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Biochemistry

Physical map of the *recA* gene

(restriction mapping/RNA polymerase binding/transcription/maxicells/*recA* promoter)

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ABSTRACT **We have cloned the *recA* gene of *Echerichia coli* K12 and some of its restriction fragments on the plasmid cloning vehicle pBR322. The *recA* gene was mapped with regard to the restriction sites of *Eco*RI, *Bam*HI, *Pst* I, *Hha* I, *Hae* III, *Hin*fI, and *Taq* I restriction endonucleases. The *recA* promoter was localized by the binding of RNA polymerase to restriction fragments. The initiation point of transcription of *recA* mRNA and the direction of transcription were determined from *in vitro* transcription of *recA* gene fragments and from analysis of the polypeptides made in maxicells that contain plasmids carrying only part of the *recA* gene.**

The *recA* gene of *Escherichia coli* is involved in genetic recombination (1), in postreplication repair (2), and in a number of other cellular functions such as mutagenesis, phage induction, and cell division (for a recent review see ref. 3). The *recA* protein has been identified (4) and purified to near homogeneity (5). Although the exact mechanism of action of the *recA* protein is not known, *in vitro* assays requiring *recA* protein for the proteolytic cleavage of the λ repressor (6) and for the nicking of undamaged DNA homologous to damaged DNA (7) have been reported. A more direct role for the *recA* protein in recombination is suggested by two recent studies that demonstrated that *recA* protein catalyzes the reannealing of complementary DNA strands (8) and the ATP-dependent pairing of superhelical DNA with homologous single-stranded fragments (9). However, it is already clear that the *recA* gene is under regulatory control because the level of *recA* protein in cells fluctuates from low basal levels to very high levels after treatments that damage DNA or stall DNA replication (4, 10–12). The regulation of *recA* protein synthesis depends on the *lexA* gene which may code for a repressor of the *recA* gene (13, 14), and functional *recA* protein itself seems to be required for the efficient expression of the *recA* gene (4, 14–16). Furthermore, several other mutations (*tif*, *zab*, and *lexB*) that confer diverse phenotypes are now thought to lie in the *recA* gene (17). Although several models (4, 14–16) have been considered to account for these and other observations, it is likely that experiments measuring the interaction of purified proteins with the promoter/operator region of the *recA* gene will be required to understand satisfactorily the expression and control of the *recA* gene.

In this communication we establish a physical map of the *recA* gene, a step essential for understanding its regulation. The location of the gene on cloned fragments of the *E. coli* chromosome was defined by inactivation with the *Pst* I and *Eco*RI restriction enzymes that have 6-base pair (bp) recognition sequences. These sites were then used as reference points to generate a more detailed map with the *Hha* I, *Hae* III, *Hin*fI, and *Taq* I restriction nucleases, whose recognition sites comprise only 4 bp. The restriction fragments binding to RNA polymerase were identified and the size of the RNAs and polypeptides synthesized from various fragments of the *recA* gene were determined. These data allow us to locate the promoter and operator of the *recA* gene.

MATERIALS AND METHODS

Bacterial Strains and Plasmids. Bacterial strains LC1842 (18), KM4104: *mtlA strA lysA argA* Δ $(lac)_{X74}$ (deletion of entire *lac* operon) Δ7 (*srl-recA*) Δ2134 (*gal-bio*) (ref. 19), and CSR603 (20) are derivatives of *E. coli* K12. The plasmids are described in Table 1.

Enzymes and Chemicals. Restriction enzymes, T4 DNA ligase, and polynucleotide kinase were obtained from New England BioLabs. *E. coli* RNA polymerase was from Enzo Biochemicals (New York). [^{35}S]Methionine (1000 Ci/mmol; 1 Ci = 3.7×10^{10} becquerels) was supplied by Amersham and [γ-^{32}P]ATP (125 Ci/mmol), by ICN.

