Reconstitution of RecBC DNase Activity from Purified Escherichia coli RecB and RecC Proteins*

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The Escherichia coli RecB protein, normally synthesized in low amounts, has been amplified by linkage of the recB gene to the phage λ leftward promoter in an expression plasmid. Strains harboring this plasmid, RecB protein has been purified to homogeneity by a simple procedure which includes affinity chromatography on a column of RecC protein bound to agarose. The purified RecB protein has DNA-dependent ATPase activity but no exonuclease activity. RecC protein alone has neither ATPase nor exonuclease activity. However, when combined together, the RecB and RecC proteins show the ATP-dependent double-stranded exonuclease properties characteristic of the RecBC DNase.

The enzyme encoded by the recB and recC genes of Escherichia coli, known as the RecBC DNase or exonuclease V, is essential for efficient DNA repair and genetic recombination (Emmerson, 1968; reviewed by Clark (1971)). Preparations of the enzyme exhibit a wide variety of catalytic activities, including ATP-dependent exonuclease, ATP-stimulated endonuclease, ATP-dependent helicase, and DNA-dependent ATPase activities (for review, see Muskavitch and Linn (1981)). The exonuclease activity on double-stranded DNA, although ATP dependent, is strongly inhibited in vitro by physiological concentrations of ATP.

The recB and recC genes have been cloned (Hickson and Emmerson, 1981; Sasaki et al., 1982; Dykstra et al., 1984) and their gene products identified as proteins of molecular mass 135 kDa and 125 kDa, respectively (Hickson and Emmerson, 1981; Dykstra et al., 1984). Our approach has been to genetically amplify and purify the proteins in order to study their individual properties and their mutual interaction. Such a study had previously been hampered by the lack of an assay for the individual subunits and by their very low intracellular level. Results presented here and elsewhere (Hickson et al., 1984) indicate that these low intracellular levels are due to low rates of transcription of both the recB and recC genes. We have previously described the molecular amplification and purification of the RecC protein (Hickson et al., 1984). Here, we describe the amplification of the RecB protein by fusion of the recB gene to the leftward promoter of phage λ (Pι) in an expression plasmid (Remaut et al., 1981). This amplification permitted the use of SDS-PAGE as an assay and led to the development of a simplified purification procedure for the RecB protein. Milligram quantities of homogeneous RecB protein were purified by affinity chromatography using a column of purified RecC protein covalently bound to agarose. We show that the RecB protein, unlike the RecC protein, is a DNA-dependent ATPase. Although neither the RecB nor the RecC protein alone exhibits exonuclease activity on native λ DNA, the combined proteins do.

EXPERIMENTAL PROCEDURES

Strains—The strains used in this work are listed in Table I.

Enzymes—Restriction enzymes were purchased from NBL Enzymes, New England Biolabs, or Bethesda Research Laboratories and used according to the makers' recommendations. T4 DNA ligase was purified in this laboratory using NM989 (XT4lig) (Murray et al., 1979) by Dr. A. E. Tomkinson.

Construction of Mud(bla lac) Fusions to the recB Promoter—Mud(bla lac) phage were isolated from the strain MAL103 by thermal induction. The phage lysate was adsorbed to strain HA1060 (Δlac) and the cells plated on LB agar containing ampicillin (50 μg/ml) to select lysogens. These isolates were screened for those carrying a phage inserted into either the recB or recC gene by measuring their sensitivity to mitomycin C and UV light, followed by assaying for the presence of the RecBC DNase in cell extracts. Strains carrying recB mutations were distinguished from those carrying recC mutations by complementation with the plasmid-coded recB+ gene on pPE235 (Hickson and Emmerson, 1983). These isolates were plated on minimal agar containing 5-bromo-4-chloro-3-indolyl β-d-galactopyranoside (X-Gal) to determine whether the orientation of the phage was such that the lacZ structural gene was transcribed from the recB promoter. The strains formed either blue or white colonies reflecting a Lac+ or Lac− phenotype, respectively. The level of β-galactosidase production in these strains was measured by the method of Miller (1972).

