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An *Escherichia coli* mutant defective in single-strand binding protein is defective in DNA replication

(DNA binding protein/helix-destabilizing protein/phage G4/conditional replication mutant)

RALPH R. MEYER*, JEFFREY GLASSBERG†, AND ARTHUR KORNBERG

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

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ABSTRACT **An *Escherichia coli* mutant, temperature-sensitive for DNA synthesis *in vivo* and *in vitro*, is defective in single-strand binding protein (SSB; DNA-binding protein). Conversion of phage G4 single strands to the duplex form is defective in crude enzyme fractions of the mutant and is complemented by pure wild-type SSB. Radioimmunoassays of mutant extracts show normal levels of material crossreacting with anti-SSB antibody. SSB purified to homogeneity from the mutant is active, with lower specific activity, in the reconstituted G4 replication assay at 30°C, but virtually inactive at 42°C. Surprisingly, the mutant protein, like the wild-type protein, survives heating at 100°C. Thus, mutant SSB is structurally heat-resistant but is functionally thermosensitive *in vitro* and *in vivo*. Both the *in vivo* and *in vitro* defects are tightly linked in transductions by phage P1. The mutation in the binding protein, designated *ssb-1*, is located between 90 and 91 min on the *E. coli* genetic map.**

The single-strand DNA binding protein of *Escherichia coli* was discovered (1) in a search for a protein analogous to the DNA-binding protein encoded by gene 32 of phage T4 (2). The *E. coli* protein by virtue of strong, cooperative binding to single-stranded DNA destabilizes helical duplexes and lowers their melting temperatures by about 40°C. The protein has been called DNA-unwinding (1, 3), DNA-binding (4, 5), or helix-destabilizing (6) protein. In this report we designate it single-strand binding protein (SSB).

SSB is required for the conversion, *in vitro*, of single-stranded phage DNA to the duplex replicative form (3) and in the conversion of the latter to single-stranded viral circles (5). Despite this dependence on SSB for phage DNA replication *in vitro*, it was not clear whether replication *in vivo* had a similar requirement. For lack of a mutant defective in SSB, or a specific inhibitor of it, the question of an essential or auxiliary role in replication, repair, and recombination remained in doubt.

We report here that SSB is an essential component in phage and cellular DNA metabolism *in vivo*. A mutant has been identified with a temperature-sensitive defect in SSB. DNA synthesis stops immediately when the temperature is raised from 30°C to 42°C. Single-stranded phage (ST-1) production is also blocked at 42°C. The isolated mutant SSB shows a similar temperature sensitivity in an *in vitro* phage DNA replication system. Additional descriptions of the biochemical and genetic features of SSB will be reported elsewhere.

MATERIALS AND METHODS

E. coli strains were obtained as follows: strain SG1635 (*thy A3, dnaM710, ssb-1*) and parent DG17 (*thyA3*) (7) from D. Glaser (University of California, Berkeley, CA), strain KY2750 (*dnaP18*) and its parent KY2053 (8) from T. Yura (Kyoto University, Kyoto, Japan), and strains AB1157 (9) and AN385 (*ubiA*$^-$, *gal*$^-$, *str*^{R}, λ^+) (10) from G. Weinstock of this department. SG1635 contains two mutations resulting in temperature-sensitive cell growth. One mutation, originally designated *dnaM710*, maps at about 75 min on the *E. coli* genetic map (ref. 7; unpublished results). This mutation is not responsible for temperature-sensitive DNA replication, has no effect on SSB (unpublished results), and thus is irrelevant to the results reported here. The effects of the other mutation (*ssb-1*) are the subject of this paper. The origins of strains JGC155 and JGC158 are described in this paper.

A set of Hfr strains was obtained from B. Bachmann (*E. coli* Genetic Stock Center, Yale University, New Haven, CT). Hfr mapping was performed as described (11).