Restriction Enzyme Analysis. Restriction enzymes were used as recommended by the supplier. Restriction fragments longer than 1500 bp were purified by centrifugation in an ethidium bromide/sucrose velocity gradient (22); smaller DNA fragments were separated by electrophoresis on acrylamide gels and were eluted from pieces of the gel that were incubated overnight at room temperature in 15 mM NaCl/1.5 mM Na citrate. The restriction sites for *Hae* III, *Hin*fI, and *Taq* I were determined by isolating DNA fragments after treatment with one enzyme and then treating with a second restriction nuclease (23). *Hha* I sites were determined by this method and also by partial digestion of terminally labeled fragments (24). Terminal labeling of DNA fragments was done as described by Maxam and Gilbert (25).

Plasmid Construction. *recA* plasmids were constructed by standard *in vitro* DNA recombination methods. Plasmid DNAs were purified by sodium dodecyl sulfate ($NaDodSO_4$) lysis (26) and ethidium bromide/CsCl gradient centrifugation.

Binding of RNA Polymerase and Transcription of Restriction Fragments. These experiments were done as described by von Gabain and Bujard (27), except that the KCl concentration was 40 mM in both binding and transcription buffers.

Protein Labeling in Maxicells. Maxicells are UV-irradiated cells carrying *recA* and *uvrA* in which plasmids are amplified and chromosomal DNA is degraded (28). Maxicells are used for specific labeling of plasmid-encoded proteins with little background synthesis of total cellular proteins (28). Cultures (5 ml) were grown in K medium to $A_{600} = 0.6$, cells were irradiated at 100 J/m^2 with a germicidal lamp, and incubation was continued overnight (14–16 hr). The cells were collected by centrifugation, washed, and starved for 1 hr in minimal medium lacking sulfate. [^{35}S]Methionine was added to 1 μCi/ml and incubation was continued for 1 hr. Cells were then col-

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Abbreviations: $NaDodSO_4$, sodium dodecyl sulfate; bp, base pairs; kb, kilobases (pairs).

Table 1. Plasmids used in this study

Plasmids	Size (kb)	Relevant genotype	Reference
pBR322	4.3	$tet^+ amp^+$	21
pLC18-42	19	$recA^+ srl^+$	18
pDR1453	13	$recA^+ srl^+ tet^+ amp^-$	This work
PDR1458	11	$srl^+ tet^+ amp^-$	This work
pDR1461	5.8	$srl^- tet^- amp^+$	This work
pDR1464	4.9	$srl^- tet^+ amp^-$	This work

kb. Kilobase pairs.

lected, washed, and lysed. The labeled proteins were analyzed on $NaDodSO_4$/10% polyacrylamide gels with a 30:0.8 ratio of acrylamide to bisacrylamide (29).

RESULTS

***Eco*RI and *Pst* I Inactivate *recA*.** pLC18-42 is a ColE1 recombinant plasmid that was reported to carry the *recA* gene (18). It has a size of about 19 kilobases (kb) and has a single *Eco*RI site (unpublished observations). We inserted pBR322 into this site by *in vitro* recombination. The plasmid so obtained, pDR1010, complemented KM4104 for *srl* but not for *recA*. This suggested that the *Eco*RI site was in the *recA* gene. Indeed, when pDR1010 was cut with *Eco*RI and religated, the new srl^+ plasmid pDR1011 obtained was $recA^+$ (Table 2) and was presumed to be identical to pLC18-42. This experiment confirmed that the entire *recA* gene sequence remained and was undamaged in pDR1010 even though its function was blocked by the insertion of pBR322.

