THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 248, No. 4, Issue of February 25, pp. 1361-1364, 1973
Printed in U.S.A.

Initiation of Deoxyribonucleic Acid Synthesis

IV. INCORPORATION OF THE RIBONUCLEIC ACID PRIMER INTO THE PHAGE REPLICATIVE FORM*

(Received for publication, October 30, 1972)

OLE WESTERGAARD,‡ DOUGLAS BRUTLAG,§ AND ARTHUR KORNBERG

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

SUMMARY

The product of *in vitro* replication of the ΦX174 or M13 viral strand is known to be a circular duplex with a discontinuity in the synthetic strand (RF II). This replication intermediate has now been converted to an alkali-stable covalent duplex (RF I) through the joint action of *Escherichia coli* DNA polymerase I and *E. coli* ligase. Failure of T4 DNA polymerase to substitute for the *E. coli* enzyme implied the presence of an RNA priming segment at the 5′ end which the unique 5′ → 3′ exonuclease function of the *E. coli* enzyme can excise. This suggestion was confirmed by conversion of the RF II product to an alkali-labile RF I through the joint action of T4 DNA polymerase and T4 ligase, an enzyme which can join RNA ends, and by location of the alkali-labile linkage of the RF I in the complementary (synthetic) strand. These results extend earlier evidence for RNA priming of DNA replication. They also call attention to a possible physiological role of *E. coli* DNA polymerase I in the excision of such RNA primers during DNA replication.

In the conversion of phage M13 and ΦX174 single-stranded DNAs to the duplex replicative form, we concluded that synthesis of a short RNA chain served as a primer for DNA replication (1–3). The evidence rested on: (*a*) action of specific inhibitors, (*b*) requirement for all four ribonucleoside triphosphates for DNA synthesis, and (*c*) a covalent linkage of DNA to RNA detected as an alkali-labile phosphodiester linkage in the isolated RF II[1] product.

We also observed that sealing of the RF II product to the covalently closed duplex (RF I) by *Escherichia coli* ligase would take place with *E. coli* DNA polymerase I present but not with T4 DNA polymerase. Both polymerases are known to be fully active in filling in gaps and thereby enabling the ligase to form a covalent seal between the 3′ and 5′ ends of a strand. However, because the *E. coli* ligase appears unable to link a DNA strand to an RNA strand (4), we suggested that the effectiveness of the *E. coli* DNA polymerase might be in excising an RNA segment (at the 5′ end of a DNA strand) through its unique 5′ → 3′ exonuclease activity.

In the present work we have obtained more direct evidence that the synthetic DNA strand does possess an RNA segment at the 5′ terminus by exploiting the capacity of T4 ligase to link DNA and RNA segments. The product, covalently sealed by T4 ligase is alkali-labile when the joining reaction is carried out by T4 polymerase, which, unlike the *E. coli* polymerase, cannot excise the RNA segment (Fig. 1).

MATERIALS AND METHODS

E. coli H560 (F^+, thy^-, endo I^-, $polAl^-$) was used in all experiments. The cells were grown, harvested, and lysed as previously described (3). The 100,000 × *g* supernatant of the lysate is Fraction I.

[^{3}H]RF I was isolated from infected cells as described by Francke and Ray (5) except that the cells were lysed in borate buffer.

We are indebted to our Stanford colleagues Dr. W. M. Huang and Dr. I. R. Lehman for the sample of T4 DNA polymerase (hydroxylapatite fraction (6)); to Mr. P. Modrich and Dr. Lehman for the sample of *E. coli* ligase (DEAE-Sephadex fraction); and to Dr. V. Sgaramella for T4 ligase (Fraction VII (7)). Fraction VII of *E. coli* DNA polymerase I (8) was used.

Synthetic RF II, labeled exclusively in the complementary strand, was prepared by incubating 0.3 ml of Fraction I with 0.2 ml of a mixture to give final concentrations of: 18 μM each of the deoxyribonucleoside triphosphates (of which dCTP, dGTP, or dATP was labeled with α-^{32}P at a specific activity of 950 cpm per pmole of nucleotide), 100 μM each of GTP, UTP, and CTP, 850 μM ATP, 4 mM $MgCl_2$, 60 mM NaCl, 3.3 mM spermidine, and 8 μM ΦX DNA (nucleotide residues). The mixture was incubated for 10 min at 30°. The reaction was stopped by addition of Sarkosyl (to 1%) and EDTA (to 10 mM). The synthesized product was sedimented in a 5 to 20% sucrose gradient in 1 M NaCl, 10 mM Tris·HCl (pH 7.4), and 1 mM EDTA in a Spinco SW 27 rotor for 18 hours at 25 Krpm and 4°. The peak of RF II was collected and precipitated from 0.3 M

* The preceding paper in this series is Reference 3. This study was supported in part by grants from the National Institutes of Health (United States Public Health Service) and the National Science Foundation.

