THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 251, No. 13, Issue of July 10, pp. 4085-4089, 1976
Printed in U.S.A.

Biochemical Characterization of Mutant Forms of DNA Polymerase I from *Escherichia coli*

II. THE *pol*Aex1 MUTATION*

(Received for publication, February 1, 1976)

DENNIS UYEMURA, DUANE C. EICHLER,‡ AND I. R. LEHMAN

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

DNA polymerase I has been purified to homogeneity from an *Escherichia coli* K12 strain bearing the temperature-sensitive conditionally lethal mutation, *pol*Aex1. The purified enzyme shows no defect in its polymerase or 3′ → 5′-exonuclease activities; however, its 5′ → 3′-exonuclease activity is abnormally low at both 30° and 43°. Although the mutant enzyme is able to catalyze the coordinated 5′ → 3′ polymerization and 5′ → 3′ exonucleolytic hydrolysis of nucleotides at a nick in duplex DNA ("nick translation") at a measurable rate at 30°, this reaction is undetectable at 43°. This defect is very likely responsible for the retarded joining of nascent DNA fragments and the consequent loss of viability that occur in the mutant at this temperature.

The isolation of the temperature-sensitive conditionally lethal mutant *Escherichia coli pol*Aex1 established that DNA polymerase I is essential for the viability of *E. coli* (1). The finding that the joining of nascent DNA fragments is greatly retarded in this and other *pol*A mutants (2, 3) indicates that DNA polymerase I is required for the discontinuous replication of the *E. coli* chromosome and suggests that the inviability of *pol*Aex1 at restrictive temperatures is a consequence of a severe defect in this process. In fact, the rate of joining of nascent DNA fragments in *E. coli pol*Aex1, in contrast to its wild type parent, occurs at a significantly lower rate at 43° than at 30° (1). Examination of the partially purified DNA polymerase I from *E. coli pol*Aex1 showed that its polymerase activity was nearly normal but that its 5′ → 3′-exonuclease activity was greatly reduced even at 30°. However, the residual 5′ → 3′-exonuclease was not abnormally thermolabile.

In an attempt to clarify the relationship of the defective 5′ → 3′-exonuclease activity to the temperature-sensitive phenotype of *E. coli pol*Aex1, we have purified the mutant DNA polymerase I to homogeneity and examined in detail its polymerase and associated exonuclease activities. These studies have confirmed that the *pol*Aex1 mutation has caused little if any change in polymerase activity. They have further demonstrated that the residual 5′ → 3′-exonuclease activity that persists at 30° is indeed thermolabile. However, the thermolability is apparent only when 5′ → 3′-exonuclease activity is measured as polymerization proceeds.

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

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

MATERIALS AND METHODS

The materials and methods used are those described in the preceding paper (4). In addition, *Escherichia coli* K12 *pol*Aex1 (RS5064) was isolated as described previously (1), and *E. coli* K12 JG112 (*pol*A1) was obtained from Dr. T. Kornberg (Massachusetts Institute of Technology). ϕX174 [^{32}P]DNA was provided by Lee Rowen (Stanford).

RESULTS

Purification of DNA Polymerase I from Escherichia coli polAex1—The procedure developed for the purification of DNA polymerase I from *E. coli pol*A12 (4) served for the purification of the enzyme from *E. coli pol*Aex1, with the following modifications. (*a*) Fraction III was dialyzed against 4 liters of 0.06 M potassium phosphate (pH 7.0), 2 mM EDTA, and 20% glycerol (v/v) for 16 hours prior to phosphocellulose chromatography. The column was equilibrated with this buffer and developed with a gradient of 0.06 to 0.2 M potassium phosphate (pH 7.0), 2 mM EDTA, and 20% glycerol (v/v). (*b*) Fraction IV was applied to the DNA-cellulose column as described for the *pol*A12 enzyme, but activity was eluted between 0.17 and 0.28 M potassium phosphate (pH 7.0), 2 mM EDTA, and 20% glycerol (v/v), which is a lower ionic strength range than that required to elute the *pol*A12 enzyme. The active fractions were pooled (9.5 ml), then concentrated to 3.0 ml by dialysis against dry Sephadex G-50 (Fraction V). This fraction was inhomogeneous as judged by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate, but could be purified further by Sephadex G-150 filtration. (*c*) A column (3 $cm^2 \times 25$ cm) of Sephadex G-150 was equilibrated with 0.2 M potassium phosphate (pH 7.0), 2 mM EDTA, and 20% glycerol (v/v). Fraction V (2 ml) was dialyzed against 2 liters of the same