Pst I cuts pLC18-42 at five sites generating fragments of 6.8, 6.3, 3.0, 1.8, and 1.2 kb. These fragments were inserted individually into the *Pst* I site of pBR322, but none of these new plasmids was $recA^+$. However, one plasmid, pDR1453, did confer recombination proficiency and UV-resistance to strain KM4104 (Table 2). Restriction analysis of pDR1453 showed that both the 6.8- and 1.8-kb *Pst* I fragments were inserted in pBR322. The simplest interpretation of this result is that each of these *Pst* I fragments contains part of the *recA* gene. Because further analysis showed that the 1.8-kb *Pst* I fragment of pDR1453 was cut by *Eco*RI about 550 bp from the junction with the 6.8-kb *Pst* I fragment, we conclude that this 550-bp segment of DNA is essential for expression of the *recA* gene. This 550-bp segment is probably part of the *recA* structural gene because there is no reason to expect that the *recA* structural gene and its promoter are not contiguous.

Construction of Plasmids Carrying Fragments of the *recA* Gene. pDR1453 contains 8.6 kb of chromosomal DNA of which only about 1 kb is the *recA* gene, because the *recA* protein has a molecular weight of about 40,000 (4). To simplify the restriction analysis of the *recA* gene, we constructed two new plasmids that had lost most of the chromosomal DNA. The 6.8-kb *Pst* I fragment of pDR1453 carries *srlC* and is cut with *Bam*HI 1800 bp from a *Pst* I site as shown in Fig. 1. pDR1461 was made by cutting pDR1453 with *Bam*HI and *Eco*RI and inserting this fragment into pBR322. Thus, pDR1461 includes the 550-bp part of the *recA* gene between the *Pst* I and *Eco*RI sites (Fig. 1). pDR1464 was constructed by digesting pDR1453 with *Eco*RI followed by ligation. It contains the smaller *Eco* RI-*Pst* I chromosomal fragment of pDR1453 that includes the segment of the *recA* gene not included on pDR1461. (A small segment of pBR322 lying between its *Eco* RI and *Pst* I sites was deleted during the formation of pDR1464.)

Table 2. Effect of *recA* plasmids on repair and recombination functions of KM4104

Plasmid	UV survival at 10 J/m²	Lys^+ transductants per 10^7 P1 phage
None	10^{-3}	<1
pLC18-42	0.5	50
pDR1453	0.3	50
pDR1010	4×10^{-4}	<1
pDR1011	0.5	100
pDR1458	5×10^{-4}	<1
pDR1461	5×10^{-5}	<1
pDR1464	7×10^{-4}	<1

Mapping of the *recA* Gene with Four Base-Specific Restriction Enzymes. Because the 550-bp *Pst* I-*Eco*RI fragment is in the *recA* gene, restriction mapping of this and adjacent fragments should include the whole *recA* gene. The 1.8-kb *Bam*HI/*Eco*RI fragment from pDR1461 and the similarly sized *Pst* I fragment from pDR1453 were isolated for cleavage with the *Hha* I, *Hae* III, *Hin*fI, and *Taq* I restriction enzymes used singly or in pairs. Reciprocal digests were also done in which fragments from the digestion with one enzyme were isolated and then digested with a second enzyme. Some of these results are shown in Fig. 2. Because it was not possible to order *Hha* I fragments unambiguously by this method, some of the *Hha* I fragments were ordered by partial digestion of terminally labeled fragments (24) (data not shown). By analysis of these and similar gels, the restriction map shown in Fig. 3 was constructed.

Synthesis of *recA* Protein Fragments in Maxicells: Orientation of the *recA* Gene. The three plasmids pDR1458, pDR1461, and pDR1464 with $RecA^-$ phenotypes are presumably missing an end of the *recA* gene with the same end missing from pDR1458 and pDR1461, whereas the other end of the gene is absent from pDR1464. We examined the proteins synthesized by these three plasmids because we anticipated that a plasmid carrying the *recA* promoter and the part of the gene coding for the amino-terminal portion of the *recA* protein would make a polypeptide shorter than intact *recA* protein, whereas a plasmid carrying only that part of the gene coding for the carboxyl terminus of the *recA* protein would not make any polypeptide because of the absence of a suitable promoter. Because we previously showed that plasmid encoded proteins can be specifically labeled in *recA* maxicells (28), we used this system to detect polypeptides made from plasmids pDR1458, pDR1461, and pDR1464. The results obtained with pDR1458