Complementation assays were carried out in 25-μl reaction mixtures containing 20 mM Tris·HCl (pH 7.5), 5 mM dithiothreitol, 1 mM EDTA, 100 μM each of ATP, CTP, GTP, and UTP, 50 μM each of dATP, dCTP, and dGTP, 15 μM [^{3}H]dTTP (specific activity, 1000 cpm/pmol), bovine serum albumin at 0.2 mg/ml, 5 mM $MgCl_2$, 5 mM spermidine, 2.5% sucrose, G4 DNA (230 pmol of nucleotide), and 1.2 μl of complementation extract (approx. 55–65 μg of protein). Incubation was for 10 min at 30°C; the reaction was stopped with 0.5 ml of 10% trichloroacetic acid containing 20 mM sodium pyrophosphate. Precipitates were collected on GFC filters, washed, dried, and assayed for radioactivity in a liquid scintillation spectrometer. In each experiment, synthesis in a control reaction lacking G4 DNA was subtracted (usually <2.0 pmol). Supplementation with purified replication proteins is indicated where it occurs. DNA polymerase III holoenzyme (12), primase (13), and SSB (3) were purified as described.

Reconstituted G4 DNA replication assays differed from the complementation assays in that spermidine was omitted, and 1 μg of SSB and 60 units each of primase and DNA polymerase III holoenzyme were included in place of the extract. Incubation was for 5 min at 30°C. A unit of activity is the incorporation of 1 pmol of total nucleotide per min.

Radioimmunoassays (14) were carried out with ^{125}I-labeled SSB and anti-SSB gamma globulin as described (3).

RESULTS

Extracts of *E. coli* Mutant SG1635 Do Not Support Phage G4 DNA Replication. This mutant was originally characterized as temperature sensitive for cell growth and DNA replication (7). Although SG1635 synthesizes DNA and supports phage ST-1 growth *in vivo* at temperatures below 41°C (unpublished observation), extracts prepared from these cells failed to support

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Abbreviation: SSB, single-strand binding protein.

* On leave of absence from the Department of Biological Sciences, University of Cincinnati, Cincinnati, OH 45221.
† Present address: The Rockefeller University, New York, NY 10021.

phage G4 DNA replication *in vitro*, even at 20°C (Fig. 1). Extracts from mutant cells were inactive at all temperatures tested (20°, 30°, and 37°C), whereas extracts from the wild-type parent, DG17, supported DNA synthesis at 20°C (Fig. 1) as well as at 30°C and 37°C. Thus, the replication defect is observed *in vitro* as well as *in vivo*.

Extracts of Mutant SG1635 Are Deficient in Functional SSB. Conversion of single-stranded G4 DNA to the duplex form requires only SSB, primase (*dnaG* protein), and DNA polymerase III holoenzyme (3). "Complementation extracts" prepared from virtually all the *dna* replication mutants, except *dnaG* (primase) and *dnaE* and *dnaZ* (DNA polymerase III holoenzyme subunits), are able to catalyze G4 DNA replication without supplementation by purified replication proteins. To determine the replication defect in SG1635, extracts were fortified with purified proteins and assayed for G4 DNA replication (Table 1). Additions of primase and DNA polymerase III holoenzyme had no effect. In contrast, addition of 0.5 μg of wild-type SSB completely restored replication activity. The inactivity of SG1635 extracts is not likely due to a dissociable inhibitor, because mixing the extract of mutant cells with that of the wild-type cells was not inhibitory (data not shown).

