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Differential Thermolability of Exonuclease and Endonuclease Activities of the *recBC* Nuclease Isolated from Thermosensitive *recB* and *recC* Mutants

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The *recBC* nuclease (also called exonuclease V) has been partially purified from *Escherichia coli* K-12 strains carrying the thermosensitive *recB270*, *recC271*, and *recB270 recC271* mutations. Of the multiple activities associated with the enzyme, only the adenosine 5'-triphosphate-dependent exonucleolytic hydrolysis of duplex deoxyribonucleic acid (DNA) is abnormally thermolabile. The exo- and endonucleolytic degradation of single-stranded DNA is no more thermosensitive than that catalyzed by the wild-type enzyme. These results suggest that the defects in genetic recombination, DNA repair, and the maintenance of cell viability observed in *recBC* mutants in vivo result primarily from the specific loss of adenosine 5'-triphosphate-dependent exonuclease active on duplex DNA.

The enzyme specified by the *recB* and *recC* genes of *Escherichia coli* K-12 appears to be a multifunctional nuclease (exonuclease V). It degrades both single- and double-stranded deoxyribonucleic acid (DNA) exonucleolytically (5, 15, 19) in an adenosine 5'-triphosphate (ATP)-dependent reaction. It also catalyzes the endonucleolytic cleavage specifically of single-stranded DNA circles in a reaction that is stimulated by, but is not absolutely dependent upon, ATP (5).

recB and *recC* mutations also produce a variety of pleiotrophic effects in vivo. Thus, *recB* and *recC* mutants are defective in their recombination proficiency, in their ability to repair damage to DNA, and show significantly reduced viability (2, 3, 6, 18).

In the previous paper (8) it was demonstrated that temperature-sensitive *recB270* and *recC271* mutants, while impaired in their ability to promote the repair of DNA at 43 C, showed no defect in recombination proficiency. However, the double mutant strain (*recB270 recC271*) was defective in recombination at this temperature. In further contrast to the single mutants, the double mutant was abnormally sensitive to ultraviolet light and mitomycin C even at 30 C.

To determine whether one or more of the catalytic activities associated with the *recBC* enzyme in vitro specifically relates to any of its functions deduced from experiments in vivo, we have examined the thermosensitivity of the partially purified enzymes derived from temperature sensitive *recB270*, *recB271*, and *recB270 recC271* mutants. These studies have shown that of the multiple activities associated with the *recBC* enzyme only the ATP-dependent exonucleolytic cleavage of duplex DNA is abnormally thermolabile, suggesting that it is this activity which is most directly involved in DNA repair, genetic recombination, and in the maintenance of cell viability.

MATERIALS AND METHODS

Bacteria strains and media. The following strains were used as sources of the *recBC* nuclease and are described in the previous paper (8): AB1157 (wild type), SK116 (*recC271*), and SK119 (*recB270*). The double mutant enzyme was obtained from SK256, a strain which also carried mutations in the structural genes for exonuclease I (*sbcB15*) (9), and exonuclease III (*xthA1*) (14). This strain was constructed by P1 transduction of *sbcB15*, *recB270*, and *recC271* into BW9091 (14). Strains were grown with forced aeration at 30 C in Luria broth (8), harvested in mid-log phase (5×10^8 cells/ml), washed in a minimal salts buffer (M56/2) (17), and stored at −20 C until used.

Materials. Reagents were obtained from the following sources: carrier-free [^{32}P]H_3PO_4, [*methyl*-^{3}H]thymidine and "Ultra Pure" $(NH_4)_2SO_4$, Schwarz/Mann; Munktell 410 cellulose, Bio-Rad Corp.; salmon sperm DNA, Calbiochem; calf thymus DNA, Sigma Chemical Co.; DE-52, Reeve Angel; Sephadex G-150, Pharmacia; bovine serum albumin, Miles; and gelatin, Difco. The gelatin was autoclaved

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at 20 lb/in² for 20 min before using. Bacteriophage M13 was the generous gift of D. S. Ray. *E. coli* exonuclease I was isolated by the method of Lehman and Nussbaum (10).

E. coli B was labeled with ^{32}P in M-70 supplemented media (7) and the DNA was extracted by the method of Marmur (13). ^{3}H-labeled *E. coli* DNA was prepared by the method of Mahler (12); ^{3}H-labeled M13 DNA was isolated by the procedure of Forsheit and Ray (4). A homogeneous preparation of closed single-stranded circles was obtained by incubation of M13 DNA with exonuclease I, under conditions that converted about 20% of the DNA to acid-soluble material. The DNA was then precipitated with 95% ethanol. The resuspended DNA contained no detectable exonuclease I. Linear M13 DNA was obtained from an aged preparation which was more than 60% susceptible to exonuclease I.

