THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 251, No. 13, Issue of July 10, pp. 4090-4094, 1976
Printed in U.S.A.

On the Processive Mechanism of *Escherichia coli* DNA Polymerase I

DELAYED INITIATION OF POLYMERIZATION*

(Received for publication, February 1, 1976)

ROBERT A. BAMBARA,‡ DENNIS UYEMURA, AND I. R. LEHMAN

From the Department of Biochemistry, Stanford University School of Medicine, Stanford, California 94305

Escherichia coli DNA polymerase I shows a delay in the initiation of polymerization after binding to the cohesive ends of λ DNA. This delay is significantly longer than the time required for the synthesis of an octanucleotide sequence on the right-hand cohesive end of the λ DNA. When the extent of polymerization is limited by omission of one or more of the deoxynucleoside triphosphates, and polymerization started again by their addition, the delay still occurs. A plausible explanation for this phenomenon is that two forms of the enzyme or enzyme·DNA complex exist, only one of which is active. The delay, therefore, represents the time necessary to convert the inactive to the active form of the enzyme or enzyme complex. One consequence of the defect in DNA polymerase I, due to the *pol*A12 mutation, is apparently to alter the equilibrium between the two forms. However, the rate of polymerization and the rate of conversion of inactive to active enzyme or enzyme complex are not changed significantly.

We have investigated the polymerization reaction catalyzed by DNA polymerase I from *Escherichia coli* using the cohesive ends of bacteriophage λ DNA as primer-template (1). This system permits measurement of the rate of polymerization at individual cohesive ends as distinguished from the kinetics of polymerization observed for an entire population of ends. This distinction is possible because the composition of nucleotides incorporated at such ends changes in a predictable manner as polymerization proceeds (1, 2). We have found that with a molar excess of enzyme relative to DNA termini, the time required to fill in any one cohesive end is much shorter than the time needed to complete polymerization at all ends.

In the work reported here, we have demonstrated that the delay in the completion of polymerization is due not to a low rate of association between enzyme and primer termini at the cohesive ends, but rather to the slow onset of polymerization at most of the cohesive ends once binding has occurred.

MATERIALS AND METHODS

The four unlabeled deoxynucleoside triphosphates were purchased from P-L Biochemicals, Inc. [^{3}H]dGTP (approximately 20 Ci/mmol) and [α-^{32}P]dCTP (60 to 100 Ci/mmol) were purchased from New England Nuclear Corp. Bacteriophage λ DNA was isolated according to the method of Wu *et al.* (3). A preparation of *Eco*RI restriction fragments of λ DNA containing the right-hand cohesive end was a gift from Dr. Michael Syvanen (Harvard University) (4). Poly(dA)$_{6000}$ was prepared with *Escherichia coli* DNA polymerase I according to the method of Riley *et al.* (5). Wild type DNA polymerase I was Fraction VII (6). *Pol*A12 DNA polymerase I was isolated as described by Uyemura and Lehman (7). Bio-Gel A-50m was purchased from Bio-Rad Laboratories.

Polymerization of nucleotides at the cohesive ends of λ DNA catalyzed by DNA polymerase I was performed as described by Wu *et al.* (3). Changes in the concentrations of components are described for each experiment. The intactness of the λ DNA was confirmed by methods described previously (1).

The concentrations of DNA are expressed as moles of duplex DNA molecules unless stated otherwise.

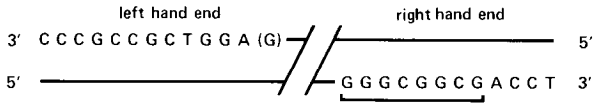
RESULTS

Kinetics of Polymerization at Right-hand Cohesive End of λ DNA—The sequences of nucleotides incorporated at the cohesive ends of λ DNA by DNA polymerase I are shown in Scheme 1. With only dGTP and dCTP, the octanucleotide sequence denoted by the *bracket* is synthesized at the right-hand cohesive end; dGMP is also incorporated at the left-hand end by an exchange reaction (8), however, the rate of exchange is slow compared to the rate at which the octanucleotide sequence is synthesized and does not interfere with the analysis (1, 8).

The kinetics of dGMP and dCMP incorporation at the right-hand cohesive end of λ DNA at 6° in the presence of an excess of DNA polymerase I are shown in Fig. 1. Also shown is the change in the dG to dC ratio during the course of the reaction. Assuming a Poisson distribution of nucleotide synthe-

* This investigation was supported in part by United States Public Health Service Grant GM-06196 and National Science Foundation Grant GB-41927.