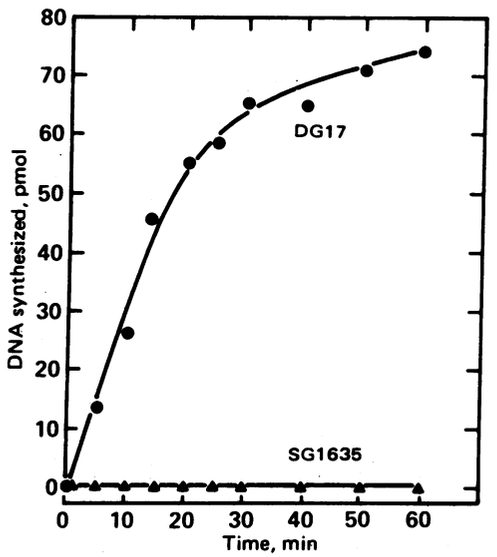

FIG. 1. Time course of phage G4 replication at 20°C with crude enzyme fractions from normal (DG17; ●) and mutant (SG1635; ▲) cells. For preparation of the crude enzyme fractions (complementation extracts), cells were inoculated into 100 ml of L broth (5 g of NaCl/5 g of yeast extract/10 g of tryptone/1.1 ml of 1 M NaOH/1000 ml of H_2O) containing 50 μg of thymidine per ml in a 1-liter flask and grown overnight at 30°–33°C. Fifty milliliters of this culture was inoculated into 2 liters of superbroth (5 g of NaCl/20 g of yeast extract/35 g of tryptone/5 ml of 1 M NaOH/1000 ml of H_2O) in a 6-liter flask and grown at 30°–33°C to OD_{595} = 2.2–2.4. The cells were harvested at 0°C, resuspended in a minimal volume of Tris/sucrose [50 mM Tris·HCl, pH 7.5/10% (wt/vol) sucrose], and adjusted to OD_{595} = 300. Ten milliliters of cell suspension (equivalent to approximately 3 g of cells) in a 40-ml centrifuge tube was frozen in liquid N_2 and stored at −70°C. To prepare complementation extracts, cells were thawed in an ice bath for 3–4 hr. The suspension was adjusted to 5% ammonium sulfate, 20 mM EDTA, and 20 mM spermidine and brought to a final volume of 20 ml with Tris/sucrose. A freshly prepared solution of lysozyme (20 mg/ml) in Tris/sucrose was added to a final concentration of 0.2 mg/ml and the suspension was incubated on ice for 30 min. Brij 58 was then added to a final concentration of 0.1%, and the tubes were centrifuged at 16,000 × *g* for 40 min at 0°–4°C. The supernatant (Fraction I) was brought to 40% saturation with a saturated ammonium sulfate solution and stirred for 30 min at 0°–4°C. The precipitate was collected by centrifugation at 16,000 × *g* for 40 min at 0°C and resuspended (in a volume equal to 1/10th that of Fraction I) in I buffer [50 mM imidazole·HCl, pH 6.8/5 mM dithiothreitol/1 mM EDTA/20% (vol/vol) glycerol] containing 40% ammonium sulfate. The precipitate was collected again and the supernatant was carefully removed. The pellet redissolved in 200 μl of I buffer is the complementation extract (Fraction II; protein concentration, ≈50 mg/ml). These fractions were kept on ice and used for 5 days or stored at −70°C for up to 6 months; about 30% of the activity was lost on freezing and thawing.

Table 1. Complementation by purified replication proteins of a crude enzyme fraction from normal and mutant cells in the replication of phage G4 DNA

Source of extract	Proteins added	Nucleotides incorporated, pmol
DG17 (wild type)	None	25
	Primase	23
	Holoenzyme	25
	SSB	30
SG1635 (*ssb-1*)	None	<0.1
	Primase	<0.1
	Holoenzyme	0.2
	SSB	31

Complementation assays were performed by using mutant SG1635 or its parent (DG17) as source of Fraction II. Indicated additions were: 60 units of primase, 60 units of DNA polymerase III holoenzyme, or 0.5 μg of wild-type SSB (prepared from HMS83).

In view of the cooperative nature of SSB binding to single-stranded DNA, assays for SSB in the crude enzyme fractions from mutant and wild-type cells were tested at several extract concentrations (Fig. 2). Up to 100 μg of crude protein of the mutant fraction (SG1635) failed to support G4 DNA replication, whereas significant activity was observed with as little as 5 μg of the wild-type fraction. Thus, extracts of SG1635 are deficient in SSB activity.

Mutant Extracts Contain Normal Levels of Protein Crossreacting with Anti-SSB Antibody. Since SSB activity was not detected by functional assays, we used competition radioimmunoassays to determine the amount of material cross-