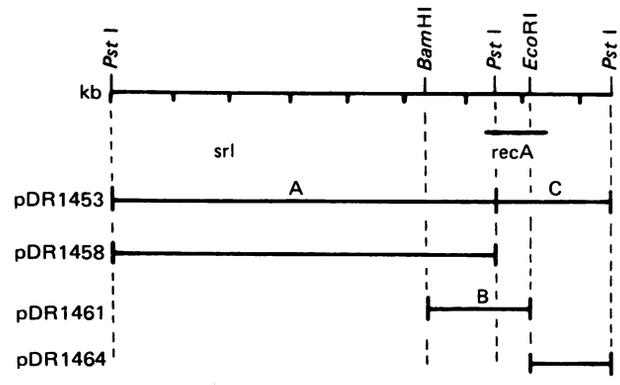

FIG. 1. Restriction sites in the chromosomal segments carried by *recA* plasmids. For pDR1453, the letters A and C designate the fragments obtained after digestion with *Pst* I. (The B fragment, not shown in the diagram, is the pBR322 cloning vehicle.) For pDR1461, B is the smaller fragment obtained after digestion with *Eco*RI and *Bam*HI, whereas the A fragment (not shown) is the cloning vehicle. The position of the *recA* gene was determined as described in the text. The exact location of the *srl* genes in pDR1458 was not determined.

