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Fine structure of the recB and recC gene region of Escherichia coli

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SUMMARY: For genetic and enzymatic study of recBC DNase, the Escherichia coli thyA, recC, recB and argA gene region cloned in a cosmid was subcloned into the pBR322 plasmid vector and its fine restriction map was made. Complementation analysis showed that these genes were located in a 19kb BamHI fragment in the order thyA recC recB argA, so far as estimated. The recA recB recC cells harboring plasmid subcloned with this BamHI fragment exhibited 12.3-fold increase in recBC DNase activity. Clones carrying the recB and the recC gene expressed recB and recC function, respectively, and the initiation sites of transcription of these genes were detected by S1 nuclease mapping, proving that the recB and recC genes consist of independent cistrons.

Studies on rec mutations have resulted in the characterization of several recombination deficient mutants that also show increased sensitivity to ultraviolet (UV) light and to cross-linking agents, such as mitomycin C (MMC) and X-ray irradiation (1). These studies have indicated the existence of three genes, recA, recB and recC (2). Much information has been obtained recently in genetic and enzymatic analysis of recA gene functions (3,4). However, investigations on the recB and recC genes, structural genes of recBC DNase (5,6), have been limited because of the poor recovery of the enzyme during its purification and lack of information on the genetic organization of the genes.

Previously we reported cloning of a large DNA fragment containing the thyA gene of Escherichia coli into a packageable ColE1 vector, pKY2662, which

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Abbreviations: kb, kilobase; UV, ultraviolet; MMC, mitomycin C; tet^r, tetracycline resistant; amp^r, ampicillin resistant.

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was developed in this laboratory and we demonstrated that the recombinant DNA obtained, named pFS11, carries recB, recC and argA genes in addition to the thyA gene (7). This paper reports studies on the fine structure of this gene region. We showed that the recB and recC genes are independent cistrons and that the coexistence of these genes enhances recBC DNase activity.

MATERIALS AND METHODS

Media: L-Broth contains 1% bacto-tryptone, 0.5% yeast extract and 0.5% NaCl, and L-agar is L-broth solidified with 1.5% agar. Minimal agar supplemented with necessary growth factors (8) was used for testing thyA and/or argA mutations.
Enzymes: BamHI was prepared and used according to the method of Smith et al. (9). HindIII, PstI, SalI, SmaI, EcoRI, BglII, PvuII and MluI were purchased from Takara Shuzo Co., Japan, XhoI, HincII and AvaII were from Bethesda Research Laboratories, and ClaI was from Boehringer Mannheim Co. T4 DNA ligase was purified by the method of Tait et al. (10). Electrophoretically pure DNase (DPFF) was obtained from Worthington Co., and made RNase-free by passage through a column of agarose-5'-(4-aminophenylphosphoryl)uridine-2'(3') phosphate (Miles) (1ml bed volume) as described (11).
Preparation of phage lysates and plasmid DNAs: Phage lysates used for marker rescue analysis were prepared by the plate method as reported elsewhere (12). Plasmid DNAs were purified as described previously (13,14).
Complementation analysis: The presence of thyA and argA genes in various clones was tested by examining their ability to grow on minimal agar in the absence of added thymine and arginine, respectively (7). The presence of recB and recC genes in various clones was demonstrated by using recB (JC5743), recC (JC5489) or recA recB recC (JC5547) cells (7). Phenotypes of these clones harboring the respective genes were identified by examining recovery of UV- or MMC-sensitivity, and sensitivity to λ red gam phage.
UV irradiation: UV was irradiated from a GL15 UV-lamp (Toshiba Co., Ltd.) at a dose of 0.3J/m^2/sec. Survival rates were estimated on cells growing exponentially in L-broth after dilution to a cell density of 0.5 x 10^4 cells/ml in $MgSO_4$ solution. Samples of 0.1ml of cell suspension spreaded on L-agar plates were irradiated for 8sec. (16). After overnight incubation at 37°C, colonies were counted.
MMC treatment: L-agar containing 1μg/ml of MMC (Kyowa Hakko Co., Ltd.) was used. Survival rates were measured as in the UV sensitivity test (15).
Assay of recBC DNase activity: ^{32}P-labeled Escherichia coli DNA was obtained as described previously (16). All enzyme assay was carried out on crude lysates prepared by the method of Goldmark and Linn (17). Protein concentration was determined by the method of Lowry et al. (18) with bovine serum albumin as a standard.
Total RNA preparation and S1 nuclease mapping: A 20ml culture of the wild type strain (AB1157) harboring pFS11-04 was grown in L-broth at 37°C to a cell density of 5 x 10^8 cells/ml, subjected to induction of recBC DNase in the presence of 1μg/ml of MMC, and then incubated for 30min. Then total RNA was isolated as described previously (19). DNA-RNA hybridization, S1 nuclease digestion and alkaline agarose gel electrophoresis were performed as described elsewhere (20,21). After electrophoresis, the gel was reduced to a film in vacuo. Autoradiography was carried out at 80°C.