DNA-cellulose was prepared by the method of Alberts (1). The calf thymus DNA (2 mg/ml) was heated to 100 C for 15 min and chilled rapidly before binding to the cellulose.

DNA concentrations are expressed in nucleotide equivalents.

The *recBC* enzyme was routinely assayed by measuring the ATP-dependent formation of acid-soluble nucleotides from duplex DNA. Incubation mixtures (0.3 ml) contained 50 mM tris(hydroxymethane)aminomethane-hydrochloride (pH 8.0), 10 mM $MgCl_2$, 0.67 mM dithiothreitol, 5 nmol of labeled DNA, 0.66 mg of gelatin per ml, and enzyme diluted just before use in the diluent described by Goldmark and Linn (5). In the fractions obtained before hydroxyapatite chromatography, the reaction mixtures contained 200 μM ATP; in those obtained after this step 33 μM ATP was used. After incubation for 20 min at 30 C the reactions were terminated by adding 0.2 ml of salmon sperm DNA (2.5 mg/ml) and 0.3 ml of 7% perchloric acid. After centrifugation, the radioactivity of the supernatant fluid was determined in a toluene-Triton X-100 scintillator. One unit of enzyme is defined as that amount which releases 1 nmol of nucleotide in 20 min at 30 C.

In measuring ATP-dependent hydrolysis of single-stranded DNA the same procedure was followed except that 1 mM ATP, 1.0 mg of gelatin per ml, and 5 nmol of ^{3}H-labeled M13 linear DNA were used. Incubations were for 10 min.

The reaction mixture for assay of ATP-stimulated endonucleolytic cleavage of single-stranded DNA contained in 0.15 ml: 50 mM glycylglycine (pH 7.0), 10 mM $MgCl_2$, 0.67 mM dithiothreitol, 1 mg of gelatin per ml, 0.33 mM ATP, 10 nmol of single-stranded M13 circles, and enzyme. After incubation for 10 min at the designated temperature, the mixture was heated for 5 min at 80 C and chilled; 0.3 ml of a mixture composed of 67 mM glycine (pH 9.5), 6.7 mM $MgCl_2$, 1.6 mM 2-mercaptoethanol, and 1 U of exonuclease I (10) was added to each tube and they were then incubated for 30 min at 37 C. Acid-soluble ^{3}H was determined as described above. One unit of enzyme is defined as the amount catalyzing the conversion of 1 nmol of M13 DNA to a form susceptible to exonuclease I in 10 min.

The *recBC* enzyme was purified as described by Goldmark and Linn (5) through the DNA-cellulose chromatography step. The specific activities (exonuclease units per milligram of protein) of the partially purified enzymes were: wild type, 12,346; *recB270*, 2,220; *recC271*, 4,200; and *recB270 recC271*, 510. All were absolutely dependent upon added ATP for the exonucleolytic digestion of DNA and showed approximately five- to 10-fold greater activity on double- than single-stranded DNA. The very low specific activity of the enzyme isolated from the double mutant was not due to the presence of an inhibitor as evidenced by an experiment in which addition of the *recB270 recC271* enzyme to wild-type enzyme produced no significant inhibition of the wild-type enzyme.

Protein concentrations were determined by the method of Lowry et al. (11).

RESULTS

Thermosensitivity of the ATP-dependent exonuclease reaction. The thermosensitivity of the exonuclease reaction was determined by measuring the rate of hydrolysis of duplex DNA at a variety of temperatures. An incubation period of 10 min was chosen after kinetic experiments at 30 C indicated that the reaction was linear during this time. Nucleotide release at a given temperature was compared to that observed at 30 C. At pH 9.0, the wild-type enzyme showed a fourfold higher activity at 43 C than at 30 C (Fig. 1C). As reported by Tomizawa and Ogawa (16), the *recB270* enzyme was more thermosensitive than the wild-type enzyme at this pH (Fig. 1C). In contrast, the *recC271* enzyme was only slightly more thermosensitive than the wild-type enzyme. The double mutant enzyme was more thermosensi-

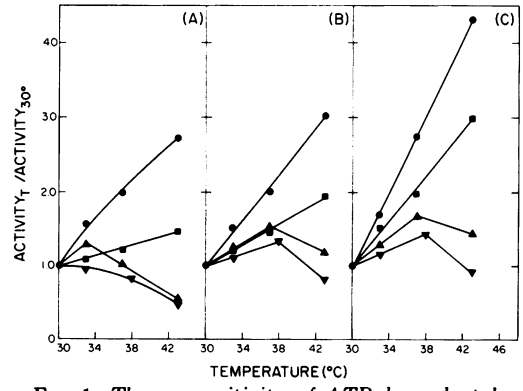

FIG. 1. *Thermosensitivity of ATP-dependent hydrolysis of double-stranded DNA. Incubations were carried out for 10 min as described. The activity observed at a given temperature was divided by the activity observed at 30 C. (A) pH 7; (B) pH 8; (C) pH 9;* (●) *wild type;* (▲) *recB270;* (■) *recC271;* (▼) *recB270 recC271.*

tive than either of the two single mutants. When the same experiments were conducted at pH 7 and pH 8 (Fig. 1A and B), the mutant enzymes showed even greater thermosensitivity than at pH 9.