Direction of Transcription of recB—The frequency of mobilization of the adjacent argA and thyA genes during F′ lac-mediated conjugation was studied to determine the direction of transcription of the recB gene. The donor was an Hfr strain carrying F lac integrated into the Mu phage in the recB gene.

Construction of pPE237 (recB+)—Plasmid pPE399 (Tomkinson, 1983), which carries the recB gene on a 7-kilobase Xhol fragment, was partially digested with SsoB3 until the majority of DNA was in the size range 3–4 kilobases and fragments ligated into the BamHI site downstream of Pι in the plasmid pPcL24 (Renaut et al., 1981). This DNA was used to transform AB2470 (recB21) (λc857) and transformants resistant to both ampicillin and mitomycin C (0.5 μg/ml) were selected at 30 °C. UV-resistant isolates were then screened for overproducing the RecB protein. This was achieved by transforming strain K12A1H1Δtrp, which carries the temperature-sensitive c857 repressor of Pι (Renaut et al., 1981), and growing transformants at 41 °C for up to 6 h before analysis of proteins by SDS–PAGE. One recombinant plasmid which directed the synthesis of elevated levels

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‡ The abbreviations used are: SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; Mops, 4-morpholinepropanesulfonic acid.
of the RecB protein was designated pPE237 and used as the starting point for the purification of RecB protein.

Preparation of a RecC Affinity Column—Construction of a column in which the RecC protein was covalently linked to an agarose gel matrix was essentially by the method of Formosa et al. (1983), except that "Affi-Gel 15" (Bio-Rad) was used because RecC protein has an acidic isoelectric point (pI approximately 5.5). To prepare a 2-ml column, 10 mg of RecC protein, previously dialyzed against 0.1 M NaHCO₃, pH 8.0, was agitated gently with 1.25 g of activated agarose matrix for 16 h at 4 °C. Any remaining active groups on the matrix were then blocked by the addition of 0.1 volume of 1 M ethanolamine HCl (pH 8.0). After stirring overnight for 4 h at 4 °C, the slurry was transferred to a column, washed extensively with coupling buffer, and equilibrated with column buffer.

Purification of RecB Protein—The strain K12ΔH1 Δtrp harboring pPE237 was grown to an adjusted OD₆0₀ of 0.8 in LB medium at 30 °C. Derepression of P₁ was achieved by the addition of an equal volume of LB medium, previously heated to 56 °C, followed by growth at 41 °C for 3 h with vigorous aeration. The cells were harvested and resuspended in 50 mM Tris·HCl, pH 8.0, 10% sucrose, and frozen at −20°C.

The frozen cell suspension was thawed slowly at 4 °C and adjusted to 0.2 mg/ml lysozyme, 10 mM 2-mercaptoethanol, 1 mM EDTA, and 200 mM NH₄Cl. After 30 min on ice, the suspension was sonicated in pulses for a total of 5 min at an amplification of 6 µ. The lysate was cleaned by centrifugation at 29,000 rpm for 1 h, made 0.2% in Polymin P, and held at 4 °C for 30 min. The precipitate of nucleic acid was removed by centrifugation at 10,000 rpm for 10 min, and solid (NH₄)₂SO₄ was added to the supernatant to 40% saturation. After 30 min at 4 °C, the precipitate was harvested, resuspended in Buffer A (20 mM Tris·HCl, pH 7.6, 0.5 mM EDTA, 10 mM 2-mercaptoethanol, and 10% glycerol), and diluted in the same buffer until the conductivity of the solution was the same as that of a 50 mM NH₄Cl solution. This sample was then loaded directly on a 30-ml heparin agarose column. The column was washed with a 30-bed volume of Buffer A containing 150 mM NH₄Cl, and proteins eluted with a 10-bed volume 150-750 mM NH₄Cl gradient. Fractions (8 ml) were assayed for the presence of RecB protein by SDS-PAGE. Peak fractions, eluting at approximately 450-500 mM NH₄Cl, were pooled and made 70% with solid (NH₄)₂SO₄. After 30 min at 4 °C, the precipitate was harvested and resuspended in 10 ml of Buffer A. After dialysis for 4 h against 2.5 liters of Buffer A containing 50 mM NH₄Cl, the sample was loaded onto the 2-ml RecC affinity column. The column was washed with buffer A containing 300 mM NH₄Cl and proteins eluted with a 300–1200 mM NH₄Cl gradient. RecB protein, assayed by SDS-PAGE, was the only protein to bind tightly under these conditions and eluted in a broad peak at 600-900 mM NH₄Cl. These fractions were concentrated by filtration through a CX30 immersible filter, adjusted to 50% glycerol, and stored at −20 °C in aliquots.

