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Replication deficiencies in *priA* mutants of *Escherichia coli* lacking the primosomal replication n′ protein

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ABSTRACT **The *priA* gene of *Escherichia coli* encodes the protein that initiates assembly of the primosome, the entity essential for the replication of phage φX174 and ColE1-like plasmids *in vitro*. We have prepared a null *priA* mutant to assess its role *in vivo* in replication of phages, plasmids, and the host chromosome. Extracts of this mutant are inert in the initial conversion of the φX174 viral strand to the duplex form, confirming the absence of the PriA activity. *In vivo*, the *priA* mutant fails to produce φX174 phage and, remarkably, is unable to maintain plasmids that depend on the *E. coli* chromosome origin as well as those of ColE1. Deficiencies in cell growth and cell division are also manifest.**

The mechanism of initiation of DNA chains in the discontinuous synthesis of the lagging strand was suggested by studies of how the complementary strand was started on the single-stranded circle of phage φX174 (1). These studies revealed that protein n′ (renamed PriA) recognizes a 55-nucleotide, hairpin-like sequence in the template, hydrolyzes ATP, and initiates the assembly of a mobile primer-forming entity, called a primosome, made of six additional proteins: n (renamed PriB) (2), n″ (renamed PriC) (3), i (renamed dnaT) (4), dnaB, dnaC, and dnaG (renamed primase) (5).

PriA protein was later found to recognize, in certain plasmids (6, 7), particular sequences that promote hydrolysis of ATP (8) and function as primosome assembly sites (*pas*). Recognition of *pas* appears to be based on a higher-order DNA structure, inferred from mutations of the sequence in pBR322 and comparisons of various plasmids (9, 10). Primosomal translocation on single-stranded DNA and its helicase action (fueled by ATP) can be directed by PriA protein, moving in the 3′→5′ direction (11) or by dnaB protein, moving in the opposite (5′→3′) direction (12). When the template strand functions in both the priming and elongation stages of discontinuous synthesis, both helicases may be engaged, operating in opposite directions.

Whereas the contribution of PriA protein to the replication of φX174 and pBR322 DNA *in vitro* had been made clear (8, 13), evidence was lacking for its role in the replication of other plasmids or of the host chromosome itself. Nor was such evidence available for certain other primosomal proteins (i.e., PriB, PriC, and dnaT), except that *dnaT* mutants were known to be deficient in the stable DNA replication that depends on RecA and the SOS response (14).

With the cloning of the *priA* gene (15, 16) it became possible to disrupt the gene by mutation. The present report describes replication defects in strains that lack the *priA* function and strains with mutations in other replication genes, in addition to *priA*.

MATERIALS AND METHODS

Reagents. Sources were as follows: unlabeled deoxynucleoside triphosphates and ribonucleoside triphosphates, Pharmacia LKB; [^{3}H]dTTP (26 Ci/mmol; 1 Ci = 37 GBq) and [α-^{32}P]dTTP (800 Ci/mmol), Amersham; bovine serum albumin (Pentex fraction V), Sigma; 4′,6-diamidino-2-phenylindole (DAPI), Boehringer Mannheim. Buffer G is 20 mM Tris·HCl, pH 7.5/180 mM potassium glutamate/9 mM $MgCl_2$/4% (wt/vol) sucrose/bovine serum albumin at 100 μg/ml.

***Escherichia coli* Strains and Plasmids.** *E. coli* strains used were DPB271 (*recD*::mini-tet) (17) and CR (*thy*$^-$, C strain harboring suppressor III). Plasmid pEL042 is pTZ18R-based and carries the *priA* gene (15); pTB101 contains a 678-base-pair (bp) *Hin*cII–*Pst* I fragment spanning *oriC* (−189 to +489) cloned in the pBluescript vector (Stratagene) (18); pCM700 is a derivative of pCM959 DNA (19) containing the chloramphenicol-resistance gene in place of the *Pvu* II fragment in pCM959 (D. S. Hwang and A.K., unpublished data); pCM800 is the same as pCM700 with the *priA* gene inserted at the *Eco*RV site; pHM6050 is a derivative of R1 plasmid (20); pBR322 is described by Bolivar *et al.* (21) (Table 1).