RESULTS AND DISCUSSION

Construction of recombinant DNA: After partial digestion of E. coli DNA with BamHI, a DNA fragment containing thyA, recC, recB and argA genes was cloned to

Table 1 Properties of the recB and recC cells harboring pFS11.

Strain	Genetic Properties	(Plasmid)	UV Sensitivities[a]	MMC Sensitivities[a]
AB1157	rec$^+$	(No)	94.5	88.4
JC5743	recB	(No)	15.2	0.1
JC5743	recB	(pFS11)	97.6	93.4
JC5489	recC	(No)	21.5	0.1
JC5489	recC	(pFS11)	96.9	91.1

UV irradiations and MMC treatments were performed as described in MATERIALS AND METHODS.

[a]UV sensitivity and MMC sensitivity were measured as percent survivals after treatments

a cosmid vector, pKY2662, and the recombinant molecule obtained was named pFS11, as described elsewhere (7). As shown in Table 1, recB or recC cells harboring pFS11 showed apparent recoveries of UV and MMC sensitivities. recBC DNase activities were studied using a recA strain (AB2463) and recA recB recC strain (JC5547). Comparison of their specific activities, obtained by subtracting the rates in the absence of ATP from those in the presence of ATP, showed that cells harboring pFS11 had 4.2 times higher specific activity than cells without the plasmid (Table 2). Thus the presence of recB and recC genes was confirmed by these phenotypical and enzymatic observations. Then the fine

Table 2 Levels of recBC DNase in recA recB recC cells harboring recombinant plasmids.

Strain	Genetic Properties	(Plasmid)	-ATP[a]	+ATP[a]	Δ[b]
AB2463	recA	(No)	5.1	14.5	9.4
JC5547	recA recB recC	(pKY2662)	6.6	5.3	-
JC5547	recA recB recC	(pBR322)	11.2	7.8	-
JC5547	recA recB recC	(pFS11)	4.3	44.1	39.8
JC5547	recA recB recC	(pFS11-04)[c]	5.6	120.9	115.3
JC5547	recA recB recC	(pFS11-05)[c]	9.6	104.2	94.6

recBC DNase activity was measured in crude lysates prepared as described in MATERIALS AND METHODS.

[a]Units recBC DNase/mg protein, assayed in the absence and presence of ATP.

[b]Δ = (+ATP) - (-ATP)

[c]pFS11-04 and -05 are subclones of pFS11, and characterized in Fig. 2 and in the text.