buffer for 4 hours and applied to the column. A flow rate of 12 ml/hour was maintained and 2.4-ml fractions were collected. The peak of activity was eluted at 42 ml, whereas the void volume was 31 ml. Fractions containing 50% of the applied activity were pooled (Fraction VI). A summary of the purification is given in Table I.

Fraction VI was stored in liquid N_2 and remained stable for 1 year. It was used in all studies to be described.

Electrophoretic Analysis of polAex1 Enzyme—Fraction VI was >90% homogeneous as determined by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate, and it co-migrated with wild type DNA polymerase I under these conditions. A single peak of DNA polymerase I activity was eluted from nondenaturing, discontinuous, polyacrylamide gels that co-migrated with the major protein peak. Unlike the *pol*A12 polymerase, the *pol*Aex1 protein was not resolved from the wild type enzyme by electrophoresis in nondenaturing polyacrylamide gels.

polAex1 Enzyme Is Defective Specifically in Its 5′ → 3′-Exonuclease Activity—The polymerase activity of the mutant enzyme measured with activated calf thymus DNA as primer-template was similar to the wild type at 30°. The same was true for the 3′ → 5′-exonuclease activity. Neither activity was abnormally temperature-sensitive (Table II). In contrast, the 5′ → 3′-exonuclease activity of the mutant enzyme was substantially reduced under all conditions examined. Furthermore, the properties of the residual 5′ → 3′-exonuclease activity were altered. Like wild type DNA polymerase I, the 5′ → 3′-exonuclease activity of the mutant enzyme at 30° was markedly increased by addition of the four deoxynucleoside triphosphates. However, at 43° the 5′ → 3′-exonuclease activity of the mutant enzyme was inhibited by their addition. Moreover, although the 5′ → 3′-exonuclease of the mutant enzyme was not abnormally thermolabile when measured in the absence of the four deoxynucleoside triphosphates, it was thermolabile in their presence.

polAex1 Enzyme Is Abnormally Temperature-sensitive in Nick Translation—With nicked PM2 DNA as primer-template, the wild type enzyme catalyzed the equimolar incorporation and release of nucleotides (nick translation) at both 30° and 43° (Fig. 1). In contrast, there was a large discrepancy between nucleotide incorporation and release catalyzed by the mutant enzyme. Thus, at 30° polymerization proceeded at 30% and 5′ → 3′-exonuclease activity at only about 10% the rate of the wild type enzyme. These results suggest that even at 30° the predominant reaction catalyzed by the mutant polymerase consists of the elongation of the 3′ primer strand with concomitant unwinding of the 5′ strand ahead of the enzyme molecule, *i.e.* strand displacement. As judged by the stimulation of the 5′ → 3′-exonuclease activity by addition of the four deoxynucleoside triphosphates (Table II), the low level of 5′ → 3′-exonuclease probably reflects residual nick translation (approxi-

TABLE I

Purification of DNA polymerase I from Escherichia coli K12 polAex1

The values shown are adjusted for the protein obtained from a total of 240 g wet weight of cells.