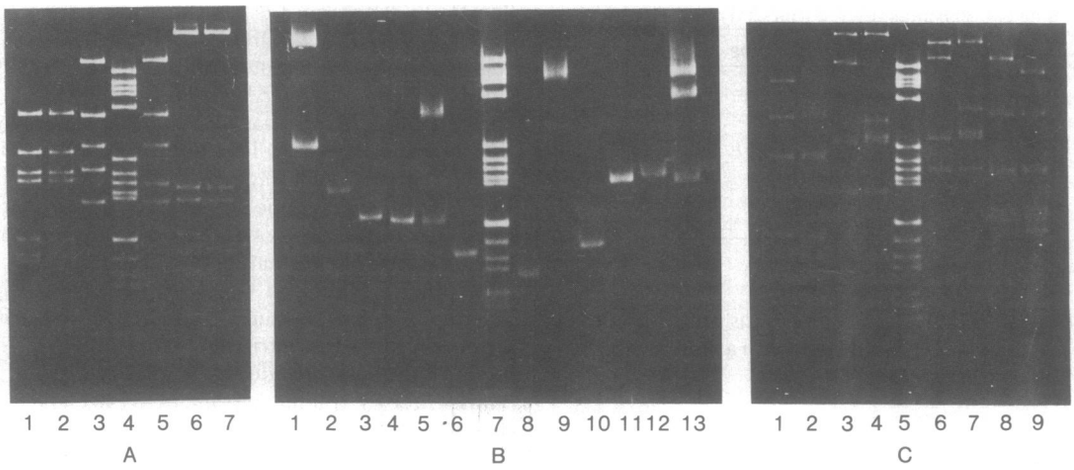

FIG. 2. Mapping of endonuclease cleavage sites in the *recA* gene by electrophoresis on 5% polyacrylamide gels. (*A*) The B fragment of pDR1461 was treated with the following enzymes: 1, *Hha* I; 2, *Hha* I and *Pst* I; 3, *Hin*fI; 4, reference (pBR322 DNA and *Hae* III); 5, *Hin*fI and *Pst* I; 6, *Taq* I; 7, *Taq* I and *Pst* I. (*B*) The B fragment of pDR1461 was treated with *Taq* I and individual fragments were isolated from a 5% polyacrylamide gel and then treated with either *Hae* III (lanes 1–6) or *Hin*fI (lanes 8–13). The *Taq* I fragments are (designated alphabetically by decreasing size): 1 and 13, A; 2 and 12, B; 3 and 11, C; 4 and 10, D; 5 and 9, E*; 6 and 8, F; 7, reference (pBR322 DNA and Hae III). (The E* fragment was obtained from digestion of pDR1461 with *Taq* I and is the fragment with the *Eco*RI site. It thus contains a segment of pBR322 joined to the E fragment at the *Eco*RI site.) (*C*) The C fragment of pDR1453 was treated with the following enzymes: 1, *Hha* I; 2, *Hha* I + *Eco*RI; 3, *Hae* III; 4, *Hae* III and *Eco*RI; 5, reference (pBR322 DNA and *Hae* III); 6, *Hin*fI; 7, *Hin*fI + *Eco*RI; 8, *Taq* I; 9, *Taq* I and *Eco*RI.

and pDR1461 are presented in Fig. 4. For comparison, known *recA* protein was made by treatment of KM4104/pDR1453 with nalidixic acid because the maxicell method for labeling proteins cannot be used with *recA*$^+$ cells. A polypeptide of M_r about 32,000 was made from pDR1461, whereas pDR1458 determined a polypeptide of M_r about 26,000. These polypeptides are different from any synthesized in maxicells with pBR322 alone; and with pDR1464, no *recA* determined protein was found (data not shown). Because the two plasmids that led to the synthesis of shortened polypeptides contain the end of the *recA* gene nearer *srl* whereas pDR1464 contains the end away from the *srl* gene, we infer that the *recA* promoter is located between *srl* and the *Eco*RI site in the *recA* gene.

If the polypeptides synthesized in maxicells are stable, their sizes can be used to locate the starting point of the *recA* structural gene. These truncated polypeptides will contain the amino-terminal amino acids of the *recA* protein to the junction of the *recA* gene with the pBR322 DNA plus those additional amino acids that are encoded by the pBR322 DNA up to the first in-phase nonsense codon. Because the relevant sequence of pBR322 is known, the number of additional amino acids can be determined for all possible reading frames. In the case of pDR1461 where the *recA* gene is joined to the pBR322 DNA at the *Eco*RI site, nonsense codons are soon encountered in all three reading frames beginning at nucleotides 5, 24, and 46 in the sequence published by Sutcliffe (30). Thus the maximum number of extra amino acids at the carboxyl terminus is 15 and the start of the structural gene for a polypeptide of M_r about 32,000 must be about 800 bp from the *Eco*RI site.

With pDR1458, the polypeptide synthesized is shorter than that synthesized with pDR1461 because the *Pst* I site in the *recA* gene lies between the promoter and the *Eco*RI site. The observed difference in sizes is less than might be anticipated from the 550-bp distance between the *Pst* I and *Eco*RI sites in the *recA* gene and is probably caused by the insertion of the *recA* gene segment in the *Pst* I site of pBR322 so that the sequence