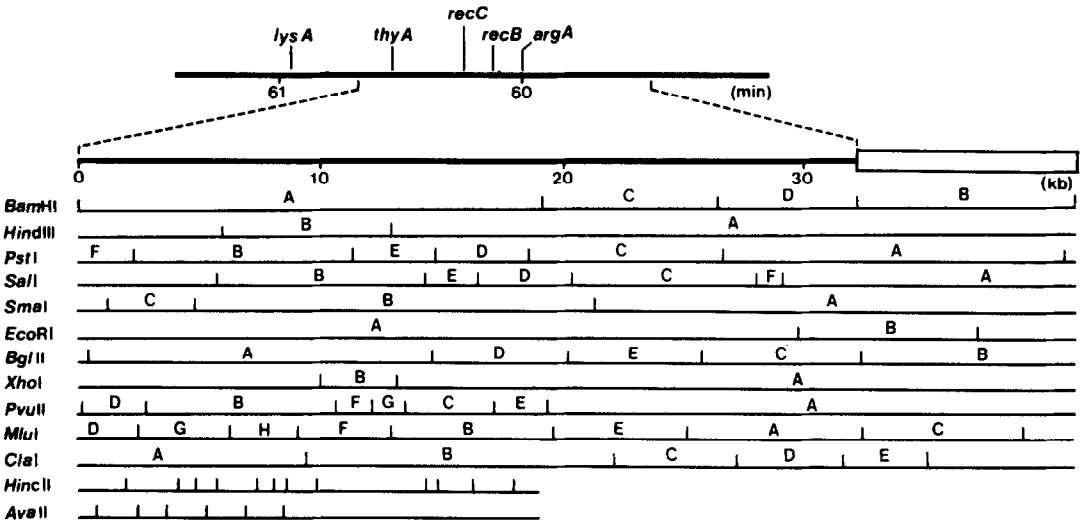

Fig. 1 Genetic map of the thyA-recC-recB-argA region of E. coli and restriction map of pFS11 DNA. The two lower lines indicate the BamHI-A fragment. The genetic map of Bachmann and Low (22) is shown at the top. The location of the chromosomal region is shown by a solid thick line. The open box represents pKY2662.

restriction map of pFS11 was constructed as shown in Fig. 1. Southern blotting showed that neither rearrangement nor deletion had occured in the insert DNA (7).

Location of the recB and recC genes within pFS11: Subcloning of pFS11 was carried out to locate the recB and recC genes more precisely. First, the DNA insert in pFS11 was found to be cleaved into 3 fragments by BamHI digestion. These fragments, named BamHI-A, -C and -D were separately ligated to the BamHI site of pBR322 and the ligates were introduced into recA recB recC cells. Among the recombinant plasmids obtained, the BamHI-A fragment-containing pFS11-04 was observed to complement thyA, recC, recB and argA mutations and to change recA recB recC cells to λ red gam resistant cells, indicating that the 19.0kb BamHI-A fragment carries not only recC and recB genes but also thyA and argA genes (Fig. 2). In addition, recA recB recC cells harboring pFS11-04 exhibited 12.3-fold higher recBC DNase activity, as calculated from the data in Table 2 by the method described above. Similarly, recA recB recC cells harboring another subclone named pFS11-05, which was obtained by partial digestion of the BamHI-A fragment with PstI, exhibited 10.1-fold higher recBC DNase activity (Table 2).