Enzymes and Proteins. T4 DNA ligase, and *Bsm* I, *Eco*RV, *Hin*dIII, *Pvu* II, *Sma* I, and *Xba* I were purchased from New England Biolabs. The enzymes required for conversion of φX174 single-stranded DNA (ssDNA) to replicative form (RF) DNA *in vitro* were prepared as described (5).

Preparation of Fraction II. Cells were grown in 2 liters of L broth (23) to OD_{600} = 1.0, harvested by centrifugation, and lysed as described (15). Cleared lysates were precipitated with ammonium sulfate (0.28 g/ml). Centrifuged pellets were dialyzed against buffer containing 50 mM Hepes–KOH at pH 7.6, 20% (vol/vol) glycerol, 20 mM NaCl, 2 mM dithiothreitol, and 1 mM EDTA for 2 hr at 0°C (fraction II).

Complementation and Replication Assay for PriA Protein. The *in vitro* complementation assay mixture (20 μl) contained creatine kinase (100 μg), creatine phosphate (5 mM), ATP (0.5 mM), CTP, GTP, and UTP (125 μM each), deoxyribonucleoside triphosphates (50 μM each) with [α-^{32}P]dTTP (137 cpm/pmol of dNTP), and 200 pmol of φX174 ssDNA (as nucleotides) in buffer G. Protein fraction II (100–300 μg) of the *priA* mutant strain was added and the mixture was incubated at 30°C for 10 min. One unit of replication activity is defined as 1 pmol of nucleotide incorporated in 1 min at 30°C. The reconstituted φX174 ssDNA to RF replication assay was used as described (5).

UV Survival Assay. Cultures were grown to midlogarithmic phase (2–5 × 10^8 cells per ml) and centrifuged. Collected pellets were resuspended in one-half the culture volume of buffer containing 50 mM Tris·HCl at pH 7.5 and 10 mM $MgCl_2$ and were irradiated at 254 nm with a germicidal lamp. To determine survival at 37°C, appropriate dilutions were plated on L plates and incubated overnight. All manipulations were under red light.

Other Methods. Immunoblot assays (22) and Southern blot hybridization (ref. 23, pp. 382–389) were performed as described.

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Abbreviations: ssDNA, single-stranded DNA; RF, replicative form.

Table 1. Bacterial strains

Strain	Host, genotype	Plasmid
DPB271	*priA*$^+$	None
EL500	DPB271, *priA*$^-$	None
EL501	DPB271, *priA*$^+$	*priA*$^+$
EL502	DPB271, *priA*$^-$	*priA*$^+$
CR	*priA*$^+$	None
EL600	CR, *priA*$^-$	None
EL601	CR, *priA*$^+$	*priA*$^+$
EL602	CR, *priA*$^-$	*priA*$^+$

DPB271 (17) was donated by S. Cohen. CR is a C strain with suppressor III. EL600 was constructed by phage P1 transduction of *priA*$^-$ from EL500.

RESULTS

Isolation of a Null *priA* Mutant. A *priA* mutant was constructed by insertion of a kanamycin marker (Fig. 1). Nine kanamycin-resistant transformants were obtained from transformation of strain DPB271 with an isolated linear DNA (*Xba* I–*Eco*RV fragment). This mutation was then transduced by P1 into strain CR with high efficiency; ≈300 P1 transductants were obtained at a multiplicity of infection of 0.1. The isolated mutant was examined by Southern hybridization (Fig. 2). A nick-translated, 2.7-kb *priA* DNA probe hybridized only to a 2.9-kb *Sma* I fragment, precisely the size expected from the nucleotide sequence of *priA* (15, 16) (Fig. 2*A*, lanes 1 and 3). On the other hand, no such band was observed with a *priA* mutant; instead distinct DNA bands of 2.4 and 1.6 kb appeared, as expected (Fig. 2*A*, lanes 2 and 4). Analysis of *Hin*dIII restriction fragments revealed sizes from the *priA* mutant distinctive from those obtained from wild-type cells (data not shown). These results indicate that *priA* has been disrupted by insertion of the kanamycin DNA fragment.