‡ Postdoctoral Fellow of the Jane Coffin Childs Fund for Medical Research.



SCHEME 1. Sequences at the cohesive ends of λ DNA. The *letters* represent the nucleotides incorporated by DNA polymerase I in the presence of all four deoxynucleoside triphosphates (2). In the absence of dATP, only the sequence denoted by the *bracket* is synthesized. The 3′-terminal dGMP of the left-hand end (*letter in parentheses*) can exchange with labeled dGTP (8). This scheme is reprinted from Uyemura *et al.* (1).

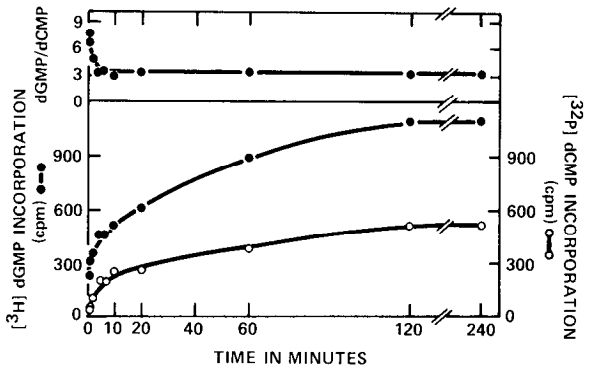


FIG. 1. Synthesis by DNA polymerase I of the octanucleotide sequence at the right-hand cohesive end of λ DNA in the presence of dGTP and dCTP. The reaction mixture (200 μl) contained 0.88 nM λ DNA, 70 mM potassium phosphate buffer (pH 7.0), 10 mM $MgSO_4$, 10 mM dithiothreitol, 80 mM NaCl, 7 μM [^{3}H]dGTP, and 5 μM [^{32}P]dCTP. The reaction was started by addition of 2 pmol of DNA polymerase I in 2 μl of polymerase diluent (50 mM Tris-Cl buffer (pH 8.5), 20% glycerol (v/v), 0.2 mg/ml of bovine serum albumin, and 0.2 M NH_4Cl) and incubated at 6°. Aliquots (15 μl) were removed at the times indicated and acid-insoluble radioactivity was determined. In the *lower panel*, values are expressed as counts per min per 15 μl. The *upper panel* shows the dG to dC ratio at the times indicated. The specific activities of [^{3}H]dGTP and [^{32}P]dCTP, determined as described previously (1), were in the range of 10 to 100 cpm/fmol.

sis at all ends, a dG to dC ratio of 4.9 is to be expected when an average of four nucleotides has been incorporated at all ends at which there is any synthesis. The time required to reach this ratio is an approximate measure of the half-time for completion of an individual octanucleotide sequence at the right-hand end. As estimated from the drop in ratio shown in Fig. 1, the approximate half-time for synthesis of the octanucleotide sequence was 1½ min. Five minutes after the addition of polymerase, when approximately 35% of all the cohesive ends had been filled in, the dG to dC ratio had reached a value of approximately 3, indicating that synthesis of the octanucleotide sequence was complete on all right-hand cohesive ends at which polymerization had occurred. Thus, at 5 min about 35% of the cohesive ends contained completed octanucleotide sequences; the remainder had sustained essentially no synthesis.

After 5 min, the rate of polymerization fell sharply, and 2 hours were required for the remainder of the right-hand cohesive ends to be filled in. Nevertheless, the dG to dC ratio remained constant at about 3 throughout this period. Hence, incorporation of nucleotides occurred at only a small fraction of the total ends at any given time after 5 min. These data are consistent with a relatively slow rate of initiation of polymerization at most cohesive ends. Once initiation has occurred, synthesis is then completed very rapidly (4 to 5 min). Therefore, initiation appears to be the rate-determining step in the