Fraction	Units	Protein	Specific activity	Yield
		mg/ml	*units/mg protein*	%
I. Extract	7,350	10.2	1.9	(100)
II. DEAE-cellulose	8,080	2.4	2.8	110
III. Ammonium sulfate	7,150	37.2	4.9	97
IV. Phosphocellulose	4,000	0.085	36.4	55
V. DNA-cellulose	1,930	0.24	2,680	26
VI. Sephadex G-150	640	0.013	3,550	9

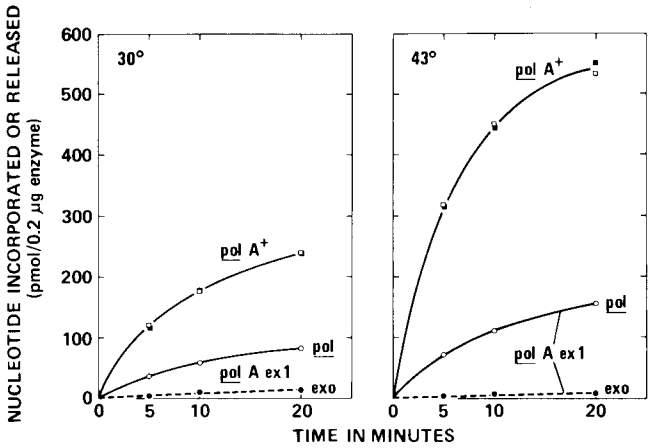

FIG. 1. Action of DNA polymerase I from *Escherichia coli polA*$^+$ and *E. coli pol*Aex1 on nicked PM2 DNA. The procedure was the same as described for the 5′ → 3′-exonuclease assays in the preceding paper (4), except that at the times indicated, aliquots (25 μl) were removed and acid-soluble (●, ■) and acid-precipitable (○, □) radioactivity were determined. Each reaction mixture (200 μl) contained 0.4 μg of enzyme, and aliquots (50 μl) were removed at the times indicated. The values shown represent picomoles of nucleotide incorporated or released by 0.2 μg of enzyme in 100 μl of reaction volume.

TABLE II

Polymerase and exonuclease activities of DNA polymerase I from Escherichia coli polA$^+$ *and E. coli polAex1*

Assays were performed as described by Uyemura and Lehman (4).

Enzyme	Temperature	Polymerase activity	5′ → 3′-Exonuclease activity		3′ → 5′-Exonuclease activity
			−dNTP	+dNTP	
		nmol/mg protein			
polA$^+$	30°	14,800	1,100	6,000	1,600
	43	41,900	5,400	11,800	3,400
*pol*Aex1	30	23,800	73	330	2,500
	43	66,000	340[a]	200[a]	3,100

[a] A portion (25 to 50%) of the apparent 5′ → 3′-exonuclease activity at 43° may be attributable to 3′ → 5′-exonuclease action. This estimate is based on the extent of hydrolysis observed after incubating the nicked PM2 DNA at 43° with T4 DNA polymerase, which has 3′ → 5′- but no 5′ → 3′-exonuclease activity.

mately 10% of wild type). At 43°, the rate of polymerization was 2-fold greater than at 30°, but nucleotide release attributable to nick translation was nearly undetectable. Hence, at 43° polymerization was accompanied only by strand displacement.

polAex1 Enzyme Is Not Defective in Polymerization at Gaps in Duplex DNA—With PM2 DNA containing gaps as primer-template, the wild type enzyme rapidly filled in the gaps to generate nicks, then continued nick translation at a normal rate at both 30° and 43° (Fig. 2). Gap-filling was complete by 5 min since the difference between nucleotide incorporation and release did not change after this time.

The mutant enzyme again showed a substantially greater rate of nucleotide polymerization than release at both 30° and 43°. The difference between the two was greater than that observed for the wild type enzyme, presumably reflecting a normal rate of polymerization at the gap followed by the reduced rate characteristic of strand displacement once the gap had been filled. A small amount of nucleotide hydrolysis was evident at both temperatures.