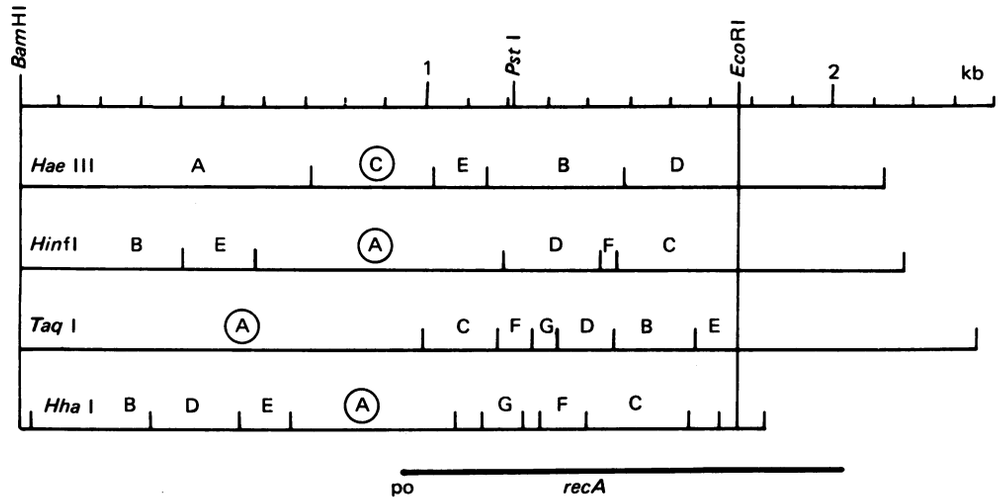

FIG. 3. Restriction map of the *recA* region. The letters refer to those fragments derived from the B fragment of pDR1461 (see Fig. 1) and are assigned according to size. The circled fragments are those that bind to RNA polymerase (see text and Fig. 5). The position and orientation of the *recA* gene were determined as described in the text. The extensions beyond the *Eco*RI site to the first cleavage site for each enzyme were determined from the digestions described in Fig. 2*C*. po, Promotor/operator.

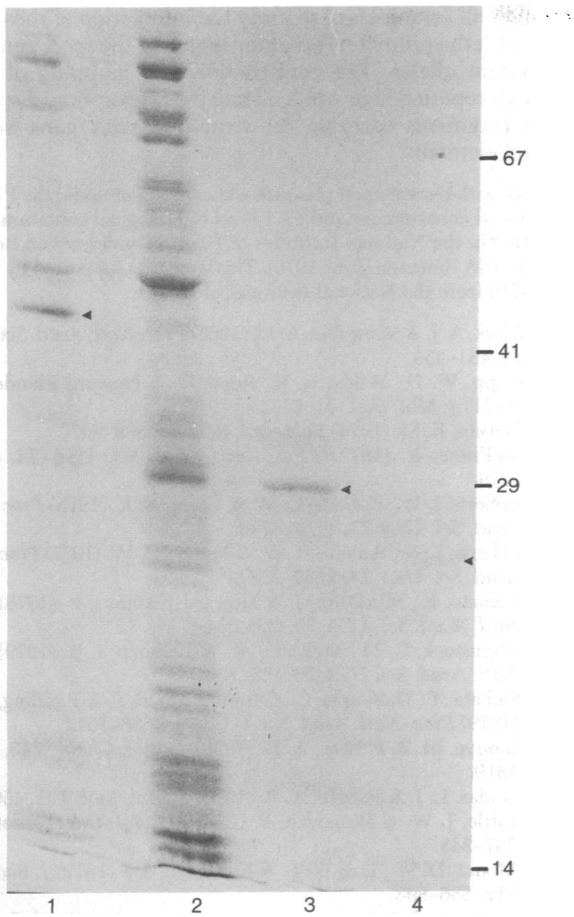

FIG. 4. Autoradiograph of *recA* protein and its fragments. Cultures were labeled with [^{35}S]methionine (1 μCi/ml) for 1 hr. Samples were electrophoresed on a $NaDodSO_4$/10% polyacrylamide gel (ratio of 30 parts of acrylamide to 0.8 parts of bisacrylamide). The autoradiograph was exposed for 3 days. Lanes: 1, total cellular proteins synthesized in KM4104/pDR1453 after induction with nalidixic acid for 30 min; 2, total cellular proteins of KM4104/pBR322 after nalidixic acid induction; 3, maxicell proteins of CSR603/pDR1461; 4, maxicell proteins of CSR603/pDR1458. The molecular weight standards were: bovine serum albumin, 67,000; ovalbumin, 41,000; carbonic anhydrase, 29,000; and lysozyme, 14,200. The triangles in lanes 1, 3, and 4 mark the positions of the intact *recA* protein (≈42,000) and the *recA* fragments (≈32,000 and ≈26,000).

coding for the carboxyl-terminal portion of β-lactamase is in the same reading frame as that coding for the amino-terminal end of the *recA* protein.