The thermosensitivity of ATP-dependent exonucleolytic cleavage of single-stranded DNA was determined with M13 linear DNA as substrate. Here again, 10-min incubations were used, since kinetic experiments at 30 C indicated that the reaction was linear during this time. In contrast to the results obtained with duplex DNA, the mutant enzymes were not significantly different from the wild-type enzyme in their thermosensitivity (Fig. 2).

Thermosensitivity of ATP-stimulated endonucleolytic cleavage of single-stranded DNA. Like the exonuclease activity on single-stranded DNA, the ATP-stimulated endonucleolytic cleavage of single-stranded DNA catalyzed by the mutant enzymes was not significantly different in its thermosensitivity from the wild-type enzyme (Fig. 3).

DISCUSSION

The *recBC* enzyme isolated from the *recB270* and the *recB270 recC271* mutants is abnormally thermosensitive when assayed by the exonucleolytic cleavage of duplex DNA. In contrast the endonuclease activity as measured with single-stranded circular DNA is no more thermosensitive than the wild type. Similarly, exonucleolytic attack of single-stranded DNA by the mutant enzymes is not abnormally thermosensitive. The nearly normal thermosensitivity of the *recC271* enzyme when measured with duplex DNA as substrate does not correspond to the in vivo findings where both *recB270* and *recC271* single mutants appeared identical. Clearly, an understanding of these effects must await a detailed picture of the active site of the *recBC* nuclease. However, the finding that the exonuclease and endonuclease activities of the mutant enzymes are affected differently by a mutational alteration suggests that these activities may be associated with different portions of the protein and that there may be more than one active site on the enzyme molecule.

The *recBC* enzyme with a defect in both the *recB* and *recC* subunits is altered only in its exonucleolytic attack of duplex DNA. It is therefore possible that the pleiotrophic effects of the *recB270 recC271* double mutation observed in vivo at 43 C, i.e., reduced ability to repair damage to DNA, decreased viability, and

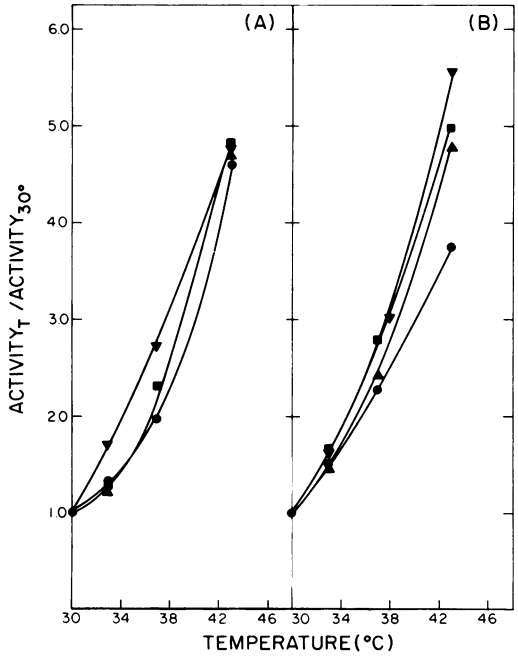

FIG. 2. *Thermosensitivity of ATP-dependent hydrolysis of single-stranded DNA. Activity was measured as described. Incubations were carried out for 10 min. Activity ratios were obtained as described in Fig. 1. (A) pH 7; (B) pH 9; (●) wild type; (▲) recB270; (■) rec C271; (▼) recB270 recC271.*

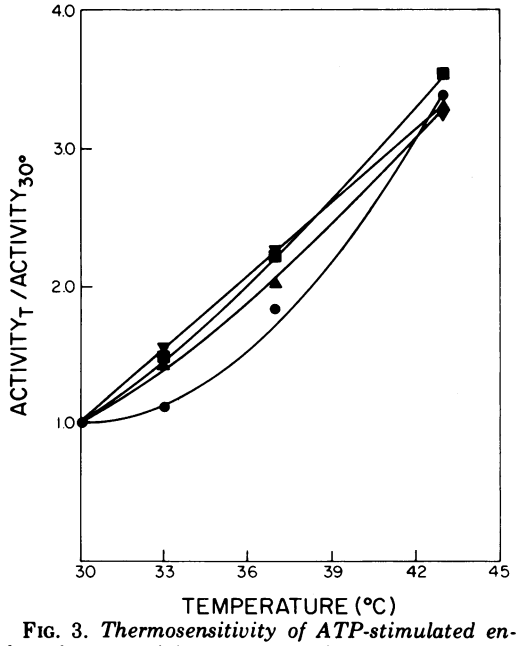

FIG. 3. *Thermosensitivity of ATP-stimulated endonuclease. Activity was assayed as described. The amount of M13 circular DNA made susceptible to exonuclease I in the absence of ATP was subtracted from that found in its presence. (●) Wild type; (▲) recB270; (■) recC271; (▼) recB270 recC271.*