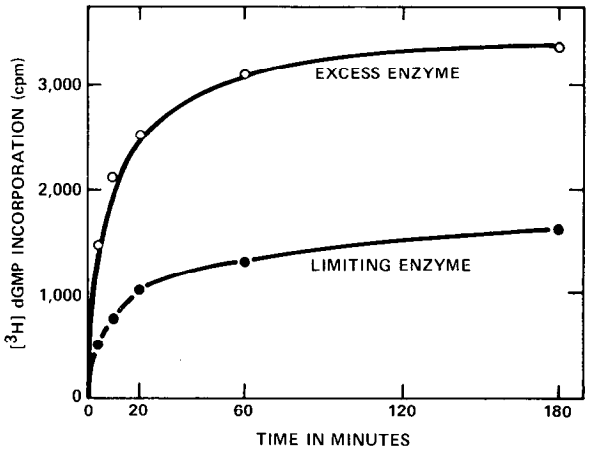


FIG. 2. Comparison of rates of nucleotide incorporation at the cohesive ends of λ DNA at excess and limiting DNA polymerase I concentrations. Reaction conditions were the same as described in the legend to Fig. 1, except that 10 μM each of unlabeled dATP and dCTP replaced the [^{32}P]dCTP. Aliquots (30 μl) of the reaction mixture were tested for acid-insoluble ^{3}H. O——O, 20 pmol of DNA polymerase I were added in 2 μl of polymerase diluent. ●——●, 0.2 pmol of DNA polymerase I was added in 2 μl of polymerase diluent.

polymerization of the first eight nucleotides at the cohesive ends of λ DNA catalyzed by DNA polymerase I at 6°.[1]

DNA Polymerase I Binds Rapidly to 3′-Hydroxyl Termini of λ DNA—The apparently slow rate of initiation of polymerization could reflect the time required for most of the enzyme molecules to find and bind correctly the 3′-hydroxyl termini at the right-hand cohesive ends. This possibility was eliminated by the following experiments.

First, dGMP and dCMP incorporation rates were measured at two widely differing enzyme concentrations. As seen in Fig. 2, there was essentially no effect on the overall kinetics observed in going from a 2-fold excess of cohesive ends over enzyme to a 50-fold excess of enzyme over ends.[2] Thus, the rate of association between enzyme and cohesive ends is rapid and does not appear to be rate-limiting for the initiation of polymerization during the slow phase of the reaction.

Secondly, a rapid rate of association of enzyme with DNA was demonstrated by direct binding measurements. DNA polymerase I was incubated with λ DNA at a 2-fold excess of ends over enzyme molecules for 4½ min at 6°, then applied to a column of Bio-Gel A-50m. Under these conditions an enzyme DNA complex can be separated from free enzyme in 2 to 3 min. As shown in Fig. 3, almost all of the enzyme was eluted together with the DNA. When DNA was omitted from the reaction, all of the enzyme was eluted together with the deoxynucleoside triphosphates (data not shown). These data demonstrate that formation of a stable enzyme·DNA complex is rapid compared to the time required for the overall polymeri-

[1] The rate of dissociation of enzyme from DNA is so slow under the conditions used (1) that the enzyme may be considered to react stoichiometrically with the cohesive ends. Thus, with excess enzyme, dissociation does not enter into the overall reaction and is not a rate-determining step.

[2] The curve of nucleotide incorporation at limiting enzyme concentrations would be expected to reach a plateau because of the low rate of dissociation of enzyme from DNA (1). However, there is a consistent, slight increase most evident by 180 min, which presumably results from the dissociation of active enzyme from the DNA, permitting it to react at other cohesive ends. With excess enzyme, the reaction is limited by exhaustion of DNA substrate, and there is no such increase.

zation reaction to reach completion.

Although complex formation was rapid, most of the enzyme molecules may have been bound nonproductively to either the double-stranded region of the λ DNA or to the single-stranded portions of the cohesive ends. The apparently low rate of initiation of polymerization would then reflect the low rate of dissociation of such complexes. This possibility was tested by determining whether changes in the concentration of double- or single-stranded DNA relative to the concentration of right-hand cohesive ends affected the overall time course of polymerization. Addition of a 10- or 150-fold excess (in nucleotides) of a single-stranded polynucleotide (poly(dA)$_{6000}$) over cohesive ends had no effect on the time course. Similarly, when the relative concentration of duplex DNA was lowered about 5-fold by replacing intact λ DNA with a mixture of *Eco*RI restriction enzyme fragments which contained the right-hand cohesive end but only one-fifth of the double-stranded λ DNA region, there was no increase in the overall rate of reaction. These data indicate that formation of inactive complexes of DNA polymerase I with internal duplex or single-stranded regions in λ DNA cannot account for the slow rate of initiation of polymerization. They further demonstrate that there is a rapid association between DNA polymerase I and the 3′-hydroxyl termini at the right-hand cohesive ends of λ DNA.