As further confirmation, a nick-translated, 1.3-kb kanamycin DNA probe was used. Hybridization occurred only with the *priA* mutant; the 2.4- and 1.6-kb bands from the *Sma* I digest were the same as those visualized with the 2.7-kb *priA* probe. Thus, the *priA* mutants (EL500 and EL600) possess an internal deletion of a *priA* sequence (residues 1216–1400) (15) replaced by a kanamycin insertion of 1.3 kb.

In the event that a *priA* mutant might prove inviable, the isolated linear DNA fragment (*Xba* I–*Eco*RV) was inserted into strain EL700, which harbors a temperature-sensitive, *priA*-containing plasmid (pSC101ts-*priA*); at 42°C this plasmid fails to replicate or produce PriA protein. Sixteen kanamycin-resistant colonies were obtained, only two of which lacked plasmid DNA as judged by Southern blot analysis (data not shown).

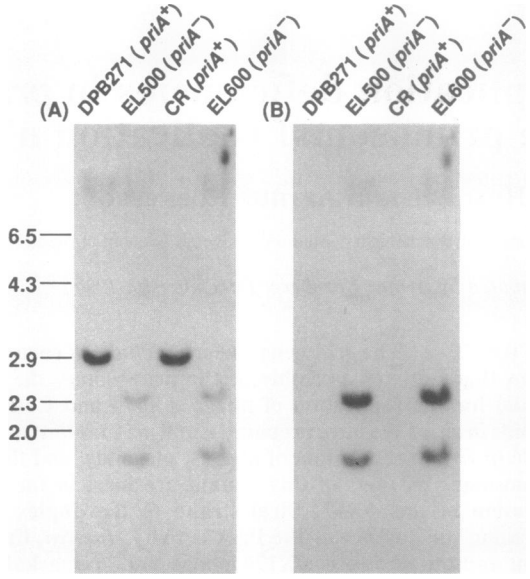

FIG. 2. Genomic analysis of *priA* mutants by Southern-blot hybridization. *E. coli* genomic DNA was prepared from the wild-type (DPB271 and CR) and mutant (EL500 and EL600) strains. *E. coli* DNAs were digested with *Sma* I restriction nuclease and subjected to an 0.8% agarose gel electrophoresis, followed by Southern-blot hybridization (23). (*A*) Nitrocellulose filters were probed with nick-translated 2.7-kb DNA carrying the *priA* gene. (*B*) Filters from experiment *A* were washed by boiling for 10 min in water and used for hybridization with a nick-translated 1.3-kb kanamycin DNA probe.

PriA Protein Activity Absent from Extracts of a *priA* Mutant. As judged by the capacity to support φX174 DNA replication, the crude extract (fraction II) prepared from a mutant strain (EL500) showed no detectable PriA activity (Fig. 3*A*). However, replication activity was fully restored when purified PriA protein complemented this mutant extract (Fig. 3*B*). Similar complementation results were obtained with the mutant strain EL600, a CR strain into which the *priA* mutation had been transduced by P1 (data not shown).

The *priA* Mutant Fails to Support a Phage φX174 Infection. In keeping with the absolute requirement for PriA protein for