recombination proficiency, result primarily from the specific loss in exonuclease activity on duplex DNA. By inference, then it is this activity which is most directly involved in these processes in vivo.

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LITERATURE CITED

1. Alberts, B., and G. Herrick. 1971. DNA-cellulose chromatography, p. 198-217. *In* L. Grossman and K. Moldave (ed.), Methods in enzymology, vol. XXI, part D. Academic Press Inc., New York.
2. Capaldo-Kimball, F., and S. D. Barbour. 1971. Involvement of recombination genes in growth and viability of *Escherichia coli* K-12. J. Bacteriol. **106**:204-212.
3. Emmerson, P. T. 1968. Recombination deficient mutants of *Escherichia coli* K-12 that map between *thyA* and *argA*. Genetics **60**:19-30.
4. Forsheit, A. B., and D. S. Ray. 1970. Conformations of the single-stranded DNA of bacteriophage M13. Proc. Nat. Acad. Sci. U. S. A. **67**:1534-1541.
5. Goldmark, P. J., and S. Linn. 1972. Purification and properties of the *recBC* DNase of *Escherichia coli* K-12. J. Biol. Chem. **247**:1849-1860.
6. Haefner, K. 1968. Spontaneous lethal sectoring, a further feature of *Escherichia coli* strains deficient in the function of *rec* and *uvr* genes. J. Bacteriol. **96**:652-659.
7. Hayes, D. H., F. Hayes, and M. F. Guerin. 1966. Association of rapidly labelled bacterial RNA with ribosomal RNA in solutions of high ionic strength. J. Mol. Biol. **18**:499-515.
8. Kushner, S. R. 1974. In vivo studies of temperature-sensitive *recB* and *recC* mutants. J. Bacteriol. **120**:1213-1218.
9. Kushner, S. R., H. Nagaishi, A. Templin, and A. J. Clark. 1971. Genetic recombination in *E. coli*: the role of exonuclease I. Proc. Nat. Acad. Sci. U. S. A. **68**:824-827.
10. Lehman, I. R., and A. L. Nussbaum. 1964. The deoxyribonucleases of *Escherichia coli*. V. On the specificity of exonuclease I. J. Biol. Chem. **239**:2628-2636.
11. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265-275.
12. Mahler, I. 1967. Preparation of tritium-labeled DNA from *Bacillus subtilis* and *Escherichia coli*, p. 693-695. *In* L. Grossman and K. Moldave (ed.), Methods in enzymology, vol. XII, part A. Academic Press Inc., New York.
13. Marmur, J. 1961. A procedure for the isolation of deoxyribonucleic acid from micro-organisms. J. Mol. Biol. **3**:208-218.
14. Milcarek, C., and B. Weiss. 1972. Mutants of *Escherichia coli* with altered deoxyribonucleases. I. Isolation and characterization of mutants for exonuclease III. J. Mol. Biol. **68**:303-318.
15. Nobrega, F. G., F. H. Rola, M. Pasetto-Nobrega, and M. Oishi. 1972. Adenosine triphosphatase associated with adenosine triphosphate-dependent deoxyribonuclease. Proc. Nat. Acad. Sci. U. S. A. **69**:15-19.
16. Tomizawa, J., and H. Ogawa. 1972. Structural genes of ATP-dependent deoxyribonuclease of *Escherichia coli*. Nature N. Biol. **239**:14-16.
17. Willetts, N. S., A. J. Clark, and B. Low. 1969. Genetic location of certain mutations conferring recombination deficiency in *Escherichia coli*. J. Bacteriol. **97**:244-249.
18. Willetts, N. S., and D. W. Mount. 1969. Genetic analysis of recombination-deficient mutants of *Escherichia coli* K-12 carrying *rec* mutations cotransducible with *thyA*. J. Bacteriol. **100**:923-934.
19. Wright, M., G. Buttin, and J. Hurwitz. 1971. The isolation and characterization from *Escherichia coli* of an adenosine triphosphate-dependent deoxyribonuclease directed by *rec* B, C genes. J. Biol. Chem. **246**:6543-6555.