These results taken together with the normal polymerase activity observed with activated calf thymus DNA, a less well defined primer-template than PM2 DNA but which presumably contains small gaps (Table II), indicate that the polymerase activity of the mutant DNA polymerase I is unaffected by the *pol*Aex1 mutation.

polAex1 Mutation Causes Severe Retardation in Joining of DNA Replication Intermediates—The *pol*Aex1 mutation causes a retarded joining of nascent DNA fragments ("Okazaki fragments") *in vivo*. Similar results have been reported for other *pol*A mutants, most of which are not conditionally lethal (2, 3, 5). To examine further the basis of the conditional lethality caused by the *pol*Aex1 mutation, the rate of joining of Okazaki fragments in *E. coli pol*Aex1 was compared with the rate in *E. coli pol*A1, a nonlethal *amber* mutant (Fig. 3). Whereas the label in the *pol*A1 cells showed a substantial increase in average size by 60 s, that from *pol*Aex1 showed little increase. Thus, the *pol*Aex1 mutation caused a further reduction in the rate of joining of nascent DNA fragments at restrictive temperatures, beyond that produced by the *pol*A1 mutation.

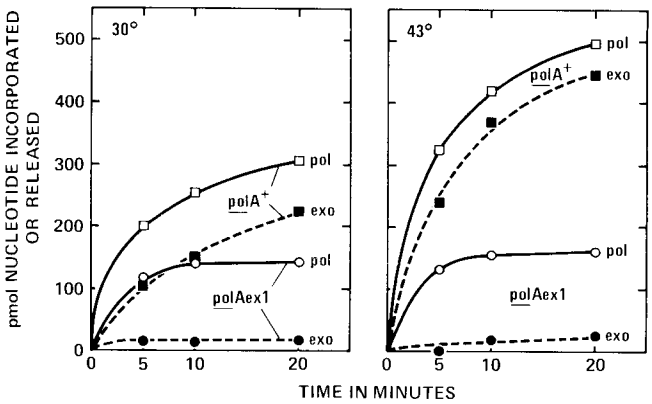

FIG. 2. Action of DNA polymerase I from *Escherichia coli pol*A+ and *E. coli pol*Aex1 on gapped PM2 DNA. The procedure was the same as that described in the legend to Fig. 1. Each mixture contained 0.1 μg of enzyme. The values shown represent the picomoles of nucleotide incorporated (○, □) or released (●, ■) by 0.05 μg of enzyme in 100 μl of reaction volume.