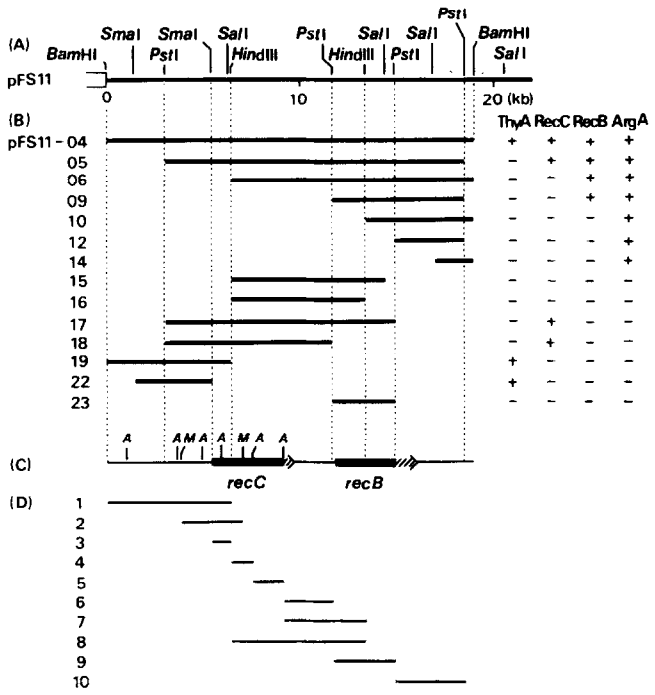

Fig. 2 Fine restriction map of the thyA-recC-recB-argA region and parts of the DNA region carried by subclones of pFS11. (A) The chromosomal DNA cloned on pFS11 is illustrated at the top with the cleavage sites for restriction endonucleases. The open box and thick solid line represent pKY2662 and chromosomal DNA, respectively. (B) Chromosomal DNAs carried by various subclones are indicated by thick lines. Ability (+) or inability (-) to complement thyA, recC, recB and argA is shown at the right. (C) The positions of recC and recB genes were determined from the results shown in Fig. 3. A and M indicate cleavage sites of restriction endonucleases AvaII and MluI, respectively. Directions of transcription are shown by arrows. Solid and shaded thick lines show certain and uncertain portions for the coding region, respectively. (D) Thick solid lines indicate the DNA fragments used for S1 nuclease mapping. (1) 5.4kb BamHI-HindIII; (2) 3.4kb MluI-G; (3) 1kb SmaI-HindIII; (4) 1kb HindIII-AvaII; (5) 1.5kb AvaII; (6) 2.5kb AvaII-PstI; (7) 4.2kb AvaII-HindIII; (8) 6.7kb HindIII-B; (9) 3.2kb PstI-E; (10) 3.6kb PstI-D.

The BamHI-A fragment was further digested completely or partially with HindIII, SalI and PstI, and the fragments obtained were subcloned into pBR322, as shown in Fig. 2. The inserts of pFS11-17 and pFS11-18 were in reverse directions in the vector, but these two subclones complemented the recC mutation similarly, suggesting that the recC gene is situated within the PstI-B fragment and its transcription is not initiated by the promoter in the vector DNA. pFS11-09 containing PstI-D and -E complemented the recB mutation, but neither PstI-D nor PstI-E fragment alone did, as observed with pFS11-12 or pFS11-23. In pFS11-06, the DNA fragment was inserted at the HindIII and