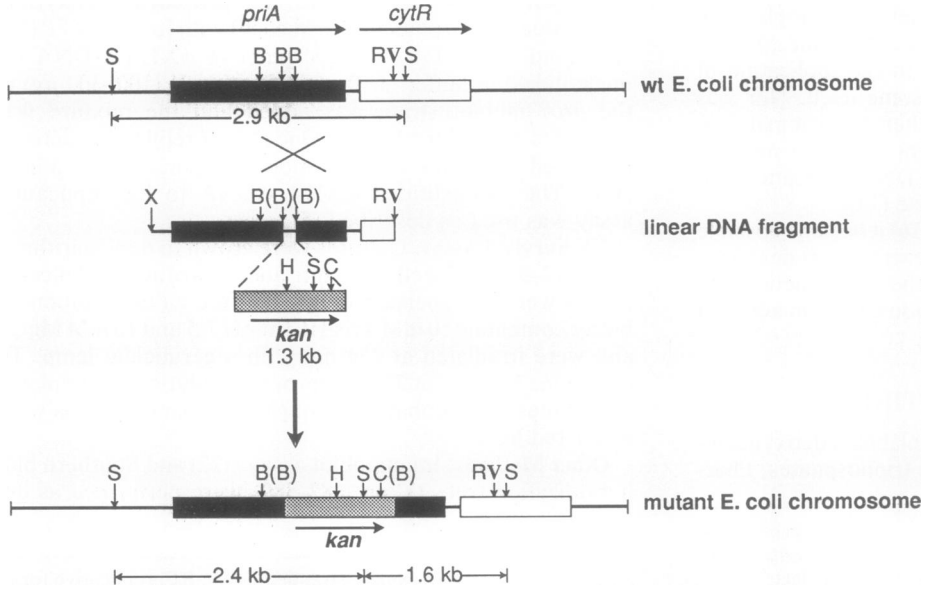

FIG. 1. Preparation of the null *priA* mutant. Restriction endonuclease sites in the *priA* and adjacent *cytR* genes in the wild-type *E. coli* chromosome are shown (*Top*). The pEL042 plasmid was partially digested with *Bsm* I restriction nuclease and ligated with a 1.3-kilobase (kb) kanamycin-resistance gene fragment; the resulting plasmid (pEL060) was digested with *Eco*RV and *Xba* I restriction enzymes. The isolated 3.8-kb linear DNA (*Middle*) was used to transform strain DPB271 (*recD*::mini-tet) and kanamycin-resistant colonies were isolated. The result of the expected recombination events between intact *priA* DNA sequences and linear disrupted *priA* DNA fragments is shown in *Bottom*. B, *Bsm* I; C, *Cla* I; H, *Hin*dIII; RV, *Eco*RV; S, *Sma* I; X, *Xba* I; (B), former *Bsm* I.

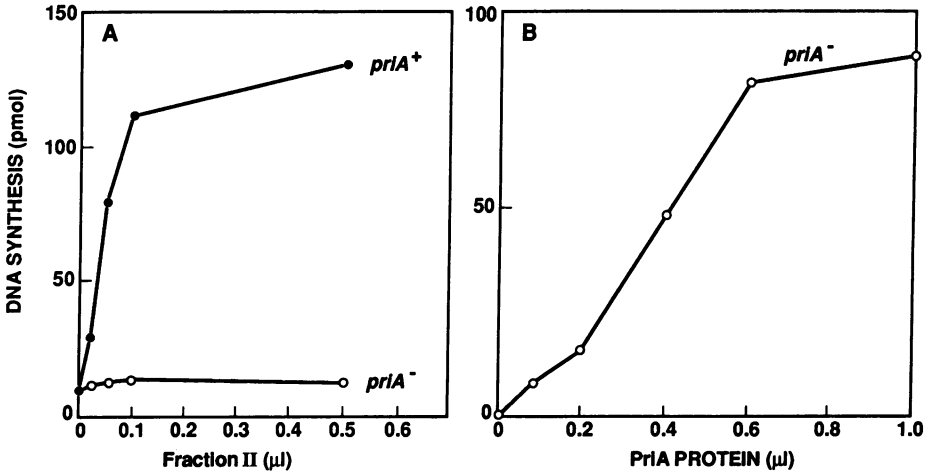

FIG. 3. Replication activity of extracts from *priA* mutant. The conversion of φX174 ssDNA to RF with the reconstituted system lacking PriA protein was assayed (5). (*A*) Crude extracts (fraction II) were prepared from the wild-type [(DPB271): *priA*$^+$; 800 μg/μl] and mutant [(EL500): *priA*$^-$; 700 μg/μl] strains. (*B*) Mutant extracts were complemented with purified PriA protein (50 ng/μl).

φX174 replication *in vitro* (5, 8) was the failure of the phage to form any plaques on a *priA* mutant strain (EL600). Plating efficiencies were reduced by greater than 10^8. Strains that harbor the multicopy *priA* plasmid (i.e., EL601 and EL602) showed a 30% higher efficiency of plating than the corresponding wild-type strains.