‡ American Cancer Society-Eleanor Roosevelt-International Cancer Fellow. Present address, Biokemisk Institut B, Juliane Marie Vej 30[2], Kobenhavns Universitet, Copenhagen, Denmark.

§ Predoctoral Fellow of the National Science Foundation. Present address, Division of Plant Industries, C.S.I.R.O., P.O. Box 1600, Canberra, ACT, Australia.

[1] The abbreviations used are: ΦX, ΦX174; RF, double-stranded DNA of circular replicative form; RF I, covalently closed RF; RF II, RF with a discontinuity in at least one strand.

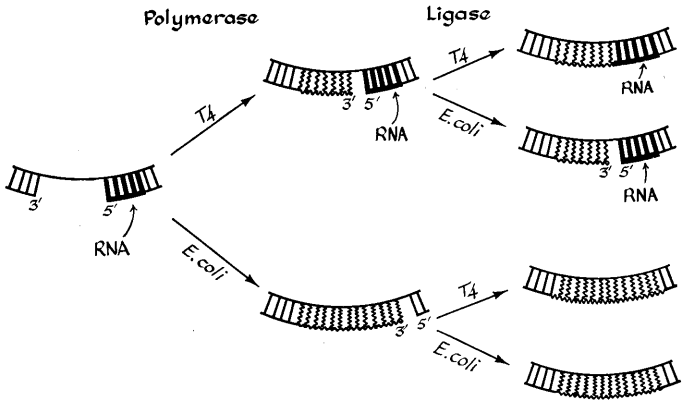

FIG. 1. Scheme illustrating joint action of DNA polymerases and ligases in sealing synthetic ΦX and M13 RF II to RF I.

sodium acetate (pH 6.5) with 2 volumes of ethanol and redissolved in 0.2 ml of 10 mM Tris·HCl (pH 7.4) and 1 mM EDTA. Of the 4 nmoles of ΦX template in the incubation mixture, 0.41 nmole was converted to RF II; 75% of the latter was recovered from the gradient and in the ethanol precipitate.

The M13 RF II was synthesized and isolated in an essentially similar way (2) and with comparable yields.

RESULTS

Alkali-stable RF I Produced When Ligases Act Jointly with E. coli DNA Polymerase—ΦX and M13 viral strands are converted by soluble extracts of *E. coli* deficient in polymerase I to RF II with a full-length linear complementary strand (1–3). This conclusion was based on several criteria including alkaline sucrose velocity sedimentation as shown in Fig. 2*a*. Purified RF II product could not be sealed by treatment with *E. coli* ligase (Fig. 2*b*) implying the presence of a small gap, or anomalous 3′ or 5′ termini at the break, or both. When *E. coli* DNA polymerase I acted jointly with *E. coli* ligase (Fig. 2*d*; Table I) or with T4 ligase (Fig. 2*e*), an alkali-stable RF was produced. T4 DNA polymerase did not substitute for the *E. coli* polymerase in the sealing reaction (Fig. 2, *c* and *f*; Table I). This suggested that the failure of ligase to act alone was not due simply to the presence of a gap. (The very small amount of RF I produced (Fig. 2, *c* and *f*) may indeed be due to a small percentage of RF II with only a gap.)

Inasmuch as both polymerases are effective in filling gaps, the unique 5′ → 3′ exonuclease function of the *E. coli* enzyme was implicated in excising an anomalous structure at the 5′ end of the RF II. Were this structure an RNA fragment then joint action with the T4 ligase, an enzyme which can seal RNA chains, might succeed (9, 10). The RF I product would, however, contain RNA and would not have survived in the alkaline sucrose gradients of Fig. 2.