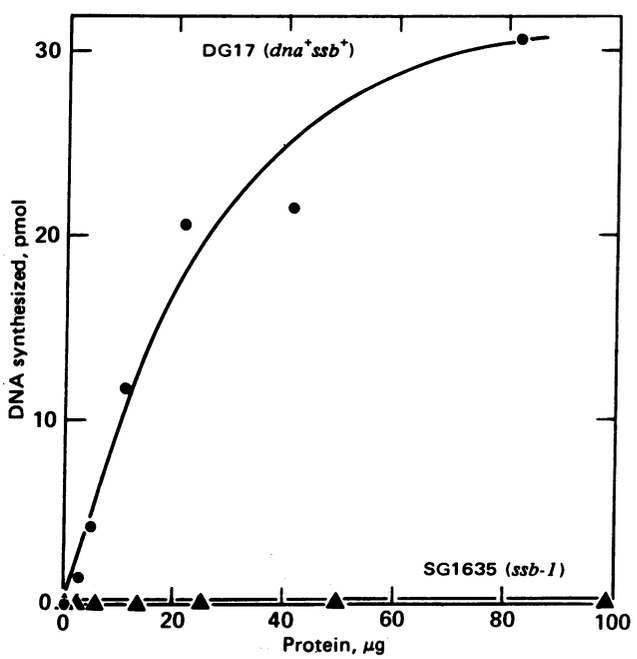

FIG. 2. Assay of SSB in crude enzyme fractions by replication of G4 DNA with pure enzymes. Phage G4 replication assays were performed as described in *Materials and Methods* except that Fraction II (see legend to Fig. 1) was the source of SSB. ●, DG17 (*dna*⁺*ssb*⁺); ▲, SG1635 (*ssb-1*).

Table 2. Levels of anti-SSB crossreacting material in crude enzyme fractions of several *E. coli* strains

Strain	Experiment	Anti-SSB crossreacting material, % of total protein
DG17 (*dna*$^+$)	1	0.67
SG1635 (*ssb-1*)	1	0.48
	2	0.72
KY2053 (*dna*$^+$)	1	0.63
KY2750 (*dnaP*)	1	0.50
AB1157 (*dna*$^+$)	1	0.54
	2	0.67

Radioimmunoassays were performed by using anti-SSB antibody bound to polystyrene tubes and ^{125}I-SSB as described (3). Several concentrations of Fraction II protein from the indicated strains were used as competitor for the bound antibody. The amount of crossreacting material in the fractions was determined from standard curves prepared with homogeneous SSB from HMS83 (3).

reacting with the anti-SSB antibody (3). Crude enzyme fractions of mutant SG1635 contained levels of crossreacting material equal to that of several other strains with normal levels of functional SSB (Table 2). The small variation from strain to strain may reflect efficiency of extracting SSB from the cells. These data suggest that strain SG1635 contains a normal level of SSB which is not functional when assayed in crude enzyme fractions.

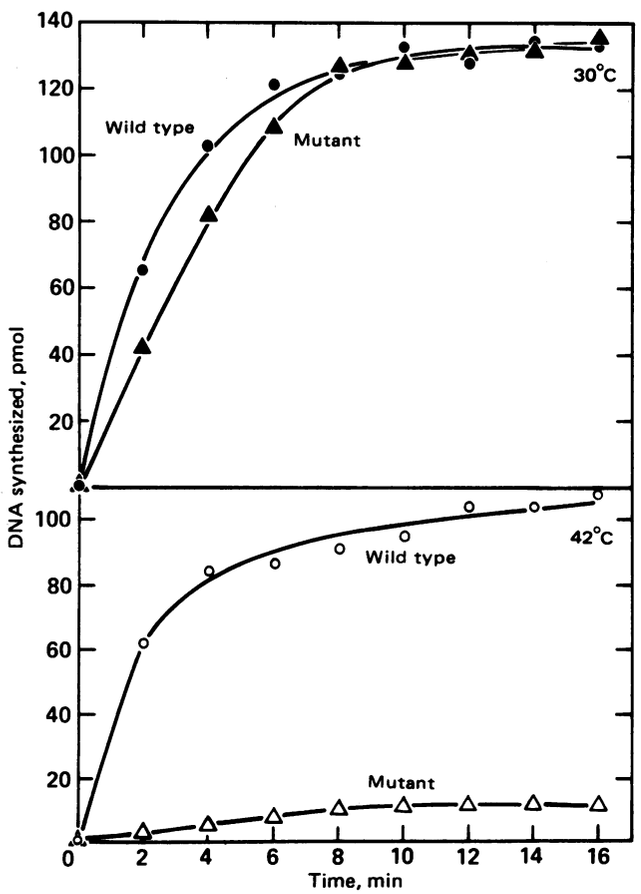

FIG. 3. Temperature sensitivity of mutant SSB for DNA replication *in vitro*. Phage G4 replication assays were as in *Materials and Methods*: either 0.5 μg of SSB from *E. coli* strain HMS83 (●, ○) or 2 μg of SSB from strain SG1635 (▲, △) was used. Duplicate samples were incubated at 30°C (*Upper*) and 42°C (*Lower*) for the indicated times.