The *priA* Mutant Fails to Support the Replication of a Variety of Plasmids. Among plasmids that depend on various replication origins, transformation of the ColE1 type in the *priA* mutant strains (EL500 or EL600) was very feeble or undetectable (Table 2). PriA protein is known to be required in the initiation of lagging-strand synthesis during pBR322 DNA replication *in vitro* (13). In a mutant strain, the transformation of a ColE1 type plasmid which also contains the *priA* gene (pEL042) reached 10–20% of the wild-type value (Table 2). Even though no need for the PriA protein in the replication of *oriC* plasmids *in vitro* has been observed (24), transformation of such plasmids was reduced as much as 10,000-fold (Table 2). Inclusion of the *priA* gene in the *oriC* plasmid (pCM800) restores transformation efficiency (Table 2). In addition, R1 plasmids, whose replication *in vitro* does not require PriA protein (25), transform with an efficiency similar to the *oriC* plasmids (Table 2).

The *priA* Mutant Grows Slowly and Is Filamentous. As judged by optical density, the *priA* mutant (EL500) grows more slowly than the parent wild type (DPB271), with a generation time of 70 min compared to 30 min. Although the mutant grows at 30°C, 37°C, and 42°C, cells grown at the high temperature tend to clump. Viability, measured by colony formation, showed a 10- to 100-fold reduction in the mutant strain (EL500) relative to optical density. However, the decreased viability was nearly completely restored by a complementing *priA*$^+$ plasmid (e.g., EL502) (data not shown).

Microscopic examination of *priA* mutant cells revealed their morphology to be highly filamentous. The cells were 2 to 50 times longer than the wild type, depending on the incubation time (Fig. 4 *C* and *D*). Strains EL502 and EL602, which contain the complementing *priA*$^+$ plasmid, appeared nearly normal; only 5–10% of the cells were longer and none reached 5 times normal length. The chromosomal DNA of filamentous cells, visualized by fluorescence microscopy (Fig. 4*D*), often appeared to be interrupted.

The *priA* mutant strain was more sensitive to mitomycin C and UV irradiation (10 J/m^2 of UV at 254 nm) by more than two orders of magnitude (data not shown).

Viability of Double Mutants. Although the *polA12ts* and *priA*$^-$ strains can grow at 30°C, 37°C, and 42°C, the *polA12ts priA* double mutant is not viable at 42°C. In addition, cells with a combination of the normally reversible *dnaA46ts* mutation (26) with *priA* are not viable when subjected to the nonpermissive temperature of 37°C for 1.5 hr.

DISCUSSION

We have generated a null mutation in the *priA* gene, which encodes the PriA protein (n′ protein) essential for conversion *in vitro* of the viral φX174 ssDNA to the duplex RF (5, 8). Such *priA* mutants permit the use of genetic and biochemical approaches to understand how and at which stage in DNA replication the PriA protein participates. The *priA* mutants, as expected, fail to support φX174 phage DNA replication *in vivo* as well as *in vitro* (Fig. 3) and fail to maintain the ColE1 plasmid *in vivo* (Table 2). Unexpectedly, the mutants are also ineffective in maintaining *oriC* plasmids (Table 2).

The inability to sustain a φX174 phage infection and the failure to obtain transformation with pBR322 plasmid DNA in these mutant strains suggest that the mutants contain no residual PriA activity. It is unlikely that the *priA* mutant contains any suppressor mutation, since the *priA* mutation can be transduced with P1 with high efficiency to another strain.

The *priA* mutant strains grow more slowly and exhibit a 10- to 100-fold reduction in viability as measured by colony

Table 2. Transformation efficiencies of various plasmids in the *priA* mutants

Strain	Transformation efficiency, % of *priA*$^+$ strain							
	pBR322 ColE1	pTZ18R ColE1	pBluescript ColE1	pEL042* ColE1	pCM700 *oriC*	pCM800* *oriC*	pTB101 ColE1 + *oriC*	pHM6050 R1
EL500 (*priA*$^-$ DPB271)	<0.001	<0.001	0.05	10	0.01	10	0.1	<0.001
EL600 (*priA*$^-$ CR)	<0.001	<0.001	0.01	20	0.01	22	0.2	0.01

Transformations of plasmid DNA into *E. coli* cells were performed as described (ref. 23, pp. 249–255). The first line of the column heading gives the plasmid; the second, its replication origin. The transformation efficiency of these plasmids to *priA*$^+$ strains (DPB271 and CR) were 2–6 × 10^5 colony-forming units/μg of DNA. Each value (average of three independent assays) indicates the transformation relative to that of the corresponding *priA*$^+$ strain.