ATPase Assay—Reaction mixtures (50 µl) contained 5 mM Mops, pH 7.0, 10 mM MgCl₂, 1 mM dithiothreitol, 1 µg/ml nuclease-free bovine serum albumin, 50 µM ATP, 1 µCi of [³²P]ATP, and 1 µg of various DNA cofactors. Following the addition of RecB protein or RecC protein, the mixtures were incubated for 1 h at 37 °C after which 4-µl samples, to which carrier ATP and ADP had been added, were spotted on thin-layer chromatography sheets (Kieselgel 60 F₂₅₄, from Merck). ATP and ADP were separated by chromatography in Solvent A (10 parts propan-1-ol, 1 part methanol, 5 parts 5% NH₄OH, 2 parts water). The spots of ATP and ADP, visible under UV illumination, were cut out and their radioactivity determined by scintillation counting.

Exonuclease Assays—Exonuclease V activity was assayed by measuring the conversion of native λ DNA to acid-soluble nucleotides. In crude cell extracts, the assay was essentially as described by Barbour and Clark (1970).

Purified RecB and RecC proteins were assayed in a reaction mixture containing 5 mM Mops, pH 7.0, 10 mM MgCl₂, 1 mM dithiothreitol, 1 µg/ml nuclease-free bovine serum albumin, and 360 ng [³²P]DNA. The RecB and RecC proteins were added as indicated and the mixtures held on ice for 1 h. Reactions were initiated by the addition of ATP, as indicated, and samples incubated at 37 °C for 30–60 min. Aliquots were then removed into an equal volume of ice-cold 10% trichloroacetic acid to precipitate large oligonucleotides. After 10 min on ice, the samples were centrifuged for 5 min in an Eppendorf microfuge and the supernatant taken for scintillation counting.

One unit of RecBC DNase activity is that amount which releases 1 nmol of acid-soluble nucleotides in 30 min (Goldmark and Linn, 1972). The exonuclease activity is known to be optimal at about pH 9 (Goldmark and Linn, 1972). However, all exonuclease assays were carried out at pH 7.0 since this pH is more physiologically relevant and the results could be more readily compared with those of Goldmark and Linn (1972), who also worked at this pH.

RESULTS

Expression of the recB Gene—The level of expression of the recB gene was determined by measuring the amount of β-galactosidase synthesized in the strain PE117, which carries a Mud(bla lac) plasmid inserted into the recB gene in the appropriate orientation to permit transcription of the phage-coded lacZ gene from the recB promoter. For comparison, the strain GW1060 uvrA:Mud(bla lac) (Kenyon and Walker, 1980) was treated identically. The results (Fig. 1) show that the constitutive level of recB gene expression is approximately half that of the uvrA gene and comparable to that previously found for the recC gene (Hickson et al., 1984). Since the intracellular level of the UvrA protein has been estimated to be approximately 20 copies/cell there would appear to be about 10 copies each of the RecB and RecC proteins. This is consistent with the previous estimate of 10 molecules of the RecBC DNase/cell (Taylor and Smith, 1980).