RNA Polymerase Binding and Transcription. To locate the *recA* promoter more precisely, the small fragment (≈1800 bp) of pDR1461 was digested with restriction enzymes and mixed with RNA polymerase, and the mixture was filtered through nitrocellulose filters. The polymerase-bound fragments were eluted from filters and run on a polyacrylamide gel (Fig. 5). From the digests with *Hha* I, *Hae* III, *Hin*fI, and *Taq* I, a single RNA polymerase-bound fragment was retained on the filter in each case. The fragments binding to RNA polymerase were those designated *Hha* I-A, *Hae* III-C, *Hin*fI-A, and *Taq* I-A in the restriction map in Fig. 3. From Fig. 3 it is seen that the sequence common to all four of these fragments, and thus containing the binding site for RNA polymerase, is the 250-bp segment of *Hae* III-C that is shared with the *Taq* I-A fragment.

Additional experiments were done to determine whether this binding site for RNA polymerase resulted in the synthesis of

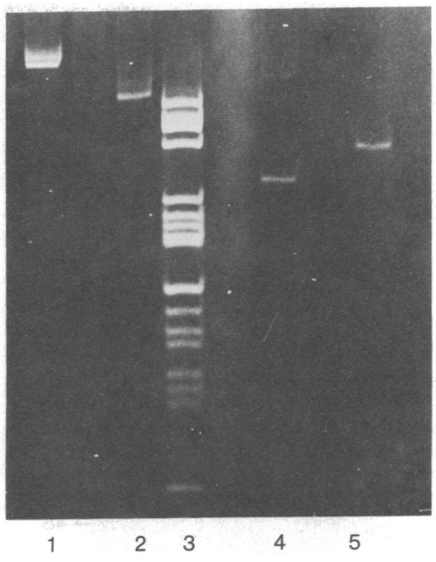

FIG. 5. Binding of restriction fragments to RNA polymerase. The B fragment of pDR1461 (see Fig. 1) was treated with a restriction endonuclease, mixed with RNA polymerase, and then passed through a nitrocellulose filter. The fragments bound to the RNA polymerase were eluted as described in ref. 27 and were electrophoresed on a 5% polyacrylamide gel and stained with ethidium bromide. The following enzymes were used: 1, *Taq* I; 2, *Hin*fI; 4, *Hae* III; 5, *Hha* I. Lane 3 is for reference and contains pBR322 DNA digested with *Hae* III.

actual transcripts. The transcripts synthesized from DNA templates cleaved with various restriction nucleases are shown in Fig. 6. The lengths of the major transcripts for the variously digested templates were *Eco*RI > *Pst* I > *Hin*fI > *Hha* I > *Hae* III > *Taq* I. These results demonstrate that RNA transcripts are initiated at or near the site identified as the RNA polymerase binding site and that synthesis proceeds toward the *Eco*RI site. By comparison with the 4S and 5S marker RNAs, the sizes of the transcripts obtained from the templates digested with *Hae* III and *Hha* I were estimated to be about 60 and 150 nucleotides, respectively, indicating that transcription is initiated at a site in the *Taq* I-A fragment near the junction with the *Taq* I-C fragment. Other studies (unpublished data) in which the sequence of the transcript of the *recA* gene was compared with the DNA sequence of the *Taq* I-A fragment showed that the initiation of the RNA chain is at a point 56 nucleotides from the end of the *Taq* I-A fragment. Because this site lies within the region common to all the restriction fragments bound to RNA polymerase (Fig. 5) and is also in the position expected from the lengths of the polypeptides synthesized in the maxicells with the plasmids carrying only part of the *recA* gene (Fig. 4), we conclude that it is the *recA* promoter.