Resumption of Polymerization after Termination Shows Delayed Initiation—After the reaction in the presence of dGTP and dCTP has reached completion, addition of dATP and dTTP will permit resumption of polymerization at those cohesive ends to which enzyme is bound (1). Under the conditions used, enzyme molecules react stoichiometrically with DNA ends, remaining bound at one end for the entire reaction. An experiment of this kind therefore permits analysis of the kinetics of polymerization after enzyme is bound to primer sites at the cohesive ends. However, since the kinetics might be influenced by the abnormally low rate of incorporation of the terminal dC and dT residues (1), dTTP was omitted, thus limiting labeled dGMP incorporation to within the first eight nucleotides at both cohesive ends (Scheme 1).

The results of such an experiment are shown in Fig. 4. The incubation mixture initially contained only [^{3}H]dGTP, and unlabeled dCTP and dATP were added at 60 min. At 20 min after polymerization had resumed, maximal incorporation had still not been achieved, indicating that initiation was slow compared to the rate of polymerization at a given cohesive end even when enzyme was prebound to the 3′-hydroxyl terminus at that end. This result is completely analogous to that found for the polymerization observed initially at the cohesive ends, and further suggests that association of enzyme and DNA is not rate-determining for most of the reaction.

Kinetics of Polymerization Catalyzed by a Mutant DNA Polymerase I—The kinetics of polymerization at the right-hand cohesive end of λ DNA were determined for DNA polymerase I isolated from a temperature-sensitive *pol*A mutant, *E. coli pol*A12 (7, 9). The *pol*A12 DNA polymerase I, although rapidly inactivated at 43°, possesses nearly normal polymerase and 5′ → 3′-exonuclease activities at 30°. However, it is markedly defective at 30° in its ability to promote the concerted polymerization and 5′ → 3′ exonucleolytic removal of nucleotides at nicks in duplex DNA (nick translation), characteristic of the wild type enzyme (7). As shown in Fig. 5, the mutant DNA polymerase I showed a slower initial rate of polymerization than the wild type enzyme. Nevertheless, the dG to dC incorporation ratio for the mutant polymerase reached a value of about 3 within 5 min, suggesting that the rate of polymerization at a given cohesive end was rapid and approximately the same as that for the wild type enzyme. Again, it is unlikely that a low rate of binding of the *pol*A12 enzyme to the cohesive ends is responsible for the apparently slow polymerization rate since the kinetics of incorporation are

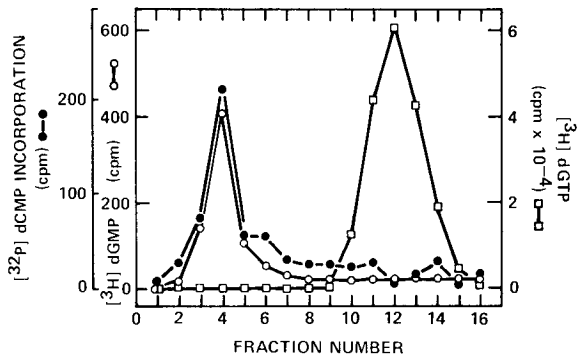


FIG. 3. Rapid binding of DNA polymerase I to the cohesive ends of λ DNA. The reaction mixture was the same as described in the legend to Fig. 1, except that [α-^{32}P]dCTP was omitted and 0.2 pmol of DNA polymerase I was added in 2 μl of polymerase diluent. After 4.5 min the reaction mixture was applied to a column (5 × 70 mm) of Bio-Gel A-50m eluted with a buffer containing 70 mM potassium phosphate (pH 7.0), 10 mM $MgSO_4$, 10 mM dithiothreitol, 80 mM NaCl, and 1 μM dTTP. A flow rate of 0.25 ml/min was maintained. Separation occurred in 2 to 3 min with complete elution by 6 to 7 min. Fractions of approximately 100 μl were collected. An aliquot (5 μl) was removed from each fraction and ^{3}H was determined. The [^{3}H]dGTP peak (□——□) marks the position where free polymerase is expected. Each fraction was then supplemented with 60 μl of a solution containing 1.6 nM λ DNA, 4 μM [^{32}P]dCTP, 4 μM dATP, dTTP, and dGTP, and 150 mM NaCl. The reaction mixtures were incubated for 4 hours at 6°. Acid-insoluble radioactivity was determined for each fraction. ^{3}H marks the position of λ DNA, O——O; ^{32}P marks the position of DNA polymerase I activity, ●——●.