Alkali-labile RF I Produced Only When T4 Ligase Acts Jointly with T4 DNA Polymerase—An RF I product can be isolated at neutral pH by CsCl equilibrium centrifugation in the presence of ethidium bromide. With this technique, conversion of ΦX or M13 RF II to RF I was observed not only with *E. coli* DNA polymerase but with T4 DNA polymerase as well (Fig. 3; Table I). Whereas the *E. coli* DNA polymerase was equally effective with either ligase, the T4 DNA polymerase produced an RF I only with the T4 ligase (data not shown). The higher conversion of RF II to RF I by *E. coli* enzymes indicated by dye-buoyant density gradient as compared with alkaline sedimentation (Table I) may be due to a longer incubation period (compare Fig. 3 with Fig. 2).

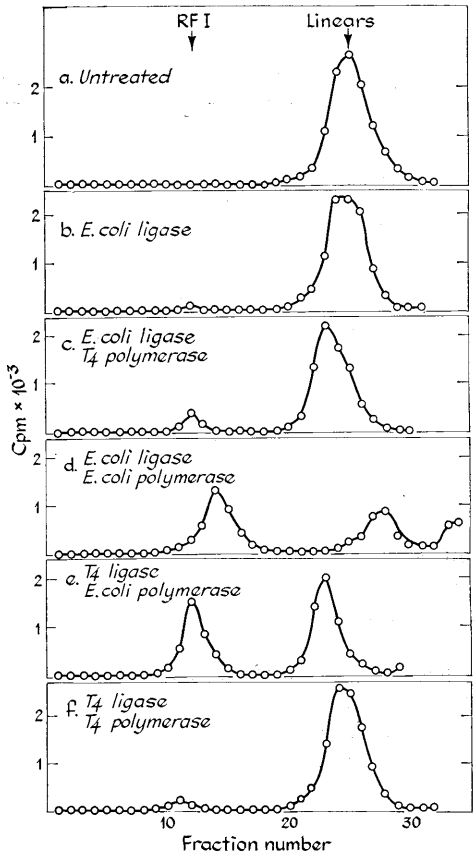

FIG. 2. An alkali-stable RF I produced by joint action of *Escherichia coli* polymerase and either ligase as judged by alkaline velocity gradients. For covalent sealing with 0.6 unit of *E. coli* ligase, 20 pmoles of the isolated ΦX RF were incubated in a reaction mixture (50 μl) containing 20 mM Tris·HCl (pH 8.1), 10 mM β-mercaptoethanol, 5 mM $MgCl_2$, 10 mM $(NH_4)_2SO_4$, 33 μM DPN, 33 μM each of the deoxyribonucleoside triphosphates and enzymes as follows: *a*, none; *b*, *E. coli* ligase; *c*, *E. coli* ligase and 0.32 unit of T4 DNA polymerase; and *d*, *E. coli* ligase and 0.18 unit of *E. coli* DNA polymerase. For sealing with 0.4 unit of T4 ligase, 20 pmoles of RF II were incubated in a reaction mixture (50 μl) containing: 65 mM Tris·HCl (pH 7.4), 10 mM dithiothreitol, 6.5 mM $MgCl_2$, 100 μM ATP, 33 μM each of the deoxyribonucleoside triphosphates, and enzymes as follows: *e*, T4 ligase and 0.18 unit of *E. coli* DNA polymerase; and *f*, T4 ligase and 0.32 unit of T4 DNA polymerase. After incubation of all samples (*a* to *f*) at 30° for 1 hour, the reactions were stopped by addition of Sarkosyl (to 1%), EDTA (to 10 mM), and NaOH (to 0.2 M). The mixtures were sedimented in 5 to 20% alkaline sucrose gradients (0.2 M NaOH, 0.8 M NaCl, and 1 mM EDTA) in a Spinco SW 56 rotor for 1½ hours at 55 Krpm and 4°. Fractions were collected and numbered from the bottom of the tube.

Upon alkaline treatment of the RF I isolated by dye-buoyant density sedimentation, 84% of the product of the action of the T4 enzymes was hydrolyzed as compared with 18% of the RF I produced by action of the *E. coli* enzymes (Fig. 4). The low level of alkali lability of the latter was about the same as the control value observed for RF I produced *in vivo*, a behavior attributable to breaks incurred during the manipulation of the DNA (11, 12).

Alkali-labile Linkage in RF I Located in Synthetic Complementary Strand—The alkali-lability of the RF I together with the known specificities of polymerases and ligases suggests that RNA linkages are present. In that case, alkali cleavage should be specific for the complementary strand where the RNA priming fragment is presumed to be. Sedimentation of the hydrolyzed

TABLE I

Conversion of ΦX and M13 RF II to RF I

The ΦX values are calculated from the data in Figs. 2 and 3. The M13 values were obtained from identical experiments except that M13 DNA replaced ΦX DNA.