DISCUSSION

In this study we located the *recA* gene by showing that insertion of pBR322 into the only *Eco*RI site in a plasmid carrying the *srl*/*recA* region of *E. coli* inactivated the *recA* gene. Various fragments surrounding this site were cloned and further mapped with the restriction nucleases *Pst* I, *Hae* III, *Hin*fI, *Hha* I, and *Taq* I. A strong binding site for RNA polymerase was found between the *srl* genes and the *Eco*RI site at a distance of 800–1000 bp from the *Eco*RI site. Transcription of restriction fragments and synthesis of polypeptides in cells containing various cloned DNA segments demonstrated that transcription and translation also began at or near the binding site of RNA polymerase and proceeded toward the *Eco*RI site. Our interpretation of these results is that the *Eco*RI site is in the *recA* structural gene and that the RNA polymerase binding site is the

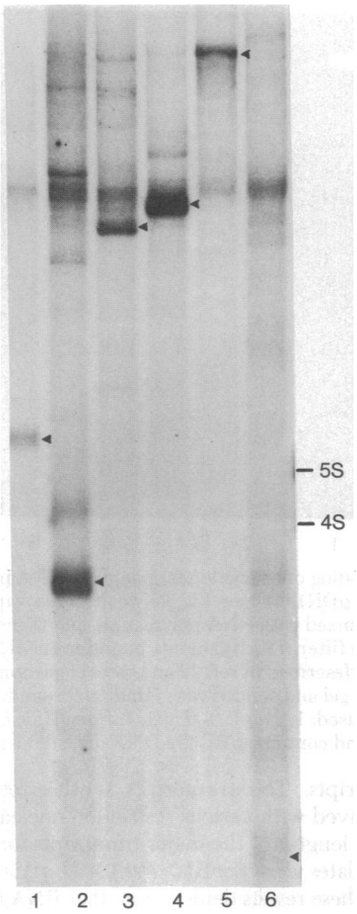

FIG. 6. Autoradiograph of transcripts synthesized from the *recA* gene digested with restriction endonucleases. The B fragment of pDR1461 (Fig. 1) was cleaved with one of the following enzymes: 1, *Hha* I; 2, *Hae* III; 3, *Hin*fI; 4, *Pst* I; 5, undigested B fragment (fragment ends at the *Eco*RI site); 6, *Taq* I. Transcription of the digested DNAs is described in the text. Transcripts were labeled with [γ-^{32}P]ATP and were electrophoresed on a 10% polyacrylamide gel containing 7 M urea. The transcripts marked with a triangle are those described in the text. In other experiments, the transcript in lane 6 marked with a triangle was much clearer than in this particular run.

promoter for the *recA* gene. Experiments are now in progress to determine sequences of DNA, RNA, and protein in this region in order to define the starting points for transcription and translation of the *recA* gene.

The concentration of *recA* protein in cells is normally very low, but treatments that damage DNA or inhibit DNA replication increase the level of the *recA* protein many fold so that it becomes one of the major proteins in the cell (4, 10–12). These extensive fluctuations indicate that the expression of the *recA* gene is tightly regulated and have led to various models to account for these observations. The properties of the *lexA* gene are consistent with the *lexA* protein's being the repressor of the *recA* gene, but the models are further complicated because of an apparent requirement for functional *recA* protein in its own induction (3, 4, 13–17).

In order to understand the molecular details of the expression of the *recA* gene and its regulation by the *lexA* and *recA* proteins, it will be necessary to study the interaction of these proteins with the promoter/operator region of the *recA* gene and its mutant alleles. The construction and mapping of *recA* plasmids reported here will facilitate the preparation of specific DNA fragments carrying the structural *recA* gene and its control elements.

Work with recombinant plasmids was carried out under the P1 level of physical containment and EK1 level of biological containment as specified in the National Institutes of Health Guidelines on Recombinant DNA Research (June 1976). This work was supported by Grant CA06519 from the National Institutes of Health.

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