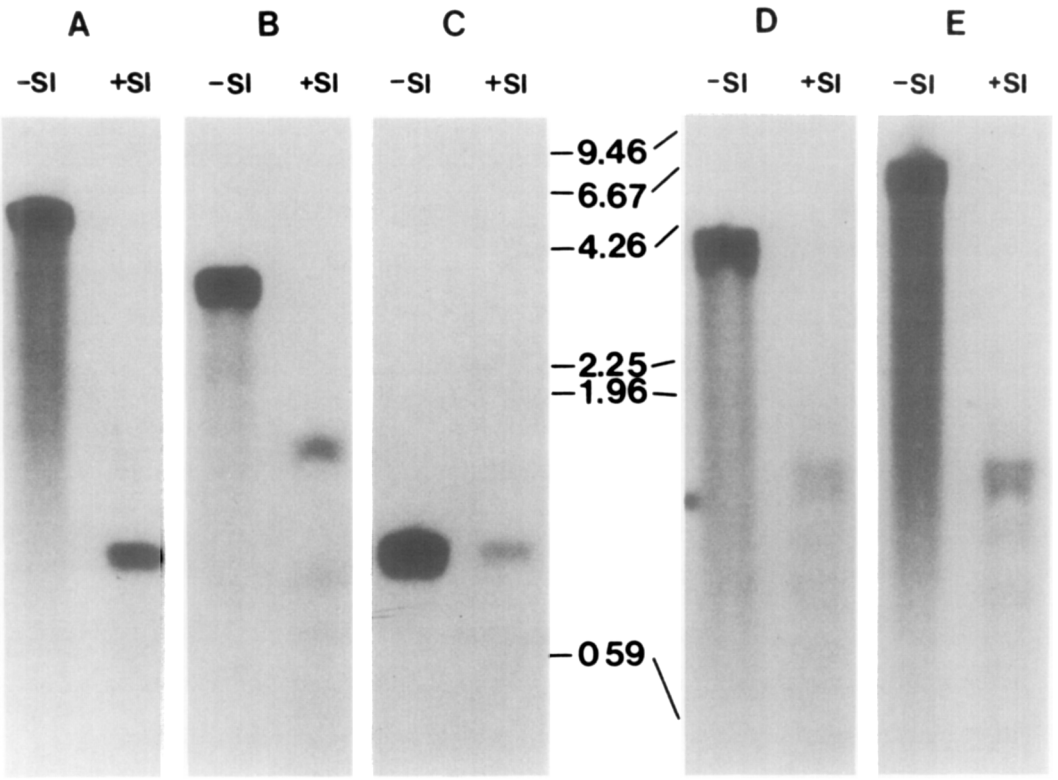

Fig. 3 Electrophoretic analysis of S1 nuclease mapping with in vivo made total RNA and various restricted DNA fragments. DNA-RNA hybridization, S1 nuclease digestion and analysis by alkaline gel electrophoresis were performed as described in MATERIALS AND METHODS. Lane (A), (B) and (C) show results on the recC gene. The DNA fragments used for DNA-RNA hybridization were (A): 5.4kb BamHI-HindIII fragment from pFS11-19 (see Fig. 2); (B): 3.4kb MluI-G fragment; (C): 1kb SmaI-HindIII fragment from pFS11-19. Lanes (D) and (E) show results on the recB gene. The DNA fragments were (D): 4.2kb AvaII-HindIII fragment from HindIII-B fragment; (E): 6.7kb HindIII-B fragment. λcI857Sam7 DNA digested with HindIII was used as a molecular size standard. Left lanes show the profiles on denaturing gel electrophoresis of the respective DNA samples without S1 nuclease treatment as controls.

BamHI sites in the tet^r gene of pBR322, but in pFS11-09, the fragment was at the PstI site in the amp^r gene. So although the directions of these two inserts were opposite with respect to the direction of transcription of the vector's marker genes (tet^r or amp^r gene), both complemented recB mutation in the same way. Thus the possibility that the recB gene is governed by the promoter in the vector could also be excluded, and the recB gene was persumed to be located within the two PstI fragments, D and E. Furthermore there are at least two ways to recover Rec^- phenotype; one is complementation and the

other is recombination between host DNA and the E. coli DNA fragment carried by the plasmid, but the latter was eliminated by a curing test of the plasmid DNA.

Next, the initiation sites and directions of transcription of recB and recC genes were analysed by the S1 nuclease mapping method of Berk and Sharp (20). In this method, length of the 5'-end labeled DNA fragment that is resistant to S1 nuclease digestion indicates the distance from one end of the DNA fragment to the initiation site of transcription or the length of the coding strand itself. Results of experiments on the recC gene are shown in Fig. 3-A, -B and -C. When the 5.4kb BamHI-HindIII fragment from pFS11-19, 3.4kb MluI-G fragment and 1kb SmaI-HindIII fragment from the PstI-B fragment (Fig. 2-D) were used for DNA-RNA hybridization, a 1kb, 1.6kb and 1kb S1 nuclease resistant DNA fragment, respectively, were detected. These results indicate that transcription of the recC gene is initiated 1kb downstream from the HindIII site in the PstI-B fragment and proceeds upstream. Similar experiments were performed with three neighboring fragments; 1kb HindIII-AvaII, 1.5kb AvaII and 2.5kb AvaII-PstI fragments in the HindIII-B fragment. A 1kb S1 nuclease resistant DNA fragment was obtained with the first DNA sample, and 1.5kb and 0.6kb fragments with the second sample, but no band was obtained with the third, indicating that transcription of recC gene terminates within the 2.5kb AvaII-PstI fragment (data not shown). It is interesting to notice that a 0.6kb band was obtained from the second DNA sample. This may be due to some unknown mechanism of expression of the recB and recC genes, such as that due to the presence of another gene.

Results of experiments with S1 nuclease on the recB gene are shown in Fig. 3-D and -E. The same 1.5kb S1 nuclease resistant DNA fragments were detected when two partially overlapping DNA fragments, a 4.2kb AvaII-HindIII and 6.7kb HindIII-B fragment, were used for DNA-RNA hybridization. Similar experiments with 3.2kb PstI-E and 3.6kb PstI-D fragments were performed; a 3.2kb S1 nuclease resistant DNA fragment was detected with the former but none with the latter (data not shown). These findings indicate that transcription

of the recB gene is initiated at a point 1.5kb downstream from the HindIII site in the PstI-E fragment, and proceeds upstream, terminating within the PstI-D fragment.

While these experiments were in progress, Hickson and Emmerson cloned the recB and recC genes separately into plasmids and identified the gene products (23). However, the locations of these two genes they reported were different from those determined from our results. The reason for this discrepancy is unknown. But in the recombinant plasmids they obtained, the DNA inserts containing the genes were larger than ours, and they studied the location in these large regions only by analysis of insertion mutants. On the other hand, in our experiments the recB and recC gene region was investigated in detail by subcloning the various fragments obtained by restriction endonuclease digestion. In addition, we examined the initiation sites and directions of transcription of these genes by the S1 nuclease mapping technique. Our results indicated that the recB and recC genes are both larger than 3kb, corresponding to the known molecular weights of the subunits of recBC DNase (17).

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