Sedimentation	Conversion of RF II to RF I	
	E. coli enzymes[a]	T4 enzymes[b]
	%	
Alkaline velocity		
ΦX	42	3
M13	38	<3
Dye-buoyant density		
ΦX	75	29
M13	73	26

[a] *E. coli* DNA polymerase + *E. coli* ligase.
[b] T4 DNA polymerase + T4 ligase.

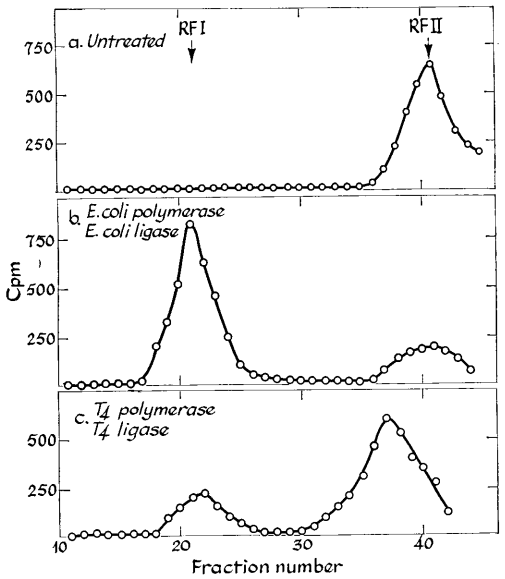

FIG. 3. Isolation by dye-buoyant density sedimentation of RF I produced by T4 DNA polymerase and T4 ligase. The experiments were performed as in Fig. 2, *a*, *d*, and *f* except that an incubation for 2 hours at 15° preceded the incubation for 1 hour at 30° in order to obtain a higher yield. The reaction was stopped by the addition of EDTA (to 10 mM) followed by 7 ml of a CsCl solution (ρ = 1.5927) in 10 mM Tris·HCl (pH 7.4), 1 mM EDTA, and ethidium bromide (0.4 mg per ml). Sedimentation to equilibrium was in a Spinco 40 rotor for 40 hours at 37 Krpm and 20°.

ΦX RF I showed 84% of the labeled complementary strand in the position of linear strands, whereas little or none is found in the region of circles (Fig. 5). The shoulder observed behind the full length linear strand zone may be in part attributable to breakage to an average of about half-size of about 16% of the molecules during the various manipulations. Alternatively, some molecules might have had more than one initiation point and would, as a result, have generated smaller products upon alkaline hydrolysis. Similar results were obtained with hydrolysis of the M13 RF I.

Buoyant Density of Alkali-labile RF—Sedimentation to equilibrium, in a neutral cesium chloride gradient, of the ΦX and M13 RF I produced from RF II by action of the T4 enzymes (see Fig. 3) showed little or no displacement of the peak to the heavier side as compared with the RF I isolated from infected cells. A one-fraction displacement attributable to RNA can

