was the A–C transcript observed, but another shortened transcript with an apparent molecular weight of 0.25×10^6 amu was observed at high molar ratios of *E. coli* RNA polymerase–rifampicin complex to DNA (data not shown). These data indicate that there is a binding site for *E. coli* RNA polymerase at 5.3% on the standard T7 map which has not previously been identified as a promoter. We have also observed transcripts corresponding in molecular weight to the A–C blocked transcript under standard assay conditions where no rifampicin was used, but high molar ratios of enzyme to DNA (>20) were employed. The amount of this blocked transcript observed on gels appears to increase with the age of the RNA polymerase preparation and suggests that there may well be RNA polymerase molecules present in enzyme preparations which are able to form open complexes but are unable to initiate or elongate.

The results presented in this paper indicate that caution should be used in interpreting experiments where high molar ratios of *E. coli* RNA polymerase to DNA are used or where rifampicin is used. The termination events that have been observed to occur at minor promoter sites in the early region of T7 DNA by Minkley and Pribnow (1973) and Minkley (1974) were almost certainly due to the blocking events observed in this paper, since both rifampicin and high molar ratios of *E. coli* RNA polymerase to DNA were used. The discontinuous transcription of the early region of T7 DNA by *E. coli* RNA polymerase at low GTP concentration observed by Darlix (1974) and Darlix and Horaist (1975), where transcription appears to stall at minor promoter sites, may well also be due to blocking caused by the high enzyme to DNA ratios and slow initiating conditions used.

The ability of transcriptionally inactive bacterial RNA polymerase to bind at promoter sites and block chain elongation through those sites may also serve as a regulatory mechanism for transcription in vivo. T7 DNA contains a number of minor promoters for *E. coli* RNA polymerase in both the early and late region (Minkley and Pribnow, 1973; Stahl and Chamberlin, 1977). Since these sites do not appear to be utilized as strong promoters in vivo (Dunn and Studier, 1973b; Bräutigam and Sauerbier, 1973; Kramer et al., 1974), it seems possible that they may serve as binding sites for inactive RNA polymerase molecules which when bound would reduce the amount of downstream transcription. During the course of T7 infection, *E. coli* RNA polymerase is inactivated via phosphorylation by a T7-induced protein kinase (the product of gene 0.7) and by the binding of a T7-induced inhibitor protein (the product of gene 2) (Schweiger et al., 1972; Zillig et al., 1975; Hesselbach and Nakada, 1977). This enzyme may still be able to bind to promoter sites.

Although it has been shown that all early transcription of T7 in vivo is initiated from the three closely spaced A promoters near the left end of T7, the T7 early mRNAs are not made in equal molar amounts (Summers et al., 1973; Hercules et al., 1976). Gene 0.3 mRNA is made at four times the rate of gene 1.0 mRNA and gene 0.7 mRNA is made at twice the rate of gene 1.0 mRNA, whereas gene 1.1 and 1.3 mRNAs are made at nearly an equal rate to gene 1.0 mRNA (Hercules et al., 1976). Since minor promoter B lies between genes 0.3 and 0.7 and minor promoter C lies between genes 0.7 and 1.0 (Minkley and Pribnow, 1975; Stahl and Chamberlin, 1977), it is possible that inactive *E. coli* RNA polymerase bound at minor promoters B and C may block elongating RNA polymerases, giving rise to the polarity observed in vivo. Another more distant possibility is that active RNA polymerase molecules bound at these minor promoters in closed complexes might slow RNA polymerase elongating through this region and allow ρ to cause termination at these sites (Darlix and Horaist, 1975).