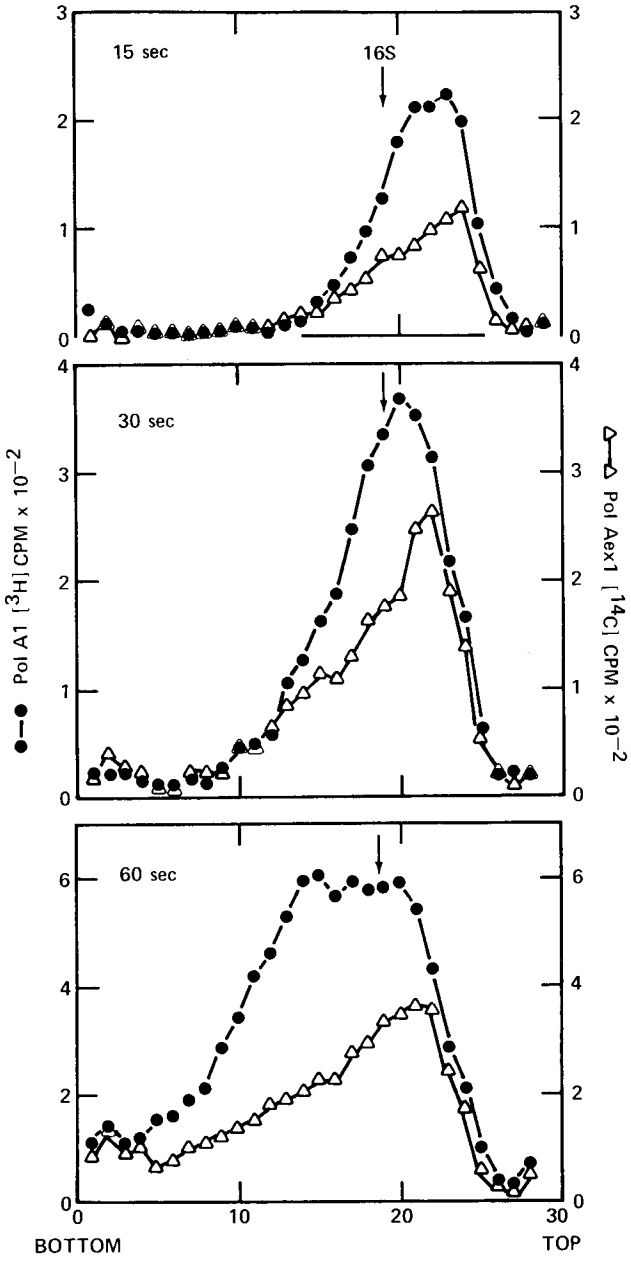

FIG. 3. Alkaline sucrose gradient centrifugation of pulse-labeled DNA isolated from *Escherichia coli pol*A1 and *E. coli pol*Aex1. *E. coli* strains JG112 (*pol*A1) and RS5064 (*pol*Aex1) were grown separately and with vigorous aeration at 30° in 20 ml of Medium A described by Okazaki *et al.* (6). At an A_{595} of 0.5, each culture was shifted to 44°. After 5 min, 20 μCi of [^{14}C]thymidine (54.6 mCi/mmol) were added to the *pol*Aex1 culture and 300 μCi of [^{3}H]thymidine (20 Ci/mmol) were added to the *pol*A1 culture. At the times shown, aliquots (6 ml) were added to 6 ml of a mixture composed of 20 mM sodium acetate, 2 mM EDTA, 75% ethanol (v/v), and 2% phenol (v/v) at 23°. The suspension was centrifuged at 8000 × *g* for 15 min at 4° and the supernatant fluid was discarded. The pellets were dissolved in 0.3 ml (*pol*Aex1) or 0.6 ml (*pol*A1) of 0.2 M NaOH containing 10 mM EDTA, and incubated at 37° for 70 min. An aliquot (300 μl) of the *pol*A1 DNA was mixed with 5 μl of the *pol*Aex1 DNA, then layed atop a 5 to 20% alkaline sucrose gradient. Phage φX174 [^{32}P]DNA was included as a sedimentation marker (*arrow*). Alkaline sucrose density gradients contained 0.2 N NaOH, 10 mM EDTA, and a linear gradient of 5 to 20% sucrose. Centrifugation was performed in the SW41 rotor for 8 hours at 40,000 rpm at 4° in a Beckman L5-65 ultracentrifuge. Fractions (0.4 ml) were collected from the bottom of the centrifuge tube. To each were added 0.1 ml of 0.53 mM calf thymus DNA (nucleotides) and 0.6 ml of 10% trichloroacetic acid. The precipitates were collected and acid-insoluble radioactivity was determined as described in the preceding paper (4).

DISCUSSION

The *pol*Aex1 mutation differentially affects the active site of DNA polymerase I. The mutant polymerase showed no defect in polymerization at gaps nor in its 3′ → 5′-exonuclease activity. In contrast, the 5′ → 3′-exonuclease activity was abnormally low under all conditions that we examined. At 30° the residual activity was stimulated by concurrent polymerization, suggesting that the mutant enzyme retains some capacity to coordinate polymerization with 5′ → 3′-exonuclease action. However, at 43° what activity remained was inhibited by addition of the four deoxynucleoside triphosphates, indicating that there is a complete loss of the capacity to promote nick translation. Thus, the only temperature-sensitive defect apparent in the DNA polymerase I isolated from *Escherichia coli* *pol*Aex1 is in 5′ → 3′-exonuclease activity that occurs during polymerization. This defect correlates with the conditionally lethal phenotype and the retarded joining of nascent DNA fragments caused by the *pol*Aex1 mutation, and suggests that the coordination of polymerase and 5′ → 3′-exonuclease may be essential for the discontinuous replication of the *E. coli* chromosome. That 5′ → 3′-exonuclease in the absence of the deoxynucleoside triphosphates is not abnormally thermolabile is probably of little physiological significance since the enzyme presumably functions only in their presence *in vivo*.