Table 3. Complementation of crude enzyme fractions from temperature-resistant and temperature-sensitive transductants by purified proteins in the replication of phage G4 DNA

Source of extract	Proteins added	Nucleotides incorporated, pmol
JGC158	None	28
	Primase	54
	Holoenzyme	43
	SSB	49
JGC155	None	<0.1
	Primase	<0.1
	Holoenzyme	0.2
	SSB	37

Complementation assays were performed as described in the legend to Table 1.

Purified Mutant SSB Is Active and Temperature-Sensitive for DNA Replication *In Vitro*. Because assays of high levels of crude enzyme fractions are complicated by the presence of inhibitory factors, the mutant SSB was purified. A purification procedure was devised that did not rely on a heating step (unpublished results), and it was monitored by gel electrophoresis. Although the crude mutant SSB is inactive in complementation assays, higher levels of the pure protein did support G4 DNA replication *in vitro* at 30°C (Fig. 3 *upper*). At 42°C the mutant protein became less active. With even 4 times as much mutant protein as the wild-type, 1/4th to 1/10th as much activity was observed (Fig. 3 *lower*).

Despite its functional temperature sensitivity, the purified mutant SSB resembles the wild-type protein in withstanding heating at 100°C for 5 min without loss of activity or characteristic properties (unpublished results).

Cotransduction of *In Vivo* and *In Vitro* DNA Replication Defects. By using Hfr crosses, we mapped a mutation causing a temperature-sensitive phenotype between 90 and 98 min on the *E. coli* genetic map. This mutation was found to be cotransducible by phage P1 with *ubiA*, a mutation located near 91 min. The mutation causing temperature sensitivity was transferred from SG1635 to AN385 (*ubiA*$^-$) by selecting for *ubiA*$^+$ recombinants after phage P1 transduction. Temperature-sensitive and temperature-resistant transductants were isolated and designated JGC155 and JGC158, respectively. JGC155 is temperature-sensitive for DNA replication *in vivo* as shown by pulse-labeling with [^{3}H]thymidine (Fig. 4). DNA synthesis stopped abruptly upon shifting the mutant to 42°C.

A crude enzyme fraction prepared from the temperature-sensitive transductant (JGC155) was defective in G4 DNA replication and was complemented by the addition of purified SSB but not by primase or DNA polymerase III holoenzyme; a crude enzyme fraction from the temperature-resistant transductant (JGC158) showed normal levels of G4 DNA replication *in vitro* in the absence of added components (Table 3). Thus the *in vivo* replication defect and the SSB deficiency, demonstrable *in vitro*, were simultaneously transduced and map in the vicinity of the *ubiA* gene.

DISCUSSION

A mutation in the structural gene for SSB of *E. coli* has been identified by screening conditional replication mutants that have not been correlated with known replication proteins. Among 13 *dna* genes whose actions are directly required for sustained DNA replication *in vivo*, only 5 (*dna B, C, E, G,* and Z) have been shown to encode proteins with specific replicative