This type of process might also be involved in regulating the transcriptional behavior of the T7 phage-specific RNA polymerase. Several minor promoters for *E. coli* RNA polymerase have been mapped in the late region of T7, and these may possibly play a role in the shut off of class II protein synthesis observed at 15 min past infection at 30 °C (Studier, 1972). Two such promoters have recently been mapped in the class II region of T7 near 17 and 23% (S. Pfeffer and M. Chamberlin, unpublished results), and it is possible that T7-inactived *E. coli* RNA polymerase can bind at these promoters and block the elongation of T7-specific RNA polymerase. We have, in fact, observed blocking of T7-specific RNA polymerase by *E. coli* RNA polymerase–rifampicin complex at these promoters in the class II region in vitro (unpublished observations).

Note Added in Proof

The increase in the amount of RNA species VI apparent in tracks A–D in Figure 7 is not due to an increase in RNA species VI promoter utilization. The apparent increase in the amount of RNA species VI on the autoradiogram results from the design of the experiment in which equal amounts of labeled RNA are run on each track. Thus the loss of radioactivity resulting from the decrease in molecular weight of RNA species IIIb from 2.0×10^6 amu to 0.82×10^6 amu is compensated by increasing the RNA synthesis reaction volume loaded on the gel tracks, which increases the amount of RNA species VI on the gel.

References

Austin, S. J., Tittawella, P. B., Hayward, R. S., and Scaife, J. G. (1971), *Nature (London), New Biol. 232,* 133–136.

Axelrod, N. (1976), *J. Mol. Biol. 108,* 753–770.

Beier, H., Golomb, M., and Chamberlin, M. (1977), *J. Virol. 21,* 753–765.

Bordier, C. (1974). *FEBS Lett. 45,* 259–262.

Bordier, C., and Dubochet, J. (1974), *Eur. J. Biochem. 44,* 617–624.

Bräutigam, A. R., and Sauerbier, W. (1973), *J. Virol. 12,* 882–886.

Chamberlin, M., and Ring. J. (1973), *J. Biol. Chem. 248,* 2235–2244.

Chamberlin, M., McGrath, J., and Waskell, L. (1970), *Nature (London) 228,* 227–231.

Darlix, J. L. (1974), *Biochimie 56,* 693–701.

Darlix, J. L., and Horaist, M. (1975), *Nature (London) 256,*

288–292.
Dunn, J. J., and Studier, F. W. (1973a), *Proc. Natl. Acad. Sci. U.S.A. 70,* 1550–1563.
Dunn, J. J., and Studier, F. W. (1973b), *Proc. Natl. Acad. Sci. U.S.A. 70,* 3296–3300.
Eilen, E., and Krakow, J. (1973), *Biochem. Biophys. Res. Commun. 55,* 282–290.
Golomb, M., and Chamberlin, M. J. (1974), *Proc. Natl. Acad. Sci. U.S.A. 71,* 760–764.
Gonzalez, N., Wiggs, J., and Chamberlin, M. (1977), *Arch. Biochem. Biophys. 187,* 404–408.
Hercules, K., Jovanovich, S., and Sauerbier, W. (1976), *J. Virol. 17,* 642–658.
Hesselbach, B. A., and Nakada, D. (1977), *J. Virol. 24,* 736–745.
Hinkle, D. C., Mangel, W. F., and Chamberlin, M. J. (1972), *J. Mol. Biol. 70,* 209–220.
Hsieh, T. S., and Wang, J. (1976), *Biochemistry 15,* 5776–5783.
Ilyina, T. S., Ovadis, M. I., Mindlin, S. Z., Gorlenko, Z. M., and Khesin, R. B. (1971), *Mol. Gen. Genet. 110,* 118–133.
Khesin, R. B., Mindlin, S. Z., Ilyina, T. S., Ovadis, M. I., and Gorlenko, Z. M. (1971), *Mol. Biol. (Engl. Transl.) 5,* 862–877.
Kramer, R. A., Rosenberg, M., and Steitz, J. (1974), *J. Mol. Biol. 89,* 767–776.
Millette, R. L., Trotter, C. D., Herrlich, P., and Schweiger, M. (1970), *Cold Spring Harbor Symp. Quant. Biol. 35,* 135–142.
Minkley, E. G. (1974), *J. Mol. Biol. 83,* 305–331.
Minkley, E. G., and Pribnow, D. (1973), *J. Mol. Biol. 77,* 255–277.
Neff, N. F., and Chamberlin, M. J. (1978), *J. Biol. Chem. 253,* 2455–2460.
Rabussay, D., and Zillig, W. (1969), *FEBS Lett. 5,* 104–106.
Schweiger, M., Herrlich, P., Scherzinger, E., and Rahmsdorf, H. J. (1972), *Proc. Natl. Acad. Sci. U.S.A. 69,* 2203–2207.
Sippel, A., and Hartmann, G. (1968), *Biochim. Biophys. Acta 157,* 218–219.
Stahl, S. J., and Chamberlin, M. J. (1977), *J. Mol. Biol. 112,* 577–601.
Studier, F. W. (1972), *Science 176,* 367–376.
Studier, F. W. (1975), *J. Mol. Biol. 94,* 283–295.
Summers, W. C., Brunovskis, I., and Hyman, R. W. (1973), *J. Mol. Biol. 74,* 291–300.
Symons, R. (1968), *Biochim. Biophys. Acta 155,* 609–610.
Thomas, C. A., and Abelson, J. (1966), *Proced. Nucleic Acid Res. 1,* 553–561.
Williams, R. C. (1977), *Proc. Natl. Acad. Sci. U.S.A. 74,* 2311–2315.
Williams, R. C., and Chamberlin, M. J. (1977), *Proc. Natl. Acad. Sci. U.S.A. 74,* 3740–3744.
Zillig, W., Fujiki, H., Blum, W., Janekovic, D., Schweiger, M., Rahmsdorf, H. J., Ponta, H., and Hirsch-Kauffmann, M. (1975), *Proc. Natl. Acad. Sci. U.S.A. 72,* 2506–2510.