*The plasmid contains the *priA*$^+$ gene.

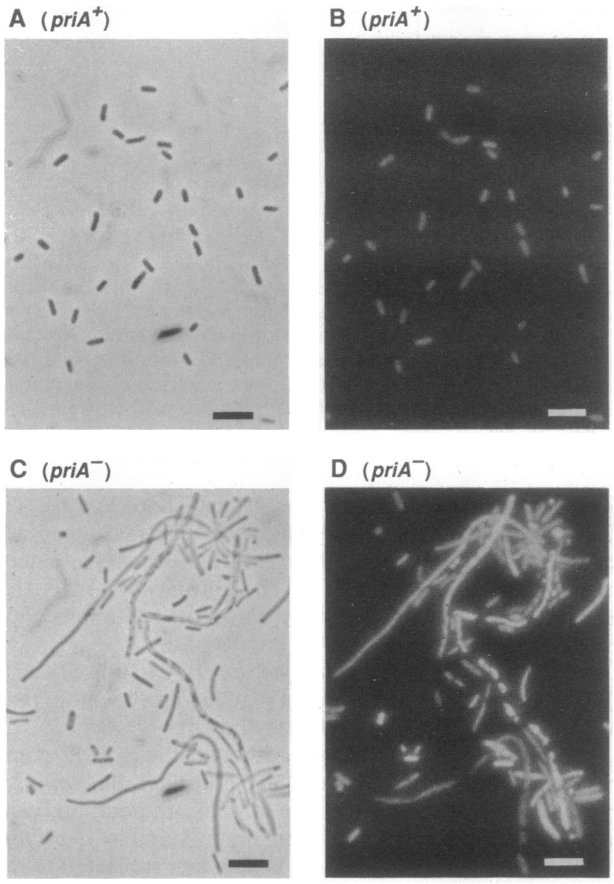

FIG. 4. Filamentous morphology of *priA* mutant cells. Cells were grown in L broth to OD_{600} of 0.5, centrifuged, resuspended in 10 mM Tris·HCl at pH 7.5, and then fixed with 10 vol of 70% (vol/vol) ethanol overnight at 4°C, centrifuged, resuspended in buffer containing 10 mM Tris·HCl at pH 7.5 and 10 mM $MgCl_2$, and stained with 4',6-diamidino-2-phenylindole (DAP; 1 μg/ml) for 2 hr at 4°C. (*A* and *C*) Phase-contrast photomicrographs showing the morphology of wild-type (DPB271) and mutant (EL500) cells, respectively. (*B* and *D*) Fluorescence micrographs corresponding to *A* and *C*, respectively. The bar is 3 μm.

formation. They produce tiny colonies and long filaments (Fig. 4) and are sensitive to UV irradiation. Introduction of a *priA* plasmid into the mutant strains restores cell viability and normal morphology, suggesting that the altered phenotype is due solely due to the lack of PriA protein. The UV sensitivity of the *priA* mutant may be attributable to its filamentous morphology rather than to a deficiency in DNA repair (27) or an SOS response (28). Extracts prepared from cells (W3110) irradiated at two different doses (120 or 240 J/m^2) showed no induction of PriA activity as judged by *in vitro* complementation, indicating that *priA* is not under SOS control (data not shown).

Inasmuch as one of the phenotypes of a defective SOS response in *E. coli* is filamentous cell growth (28), we wondered whether the filamentous morphology of the *priA* mutant may manifest a constitutive SOS response. When the level of LexA protein was examined by immunoblot assay with LexA antibody, both $priA^+$ and $priA^-$ strains showed the same level of intact LexA protein. Also, upon UV irradiation, two distinct peptide fragments (N-terminal and C-terminal) cleaved by SOS-induced RecA protein were detected in both strains (data not shown). These results demonstrate that the filamentous morphology of the *priA* mutant is not due to a constitutive SOS response.