The strains were also treated with DNA-damaging agents to determine the level of expression under conditions which induce the SOS response. Whereas a 4-fold induction of β-galactosidase activity was observed with GW1060 uvrA:Mud(bla lac) after treatment with 1 µg/ml of mitomycin C, there was no detectable induction in strain PE117 recB::Mud(bla lac) after treatment with mitomycin C (Fig. 1), UV or nalidixic acid (data not shown).
The possibility that RecB protein might act as a regulator of recB gene expression was investigated by measuring β-galactosidase synthesis in strain PE117 harboring a plasmid-coded recB gene. However, no change in expression was observed (data not shown).

**Direction of Transcription of recB—**Strains PE124 recB::Mud(bla lac) and PE117 recB::Mud(bla lac) have a Lac− and Lac+ phenotype, respectively, reflecting the direction of transcription of the phage within recB. The frequency of mobilization of the adjacent chromosomal markers argA and thyA during F′ lac-mediated conjugation (Table II) showed that thyA was mobilized at a much higher frequency than argA in PE124, while the opposite was true in PE117. This indicates that transcription of recB is toward argA, in the anticlockwise direction on the standard E. coli map, in agreement with the results of Sasaki et al. (1982) and Dykstra et al. (1984).

**Construction of pPE237 (recB+)—**Random Sau3A-generated fragments of pPE399 (Tomkinson, 1983) were ligated into the BamHI site downstream of PL in the expression plasmid pPLc24 (Remaut et al., 1981). This DNA was used to transform AB2470 (recB21)(λcI857), and transformants were selected in which the recB mutation was complemented. A λ lysogen was used to provide the cI857 repressor of PL. All plasmids carrying recB+ were tested for ability to direct the expression of the recB gene.
Purification procedure, protein samples were removed and prepared for SDS-PAGE. The proteins were separated on a 9% SDS-polyacrylamide gel and visualized by staining with Coomassie Blue. Tracks: a, b, and c, total cellular proteins of strain K12ΔH1Δtrp; d, 2-μg sample of the RecB protein peak from the RecC affinity column. A sample of the RecC protein used to prepare the affinity column is shown (track a). The molecular weights are those of proteins run in parallel: RNA polymerase (155,000), RecC (125,000), phosphorylase b (96,000), and bovine serum albumin (68,000). The position of RecB protein is indicated.

Synthesis of elevated levels of the RecB protein in strain K12ΔH1Δtrp, as described under “Experimental Procedures.” Strain K12ΔH1Δtrp carries a defective noninducible λ prophage which codes for the temperature-sensitive cI857 repressor. Repression is, therefore, lifted by temperature shift from 30 to 41 °C (Renaut et al., 1981). A plasmid, pPE237 (recB+), was isolated that directed the synthesis of elevated levels of RecB protein. This plasmid conferred wild-type levels of UV resistance (data not shown) and RecBC DNase activity (Fig. 2) on strain AB2470 (recB).

The plasmid pPE237 was used as the starting point for the purification of the RecB protein. To determine the optimal length of the thermal inducing treatment, strain K12ΔH1Δtrp harboring pPE237 was grown at 41 °C for up to 6 h before analysis of proteins by SDS-PAGE. The level of RecB protein was found to rise to a maximum after growth at 41 °C for 3 h (Fig. 3). Similar levels of RecB protein were observed for up to 6 h, after which general protein synthesis stopped. At maximal induction, RecB protein constituted about 1% of total cellular protein. Assuming a normal level of 10 molecules/cell (Taylor and Smith, 1980), this represents an approximately 1,500-fold amplification.

**Purification of RecB Protein**—Purification of RecB protein was monitored by SDS-PAGE, as shown in Fig. 4. Total cellular proteins from uninduced and induced K12ΔH1Δtrp harboring pPE237 are shown (tracks b and c, respectively).