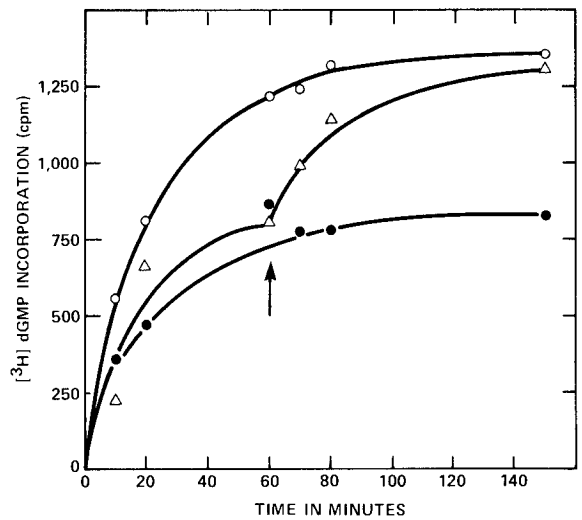


FIG. 4. Resumption of synthesis after termination. Three reaction mixtures (240 μl) were prepared as described in the legend to Fig. 1. Two contained only [^{3}H]dGTP (8 μM) (●——●, Δ——Δ), and one contained [^{3}H]dGTP (8 μM) and unlabeled dCTP and dATP (each at 10 μM) (O——O). The reaction was started by addition of 0.25 pmol of DNA polymerase I. At the times indicated, 35-μl aliquots were removed to determine acid-insoluble radioactivity. At 60 min (*arrow*), dATP and dCTP were added to 10 μM to one of the two reaction mixtures containing only [^{3}H]dGTP (Δ——Δ). The incubation was continued and samples taken as indicated.

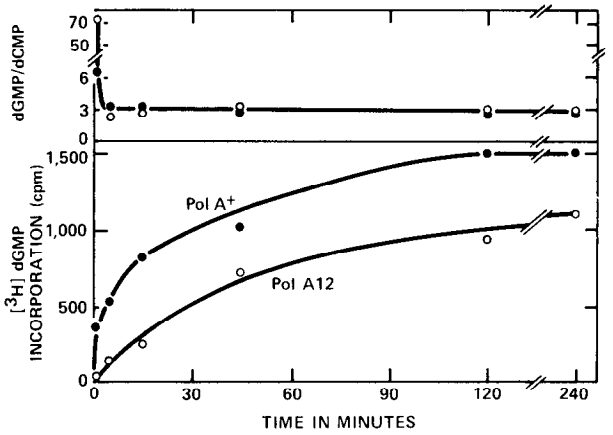


FIG. 5. Comparison of rates of synthesis of the octanucleotide sequence at the right-hand cohesive end of λ DNA by DNA polymerase I from *Escherichia coli polA*$^+$ and *E. coli polA12*. Reaction mixtures were the same as described in the legend for Fig. 1, except that 2 pmol of DNA polymerase I from *E. coli polA*$^+$ (●——●) were added to one and 2 pmol of DNA polymerase I from *E. coli polA12* (O——O) were added to the other. Incorporation is expressed as counts per min per 25-μl aliquot (*lower panel*). The *upper panel* shows the dG to dC ratio at the times indicated.

essentially the same at levels of mutant enzyme corresponding to 15 and 0.5 enzyme molecules per cohesive end. These data suggest that the reduced overall rate of polymerization observed for the *polA12* DNA polymerase I is a result of an abnormally low rate of initiation.