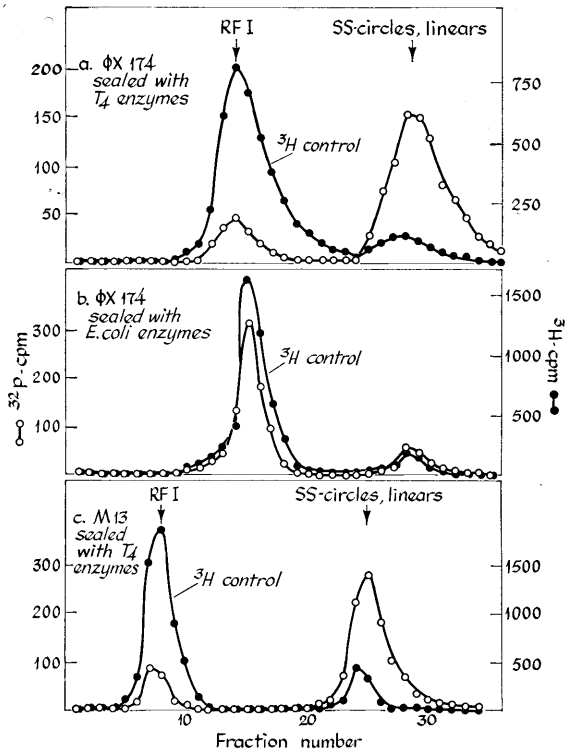

FIG. 4. Alkali-lability of RF I produced by T4 DNA polymerase and T4 ligase. Synthetic [^{32}P]ΦX RF I closed with T4 DNA polymerase and T4 ligase or with *Escherichia coli* DNA polymerase and *E. coli* ligase were isolated as described in Fig. 3. After removal of the ethidium bromide by shaking with 2-propanol, the DNA was precipitated by ethanol. [^{3}H]ΦX RF I (6,000 cpm; isolated from infected cells and further purified by dye-buoyant density sedimentation) was mixed with 0.8 pmole of the [^{32}P]ΦX RF I sealed by the T4 or the *E. coli* enzymes. To the samples were added 25 μg of bovine serum albumin and NaOH (to 0.3 M). Hydrolysis was for 1 hour at 37°. The hydrolysates were treated with Sarkosyl (to 1%) and EDTA (to 10 mM) and sedimented for 1½ hours in an alkaline gradient as in Fig. 2. A 200-μl sample was applied on gradient *a* and 100-μl samples on *b* and *c*. The experiment with M13 in *c* was identical with that with ΦX except that 1.1 pmoles of M13 RF I (sealed with T4 enzymes) were mixed with [^{3}H]M13 RF I (7,000 cpm; isolated from infected cells and further purified as for ΦX).

be calculated to represent an amount equal to about 1% of the DNA. This value, equivalent to an RNA primer length of about 100 nucleotide residues, would appear to be an upper limit.

DISCUSSION

The further evidence presented here for an RNA priming fragment in conversion of phage single-stranded DNA to the duplex RF II is based on the enzymatic requirements for closing the RF II to RF I (Fig. 1). *E. coli* ligase was effective with the DNA polymerase I of *E. coli* and not with that of T4, presumably because of the unique capacity of the *E. coli* enzyme to excise RNA at the 5′ end of a strand. However, the T4 DNA polymerase did work jointly with T4 ligase, an enzyme which can link RNA segments. This joint action by the T4 enzymes thereby produced an RF I with a complementary strand labile to alkali.

These results indicate not only the presence of an RNA fragment in the synthetic strand, but by implicating a 5′ → 3′ nuclease excision to produce an alkali-stable RF I and a ligase union of DNA and RNA to produce an alkali-labile RF I, localize the RNA fragment at the 5′ end of the synthetic strand. A

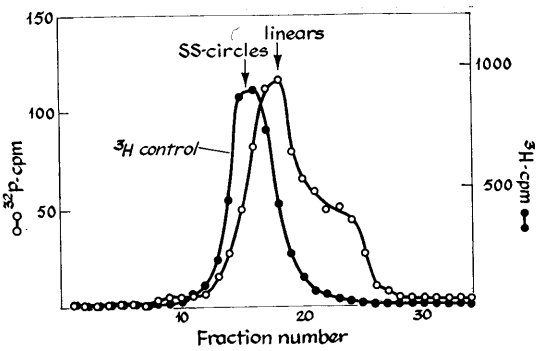

FIG. 5. Alkali-lability of the complementary strand in RF I. The synthetic [^{32}P]RF I (0.8 pmole) joined by T4 enzymes was isolated as in Fig. 3 and mixed with [^{3}H]ΦX DNA marker (6,000 cpm). The mixture was hydrolyzed in 0.3 M NaOH for 1 hour at 37°, applied to an alkaline sucrose gradient (as in Fig. 2), and centrifuged in a Spinco SW 56 rotor for 4½ hours at 55 Krpm and 4°.

similar conclusion was drawn from earlier experiments (3) showing the covalent linkage of DNA to RNA.

The cooperative action of T4 polymerase and T4 ligase in closing the RF II to RF I also indicates that the nucleotide residue at the 5′ terminus is a monophosphate and not a triphosphate. Assuming that a 5′-triphosphate initiates the synthesis of the RNA primer, why is it missing from the molecules that were converted to RF I? A possible explanation is a contamination of the T4 ligase preparation with an enzyme that removes such a terminus. Another possibility is that the triphosphate was removed, for the most part, by an enzyme which attacks the RF II during or soon after its synthesis in the crude extract (Fraction I).