The recovery of RecB protein from cell lysates was found to be very low when lysozyme alone was used for cell lysis, as described for the purification of the RecBC DNase (Eichler and Lehman, 1977). Better yields were obtained when the cells were lysed by sonication and when 0.2 M NH₄Cl was added to eliminate binding of RecB protein to cellular DNA. The cleared lysate was treated with Polymyx P to remove nucleic acids which were otherwise found to interfere with subsequent chromatographic steps.

Following (NH₄)₂SO₄ precipitation (track d), the proteins were applied to a heparin agarose column to which RecB protein bound very tightly and was eluted at approximately 0.5 M NH₄Cl (track e), unlike the RecBC DNase which eluted at salt concentrations around 0.2 M.

The RecB protein peak from the heparin agarose column was further chromatographed on a RecC affinity column (track f). A sample of the RecC protein used to prepare the affinity column is shown (track a). RecB protein was the only protein to bind tightly to this column in the presence of low salt concentrations. Following elution with up to 1 M NH₄Cl, the RecB protein appeared to be homogeneous and electrophotothetically pure. Approximately 1.5 mg of RecB protein was purified from 70 g of cells.

**Hydrolysis of ATP by RecB Protein**—The purified RecB protein was assayed for ability to hydrolyze ATP to ADP and Pi, in the presence and absence of DNA. The results (Table III) show that RecB protein has an ATPase activity that is strongly dependent on DNA. Heat-denatured calf thymus DNA is the best cofactor for the reaction, while native calf thymus DNA is approximately half as effective. Although covalently closed circular plasmid DNA is a very poor cofactor, single-stranded circular M13 DNA is effective. The reaction also has an absolute requirement for the divalent cations Mg²⁺ or Ca²⁺. As previously reported (Hickson et al., 1984), RecC protein does not have ATPase activity (Table III) under these conditions.

**DNase Activity of Combined RecB and RecC Proteins**—The RecBC enzyme has a number of DNase activities which are either stimulated by or completely dependent upon concomitant hydrolysis of ATP. The ability of either of the individual subunits of the RecBC enzyme or of the combined proteins to degrade native phage λ DNA was determined in the presence and absence of ATP. The results (Fig. 5) show that while neither RecB nor RecC protein alone degrades double-stranded λ DNA the combined proteins do. This activity of the apparently reconstituted RecBC DNase requires prein-
cubation of the RecB and RecC subunits together at presence of ATP. The specific activity of the reconstituted both the RecB and RecC proteins were present in all cases, and the was further studied by repeating the assay with different DNA. Assays were performed as described for Fig. 6, ATP concentration was varied as indicated. aliquots were removed and trichloroacetic acid-soluble radioactivity determined.

Fig. 5. DNase activity. The assay mixtures (60 µl) were prepared as described under "Experimental Procedures." To this mixture were added 4 ng of RecB protein (■), 10 ng of RecC protein (▲), or both proteins (●). After 1 h on ice, reactions were initiated by the addition of ATP to 100 mM and the samples incubated at 37 °C. At the times indicated, aliquots were removed and trichloroacetic acid-soluble radioactivity determined.

FIG. 6. Effect of ATP concentration on hydrolysis of duplex DNA. Assays were performed as described for Fig. 5, except that both the RecB and RecC proteins were present in all cases, and the ATP concentration was varied as indicated.

The dependence of the DNase activity upon ATP hydrolysis was further studied by repeating the assay with different concentrations of ATP. The DNase activity of the reconstituted enzyme is completely dependent upon ATP (Fig. 6), with an optimum concentration of about 100 µM. At concentrations of 500 µM or above the activity is progressively inhibited and is reduced to background levels in the presence of 5 mM ATP. This inhibition is somewhat higher than that observed by Eichler and Lehman (1977) with the native enzyme.