DISCUSSION

By using the cohesive ends of λ DNA as primer-template, we have demonstrated that the polymerization reaction catalyzed by *Escherichia coli* DNA polymerase I is processive; that is, many nucleotides are polymerized before the enzyme dissociates from the primer-template. This mechanism should be distinguished from one in which dissociation following addition of each nucleotide is an obligatory component of the polymerization reaction. We have further been able to separate the polymerization reaction into several of its component steps, and to determine their rates relative to each other (1). These include (*a*) association of enzyme with a 3′-hydroxyl terminus of the primer-template, (*b*) initiation of polymerization, (*c*) polymerization or chain elongation, and (*d*) dissociation of enzyme from DNA. Our results suggest that the initiation of polymerization is a slow step compared to rates of association and polymerization. Regardless of whether enzyme is present in excess or is limiting, about 30 to 40% of the enzyme molecules begin polymerization rapidly, but the remaining molecules are very significantly delayed. These data are consistent with the existence of two forms of DNA polymerase I or of the polymerase-DNA complex. The latter could, in turn, result from two modes of binding of polymerase to DNA, or two forms of the cohesive ends. While it is not possible to determine unambiguously which of these possibilities applies, we would suggest that the last is the least likely. First, the time course of polymerization after resumption of synthesis is not detectably different from that for the initial polymerization reaction. Thus, if two different structures of the cohesive end did exist, their proportion would have to remain unchanged after the right-hand cohesive end had been partially filled in. Secondly, given the same cohesive ends, DNA polymerase I from *E. coli polA12* showed a slower initial rate of polymerization than the wild type enzyme.

A simple interpretation that is consistent with our findings is that there are two interconvertible forms of DNA polymerase I, only one of which is active. Since approximately one-third of the enzyme molecules begin polymerization immediately, the normal equilibrium would give a ratio of inactive to active molecules of two. By this argument, the rate of conversion from inactive to active enzyme is the rate-determining step for the initiation of polymerization. The *polA12* enzyme might then be altered in such a way that only a very small fraction of active molecules would be present at any time, *i.e.* the equilibrium of the reaction, active ⇌ inactive, is far in the direction of inactive enzyme. However, the rate of conversion from the inactive to the active form would not be significantly altered by the mutation.

An alternative and equally plausible explanation is that two forms of polymerase·DNA complex exist and that the *polA12* mutation has reduced the relative amount of active complex.

No direct determination of the polymerization rate at individual cohesive ends during the latter stage of the reaction is possible. However, it also appears to be rapid, as evidenced by the stable dG to dC ratio of 3 during this period. In addition, the *polA12* polymerase, which does not show an initially rapid phase, nevertheless catalyzes a rapid polymerization at individual cohesive ends.

In examining the kinetics of the polymerization reaction catalyzed by DNA polymerase I, McClure and Jovin (10) observed an initial burst of nucleotide incorporation stoichiometric with enzyme concentration, followed by a second slower rate of polymerization. There is an obvious analogy between this burst phenomenon and the rapid initial phase of polymerization followed by a slow phase described here. McClure and Jovin proposed that some microscopic step subsequent to binding and nucleotide incorporation is rate-limiting and predicted that the magnitude of the burst would be stoichiometric with enzyme concentration. Implicit in their proposal is that no 3′ terminus has more than a single nucleotide added to it during the burst. Our results using λ DNA cannot be interpreted in this way, since appreciable dCMP was incorporated rapidly, indicating that at least 4 residues had been incorporated at some of the ends during the rapid phase of the reaction.

On the other hand, we have found the rate of association between enzyme and DNA termini to be rapid compared to the time course for the overall polymerization reaction, a result that is consistent with the finding by McClure and Jovin (10) that the rate constant for association is at least 1 order of magnitude greater than would be expected for a diffusion-limited interaction.

Finally, it should be pointed out that the delay in initiation of polymerization described here may not be a property of all DNA polymerases, or even of DNA polymerase I under all conditions.

REFERENCES

1. Uyemura, D., Bambara, R., and Lehman, I. R. (1975) *J. Biol. Chem.* **250,** 8577–8584
2. Wu, R., and Taylor, E. (1971) *J. Mol. Biol.* **57,** 491–511
3. Wu, R., Padmanabhan, R., and Bambara, R. (1974) *Methods Enzymol.* **29,** 231–253

4. Syvanen, M. (1975) *J. Mol. Biol.* **91,** 165–174
5. Riley, M., Maling, B., and Chamberlin, M. J. (1966) *J. Mol. Biol.* **20,** 359–389
6. Jovin, T. M., Englund, P. T., and Bertsch, L. L. (1969) *J. Biol. Chem.* **244,** 2996–3008
7. Uyemura, D., and Lehman, I. R. (1976) *J. Biol. Chem.* **251,** 4078–4084
8. Donelson, J. E., and Wu, R. (1972) *J. Biol. Chem.* **247,** 4654–4660
9. Monk, M., and Kinross, J. (1972) *J. Bacteriol.* **109,** 971–978
10. McClure, W. R., and Jovin, T. M. (1975) *J. Biol. Chem.* **250,** 4073–4080