The reduced transformation of *oriC* plasmids (pTB101 and pCM700) in *priA* mutants, and the lowered cell growth suggest that *priA* contributes at some stage of cellular DNA replication. Undisclosed alternative pathways permit the *priA* mutant to remain viable. How the PriA protein participates in chromosomal DNA replication remains to be determined.

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1. Kornberg, A. (1982) *Supplement to DNA Replication* (Freeman, New York).
2. Low, R. L., Shlomai, J. & Kornberg, A. (1982) *J. Biol. Chem.* **257**, 6242–6250.
3. Shlomai, J. & Kornberg, A. (1980) *J. Biol. Chem.* **255**, 6794–6798.
4. Arai, K., McMacken, R., Yasuda, S. & Kornberg, A. (1981) *J. Biol. Chem.* **256**, 5281–5286.
5. Shlomai, J., Polder, L., Arai, K. & Kornberg, A. (1981) *J. Biol. Chem.* **256**, 5232–5238.
6. Nomura, N., Low, R. L. & Ray, D. S. (1982) *Proc. Natl. Acad. Sci. USA* **79**, 3153–3157.
7. Imber, R., Row, R. L. & Ray, D. S. (1983) *Proc. Natl. Acad. Sci. USA* **80**, 7132–7136.
8. Shlomai, J. & Kornberg, A. (1980) *Proc. Natl. Acad. Sci. USA* **77**, 799–803.
9. Abarzúa, P., Soeller, W. & Marians, K. J. (1984) *J. Biol. Chem.* **259**, 14286–14292.
10. Masai, H., Nomura, N., Kubota, Y. & Arai, K. (1990) *J. Biol. Chem.* **265**, 15124–15133.
11. Lasken, R. S. & Kornberg, A. (1988) *J. Biol. Chem.* **263**, 5512–5518.
12. Matson, S. W. & Kaiser-Rogers, K. A. (1990) *Annu. Rev. Biochem.* **59**, 289–329.
13. Minden, J. S. & Marians, K. J. (1985) *J. Biol. Chem.* **260**, 9316–9325.
14. Lark, C. A., Riazi, J. & Lark, K. G. (1978) *J. Bacteriol.* **136**, 1008–1017.
15. Lee, E. H., Masai, H., Allen, G. C., Jr., & Kornberg, A. (1990) *Proc. Natl. Acad. Sci. USA* **87**, 4620–4624.
16. Nurse, P., Digate, R. J., Zavitz, K. H. & Marians, K. J. (1990) *Proc. Natl. Acad. Sci. USA* **87**, 4615–4619.
17. Russell, C. B., Thaler, D. S. & Dahlquist, F. W. (1989) *J. Bacteriol.* **171**, 2609–2613.
18. Baker, T. A. & Kornberg, A. (1988) *Cell* **55**, 113–123.
19. Meijer, M., Beck, F., Hansen, F. G., Bergman, H. F., Messer, W., von Meyenburg, K. & Schaller, H. (1979) *Proc. Natl. Acad. Sci. USA* **76**, 580–584.
20. Masai, H. & Arai, K. (1989) *J. Biol. Chem.* **264**, 8082–8090.
21. Bolivar, F., Rodriguez, R. L., Greene, P. J., Betlach, M. C., Heynecker, H. L., Boyer, H. W. (1977) *Gene* **2**, 95–113.
22. Sassanfar, M. & Roberts, J. W. (1990) *J. Mol. Biol.* **212**, 79–96.
23. Maniatis, T., Fritsch, E. F. & Sambrook, J. (1982) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Lab., Cold Spring Harbor, NY), pp. 68–73.
24. van der Ende, A., Baker, T. A., Ogawa, T. & Kornberg, A. (1985) *Proc. Natl. Acad. Sci. USA* **82**, 3954–3958.
25. Masai, H. & Arai, K. (1989) *J. Bacteriol.* **171**, 2975–2980.
26. Hirota, Y., Mordoh, J. & Jacob, F. (1970) *J. Mol. Biol.* **53**, 369–387.
27. Houten, B. J. (1990) *Microbiol. Rev.* **54**, 18–51.
28. Walker, G. C. (1985) *Annu. Rev. Biochem.* **54**, 425–457.