DISCUSSION

Studies with strains in which a Mud interrupter (bla lac) phage is fused to the recB promoter show that the constitutive level of recB transcription is very low and comparable with the level of expression of the recC gene (Hickson et al., 1984). These levels are consistent with the estimate of only 10 molecules of the RecBC DNase/cell, assuming efficient association of the RecB and RecC subunits. Moreover, in contrast to uraA gene expression, which was studied for comparison, the level of expression of recB is not increased following damage to cellular DNA. This suggests that, unlike several other gene products involved with DNA repair and genetic recombination (reviewed by Little and Mount (1982)), the RecBC DNase is not induced as part of the SOS response. This view is supported by the absence of a LexA protein-binding site in the nucleotide sequence of the recB control region.3

Because of its very low intracellular level, the lack of inducibility by DNA damage, and because no assay has been available for it, purification of the RecB protein has hitherto been extremely difficult. The cloning and genetic amplification of the recB gene have permitted the use of SDS-PAGE as an assay for the RecB protein. The strain K12ΔH1Δtrp (Remaut et al., 1981) harboring the plasmid pPE237, in which transcription of recB is linked to λ P3, is an excellent source of RecB protein. Despite the availability of this RecB over-producing strain, some difficulties were encountered in developing a simple purification procedure for the RecB protein, partly because a number of other DNA-binding proteins co-purified with the RecB protein through several different chromatographic steps. The best purification procedure proved to be affinity chromatography which capitalized on the availability of purified RecC protein and on the apparently highly specific interaction between the RecB and RecC proteins. Electrophoretically pure RecB protein was prepared relatively easily using a column of RecC protein covalently bound to agarose. The highly specific interaction between enzyme subunits such as the RecB and RecC proteins can thus be exploited in their purification.

The ATPase activity of the RecBC DNase appears to reside in the RecB subunit since purified RecB protein but not RecC protein has this activity. In contrast to the activity of other DNA-dependent ATPases, such as the UvrD (helicase II) (Abdel-Monem et al., 1977) and RecA proteins (Ogawa et al., 1975), the ATPase activity of the RecB protein is effectively stimulated by both double-stranded and single-stranded DNA. This is similar to the ATPase activity of the RecBC DNase itself except that double-stranded DNA is the best cofactor for the RecBC DNase while single-stranded DNA is most effective for the RecB protein. That the ATPase activity of the RecBC DNase resides in the RecB subunit is supported by DNA sequence analysis of the recB gene. Walker et al. (1982) have shown that several adenine binding enzymes, such as ATPases, have one and sometimes two conserved amino acid sequences which contribute to the nucleotide binding. In experiments to be published elsewhere,4 we have determined the entire nucleotide sequence of the recB gene. The deduced amino acid sequence appears to include two regions similar to the proposed binding sites found in ATP synthase, myosin, adenylate kinase, phosphofructokinase, the E. coli RecA protein (Walker et al., 1982), and also in the E.

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Reconstituted RecBC Enzyme

coli UvrD protein (Finch and Emmerson, 1984).

Substantial RecBC DNase activity can be reconstituted from the individually purified RecB and RecC subunits. The specific activity of the reconstituted enzyme, 24,500, is about one-half of the 57,000 reported by Goldmark and Linn (1972) for the native enzyme when assayed at the same pH, 7.0. It is about 9-fold lower than the 218,000 reported by Eichler and Lehman (1977), a value obtained at pH 8.5, where the exonuclease activity is known to be higher. However, these figures are not strictly comparable since experimental conditions and the source of the substrate DNA were different. Whereas we used phage λ DNA in our assay, Goldmark and Linn (1972) used E. coli DNA and Eichler and Lehman (1977) used phage T7 DNA.

Further work will be needed to compare the activity of the reconstituted enzyme with that of the native enzyme under identical conditions and to determine the optimal conditions for the reconstitution process. However, the observation that considerable activity can be reconstituted from the RecB and RecC subunits alone suggests that no other catalytic subunits, such as the 60-kDa protein reported by Lieberman and Oishi (1974), are required in stoichiometric amounts for double-strand exonuclease activity of the RecBC DNase.

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