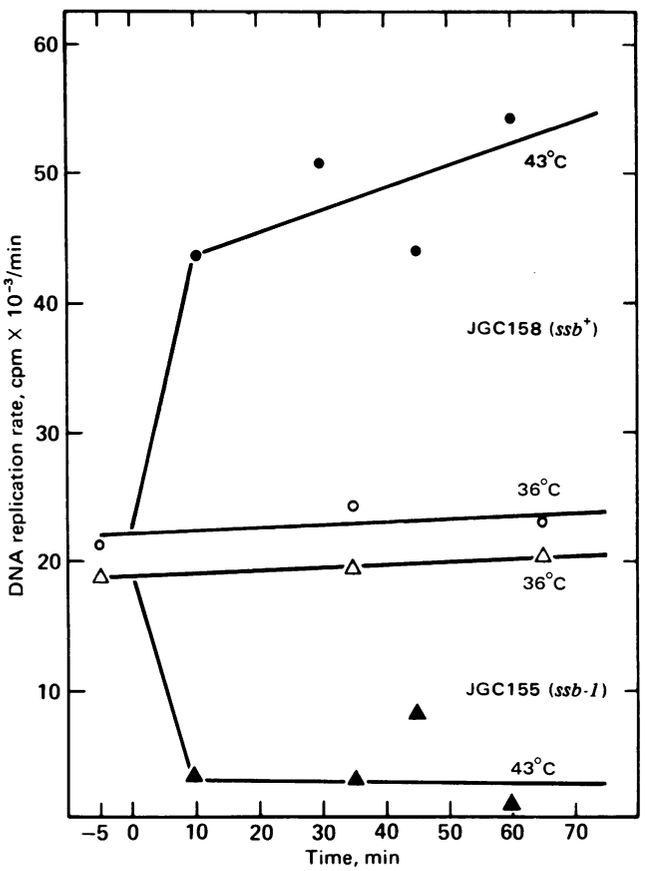

FIG. 4. Temperature-sensitivity of *ssb*$^-$ mutant for DNA replication *in vivo*. Cells were inoculated into L broth containing 50 μg of thymidine per ml at 2×10^7 per ml and grown at 36°C to a density of 2×10^8 cells per ml. Each culture was divided between two flasks, one for incubation at 36°C, the other at 43°C. At indicated times, 0.5-ml portions of the culture were added to tubes containing 0.1 ml of [^{3}H]thymidine (5 μCi/ml; 1 Ci = 3.7×10^{10} becquerels) at the appropriate temperature. DNA replication rates were determined after 5 min at 36°C or 2 min at 43°C. Cells were kept in exponential growth by periodic dilution with preheated culture medium. JGC155 (*ssb-1*): △, 36°C; ▲, 43°C. JGC158 (*ssb*$^+$): ○, 36°C; ●, 43°C.

functions *in vitro*. None of the mutants in the eight other genes (*dna A, I, J, K, L, M, P,* and *Q*) had yet been associated with a defective replication protein. Furthermore, at least seven polypeptides isolated in enzyme fractionation studies and shown to be essential factors in phage DNA replication had not been connected with any of the replication genes (15). Prominent among these proteins was SSB, which is needed *in vitro* in the conversion of phage single strands to duplex replicative form (3, 16) and in the rolling circle synthesis of single strands from replicative form (5).

Replication mutants were screened for SSB deficiency by using the relatively simple phage G4 system, which requires only SSB, primase, and DNA polymerase III holoenzyme for conversion of single strands to replicative form. One mutant strain, SG1635, was found to be defective in SSB in crude extracts. The SSB purified from this strain was functionally thermolabile in the reconstituted G4 replication system.

Preliminary genetic mapping data (7) had suggested that the temperature-sensitive replication defect previously designated *dnaM710* was located between 74 and 79 min. However, we have found that the conditional-lethal mutation located in this region does not result in conditional DNA replication when transduced by phage P1 into other genetic backgrounds. The temperature-sensitive replication defect in strain SG1635 is due to a separate mutation, designated *ssb-1*, which has been located between 90 and 91 min. Thus the *dnaM710* mutation is not in a *dna* gene.

The evidence presented here indicates that the *ssb*-1 mutation is responsible for a temperature-sensitive mutation in SSB and that this defect is simultaneously transduced by phage P1 with temperature sensitivity of replication. Were the *ssb*-1 mutation not responsible for one of these phenotypic features, then a defect in still another genetic locus mapping within about 1 min of it would have to be postulated. It is unlikely to be *dnaB*, which is located in the vicinity but behaves as a distinctly different locus genetically and biochemically (unpublished results).

Availability of the *ssb-1* mutant makes it possible to determine the participation of SSB in various DNA metabolic processes, including replication, repair, and recombination. As a quick-stop replication mutant, it seems likely that SSB function is essential at the replication fork. As a requirement for phage ST-1 replication, SSB probably plays the same role *in vivo* as it does *in vitro* in the replication of DNAs of the closely related phages G4 and φX174. The significance of SSB in other phage DNA replication systems (e.g., T7, T4, and λ) can now be evaluated.

Does SSB have a role in repair? The *ssb-1* mutant shows exquisite sensitivity to ultraviolet irradiation and may be allelic with mutations in the *lexC* locus (17) (unpublished results). The observations that recombination proteins generally function in DNA repair, and the analogy with the gene 32 protein of phage T4, known to be essential for both replication and recombination, suggest that SSB will prove to have a crucial role in virtually all aspects of DNA metabolism.

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