With the recognition that RNA fragments prime DNA synthesis it is important to consider mechanisms for excising such fragments. A prominent candidate for such a role is, of course, DNA polymerase I with its built-in 5′ → 3′ exonuclease activity. The enzyme has been demonstrated in model experiments[2] to be effective in removing the RNA priming fragments synthesized by RNA polymerase on M13 viral DNA. Furthermore, when the cell extract (Fraction I) was prepared from wild type *E. coli* rather than from the pol A mutant (13), 50% of the product was in the form of an alkali-stable RF I. This result suggests that the lack of polymerase I in the extract from mutant cells is responsible for the accumulation of RF II with an RNA fragment at the 5′ end. Finally, *in vivo* results also indicate an essential function for polymerase I that may exploit its capacity for coordinated 5′ → 3′ excision and gap filling. This function could account for the excision of the RNA fragments now known to initiate the nascent 10 S DNA pieces in discontinuous replication (14) and for filling the gaps that this excision creates. Pol A mutants, deficient in polymerase I, accumulate abnormal amounts of 10 S DNA and gaps (15, 16). The fact that such mutants previously regarded as lacking polymerase I (13) remain viable may be attributable to residual levels of the enzyme activity (17).

There are other possibilities for removal of part or all of the priming RNA. The triphosphate might be degraded by the 5′-triphosphatase that acts on the origins of RNA transcription (18). The RNA itself might be removed by nucleases such as RNase III (19) and RNase H[3] which degrade the RNA of RNA-DNA duplex hybrids. A precise characterization of the full size and composition of the priming fragment as well as the mechanisms for excising it must await further purification of the enzymes responsible for the single strand conversion to RF.

[2] L. L. Bertsch and A. Kornberg, unpublished results.

[3] I. Berkower, J. Leis, and J. Hurwitz, personal communication.

REFERENCES

1. BRUTLAG, D., SCHEKMAN, R., AND KORNBERG, A. (1970) *Proc. Nat. Acad. Sci. U. S. A.* **68,** 2826
2. WICKNER, W., BRUTLAG, D., SCHEKMAN, R., AND KORNBERG, A. (1972) *Proc. Nat. Acad. Sci. U. S. A.* **69,** 965
3. SCHEKMAN, R., WICKNER, W., WESTERGAARD, O., BRUTLAG, D., GEIDER, K., BERTSCH, L., AND KORNBERG, A. (1972) *Proc. Nat. Acad. Sci. U. S. A.* **69,** 2691
4. OLIVERA, B. M., AND LEHMAN, I. R. (1968) *J. Mol. Biol.* **36,** 261
5. FRANCKE, B., AND RAY, D. S. (1971) *Virology* **44,** 168
6. HUANG, W. M., AND LEHMAN, I. R. (1972) *J. Biol. Chem.* **247,** 3139
7. WEISS, B., JACQUEMIN-SABLON, A., LIVE, T. R., FAREED, G. C., AND RICHARDSON, C. C. (1968) *J. Biol. Chem.* **243,** 4543
8. JOVIN, T. M., ENGLUND, P. T., AND BERTSCH, L. L. (1969) *J. Biol. Chem.* **244,** 2996
9. KLEPPE, K., VAN DE SANDE, J. H., AND KHORANA, H. G. (1970) *Proc. Nat. Acad. Sci. U. S. A.* **67,** 68
10. FAREED, G. C., WILT, E. M., AND RICHARDSON, C. C. (1971) *J. Biol. Chem.* **246,** 925
11. SIEGEL, J. E., AND HAYASHI, M. (1967) *J. Mol. Biol.* **27,** 443
12. BODE, V. C. (1967) *J. Mol. Biol.* **26,** 125
13. DELUCIA, P., AND CAIRNS, J. (1969) *Nature* **224,** 1164
14. SUGINO, A., HIROSE, S., AND OKAZAKI, R. (1972) *Proc. Nat Acad. Sci. U. S. A.* **69,** 1863
15. KUEMPEL, P. L., AND VEOMETT, G. E. (1970) *Biochem. Biophys. Res. Commun.* **41,** 973
16. OKAZAKI, R., ARISAWA, M., AND SUGINO, A. (1971) *Proc. Nat. Acad. Sci. U. S. A.* **68,** 2954
17. LEHMAN, I. R., AND CHIEN, J. (1972) in *DNA Synthesis In Vitro* (WELLS, R. D., AND INMAN, R. B., eds) University Park Press, Baltimore
18. MAITRA, U., COHEN, S. N., AND HURWITZ, J. (1966) *Cold Spring Harbor Symp. Quant. Biol.* **31,** 113
19. ROBERTSON, H. D. (1971) *Nature* **229,** 169