The effects of the lethal *pol*Aex1 mutation on the purified DNA polymerase I should be contrasted with those resulting from the nonlethal mutation *pol*A12 (4). All three activities associated with the *pol*A12 enzyme are abnormally thermolabile, in agreement with the temperature-sensitive defect in DNA repair seen *in vivo*. In addition, the *pol*A12 enzyme shows a reduced capacity to promote nick translation at 30° despite nearly normal polymerase and 5′ → 3′-exonuclease activities at this temperature. The lack of an effect of the *pol*A12 mutation on viability, even at 43°, suggests that cells can withstand a substantial loss (>90%) in their ability to promote nick translation. Furthermore, the *pol*A12 enzyme is to some extent stabilized by the ionic strength and DNA concentrations that exist *in vivo*.

Two other *pol*A mutations which affect 5′ → 3′-exonuclease of DNA polymerase I have been described, only one of which is conditionally lethal. The temperature-sensitive, conditionally lethal *pol*A mutation in strain *E. coli* BT4113 causes a substantial decrease in both polymerase and 5′ → 3′-exonuclease activities of the partially purified enzyme at 30°, and both activities are abnormally thermolabile (7). Although such studies have not yet been reported, it is reasonable to suppose that the capacity to promote nick translation in *E. coli* BT4113 is reduced at 30° and largely eliminated at 43°. The DNA polymerase I from cells bearing the nonlethal *pol*A′107 mutation has been purified to homogeneity and characterized (8). With a nicked primer-template (φX RF II), the purified enzyme showed a tendency toward strand displacement, presumably because of the low 5′ → 3′-exonuclease activity. Inasmuch as 5′ → 3′-exonuclease of the *pol*A′107 polymerase was approximately 25% of the wild type enzyme at 37°, a value comparable to that for the *pol*Aex1 polymerase at 30°, it would appear that the decrease is insufficient to cause lethality.

Why should nick translation be essential for discontinuous DNA replication? One possibility is that it is required for the coordinated 5′ → 3′ exonucleolytic removal of an RNA primer at the 5′ termini of nascent DNA fragments, and the filling in of the gap thus created, to permit their ligation to the replicating chromosome.

There is now strong circumstantial evidence that RNA primes the synthesis of nascent DNA fragments in *E. coli*. The *dna*G gene product, which is required for the initiation of Okazaki fragments both *in vivo* and *in vitro* (9), is now known to be a rifampicin-resistant RNA polymerase (10) that synthesizes a unique 25- to 30-residue polyribonucleotide that primes the synthesis of the duplex replicative form of phage G4 from the single-stranded circular parental DNA *in vitro*.[1] Furthermore, a 10-residue polyribonucleotide has been clearly demonstrated at the 5′ end of nascent polyoma DNA fragments synthesized in isolated nuclei of virus-infected mouse cells (11, 12). On the other hand, there has been no substantiated demonstration of a covalent linkage of ribonucleotides to deoxyribonucleotides at the 5′ end of nascent DNA fragments isolated from *E. coli*. The density shifts, in $CsSO_4$ gradients, of DNA pulse-labeled with [^{3}H]thymidine or [^{3}H]uridine reported by Sugino *et al.* (13) are most likely accounted for by noncovalent RNA-DNA aggregates (12, 14). Moreover, the demonstration of an RNA-DNA linkage by alkali treatment of nascent fragments and estimation of the 5′-hydroxyl groups so created by means of the polynucleotide kinase reaction (15) was complicated initially by the exchange of 5′-phosphoryl termini of DNAwith [γ-^{32}P]ATP (16). Under conditions where little or no exchange occurred, Okazaki *et al.* (17) have recently reported such a linkage in nascent DNA fragments from *E. coli* *pol*Aex1 but not wild type cells. However, we have analyzed the fragments isolated from *E. coli* *pol*Aex1 by this technique, again under conditions where exchange is negligible, and have been unable to detect such a linkage.[2] The discrepancy may reflect a difference in the extent of RNA-DNA aggregation in the preparations analyzed. Thus, if the 5′-hydroxyl terminus of a nascent DNA fragment were complexed with RNA, it might not be accessible to polynucleotide kinase until after treatment with alkali. A noncovalent association of this kind could be mistakenly interpreted to indicate a covalent RNA-DNA linkage. A similar reservation holds for experiments in which the generation of a 5′-hydroxyl group after alkali treatment of nascent DNA fragments was monitored with the use of spleen exonuclease (18).