Involvement of the Mature Domain in the in Vitro Maturation of *Bacillus subtilis* Precursor 5S Ribosomal RNA†

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ABSTRACT: A precursor of 5S ribosomal RNA from *Bacillus subtilis* ($p5_A$ rRNA, 179 nucleotides in length) is cleaved by RNase M5, a specific maturation endonuclease which releases the mature 5S rRNA (m5, 116 nucleotides) and precursor fragments derived from the 5′ (21 nucleotides) and 3′ (42 nucleotides) termini of $p5_A$ rRNA. Previous results (Meyhack, B., et al. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75,* 3045) led to the conclusion that recognition elements in potential RNase M5 substrates mainly reside in the mature moiety of the precursor. Limited digestion of $p5_A$ rRNA with RNase T_1 permitted the isolation of a number of test substrates which contained both precursor-specific segments and were unaltered in the immediate vicinity of the cleavage sites, but which differed in that more or less extensive regions of the mature moiety of the $p5_A$ rRNA were deleted. Tests of the capacity of these partial molecules to serve as substrates for RNase M5 indicate clearly that the enzyme recognizes the overall conformation of potential substrates, neglecting only the double-helical "prokaryotic loop" (Fox, G. E., & Woese, C. R. (1975) *Nature (London) 256,* 505).

The maturation of most RNA molecules, both in prokaryotes and in eukaryotes, includes a series of scissions which reduce the chain lengths of precursor RNA molecules to their mature, functional forms. The nucleases which effect these maturation cleavages are highly selective in their action and therefore allow the exploration of mechanisms involved in specific interactions between proteins and polyribonucleotides. Most known RNA precursors are too large to study in the requisite detail. However, the precursors of 5S ribosomal RNA (rRNA) of *Bacillus subtilis* are relatively simple in structure (Pace et al., 1973) and consequently are reasonably amenable to manipulation and structural analysis. Additionally, the endonuclease responsible for the maturation of these precursors has been isolated in substantial purity (Sogin et al., 1977). We have undertaken to define in detail the interactions of this maturation endonuclease with its precursor RNA substrates.

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