We conclude that while a direct demonstration of RNA priming of nascent DNA fragments in the discontinuous replication of the *E. coli* chromosome is still lacking, the available evidence indicates that such primers do exist and that an essential role *in vivo* of DNA polymerase I is the concerted removal of such primers and filling in of the resulting gaps via nick translation. When this process is sufficiently defective, discontinuous replication ceases, leading ultimately to the death of the cell.

REFERENCES

1. Konrad, E. B., and Lehman, I. R. (1974) *Proc. Natl. Acad. Sci. U. S. A.* **71,** 2048–2051
2. Kuempel, P. L., and Veomett, G. E. (1970) *Biochem. Biophys. Res. Commun.* **41,** 973–980
3. Okazaki, R., Arisawa, M., and Sugino, A. (1971) *Proc. Natl. Acad. Sci. U. S. A.* **68,** 2954–2957
4. Uyemura, D., and Lehman, I. R. (1976) *J. Biol. Chem.* **251,** 4078–4084
5. Monk, M., Kinross, J., and Town, C. (1973) *J. Bacteriol.* **114,** 1014–1017
6. Okazaki, R., Okazaki, T., Sakabe, K., Sugimoto, K., and Sugino,

[1] J.-P. Bouche, S. Rowen, and A. Kornberg, personal communication.

[2] D. C. Eichler, J. Thorner, and I. R. Lehman, unpublished results.

A. (1968) *Proc. Natl. Acad. Sci. U. S. A.* **59,** 598–605
7. Olivera, B. M., and Bonhoeffer, F. (1974) *Nature* **250,** 513–514
8. Heijneker, H. L., Ellens, D. J., Tjeerde, R. H., Glickman, B. W., van Dorp, B., and Pouwels, P. H. (1973) *Mol. Gen. Genet.* **124,** 83–96
9. Lark, K. G. (1972) *Nature New Biol.* **240,** 237–240
10. Bouché, J.-P., Zechel, K., and Kornberg, A. (1975) *J. Biol. Chem.* **250,** 5995–6001
11. Eliasson, R., Martin, R., and Reichard, P. (1974) *Biochem. Biophys. Res. Commun.* **59,** 307–313
12. Reichard, P., Eliasson, R., and Söderman, G. (1974) *Proc. Natl. Acad. Sci. U. S. A.* **71,** 4901–4905
13. Sugino, A., Hirose, S., and Okazaki, R. (1972) *Proc. Natl. Acad. Sci. U. S. A.* **69,** 1863–1967
14. Probst, H., Gentner, P. R., Hofstätter, T., and Jenke, S. (1974) *Biochim. Biophys. Acta* **340,** 361–373
15. Hirose, S., Okazaki, R., and Tamanoi, F. (1973) *J. Mol. Biol.* **77,** 501–517
16. van de Sande, J. H., Kleppe, K., and Khorana, H. G. (1973) *Biochemistry* **12,** 5050–5055
17. Okazaki, R., Hirose, S., Okazaki, T., Ogawa, T., and Kurosawa, Y. (1975) *Biochem. Biophys. Res. Commun.* **62,** 1018–1024
18. Kurosawa, Y., Ogawa, T., Hirose, S., Okazaki, T., and Okazaki, R. (1975) *J. Mol. Biol.* **96,** 653–664