Biochemical and Genetic Studies of Recombination Proficiency in *Escherichia coli*, I. Enzymatic Activity Associated with *recB*⁺ and *recC*⁺ Genes*

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Abstract. An adenosine triphosphate-dependent deoxyribonuclease activity has been detected in lysates of *recB*⁺ *recC*⁺ strains. Mutations in *recB* or *recC* lead to loss of this activity, suggesting that these two genes determine the nuclease activity. The over-all reaction in crude lysates digests native DNA to nucleoside monophosphates. Complementation between *recB21* and *recC22* *in vivo* leads to normal levels of ATP-dependent nuclease activity. No complementation *in vitro* has been detected. Mutations in a third recombination gene (*recA*) do not alter significantly the wild-type levels of this nuclease activity.

Introduction. Recombination-deficient (*Rec*⁻) mutants of *E. coli* have been isolated in several laboratories.¹ These mutants are defective in their ability to produce genetic recombinants by conjugation or transduction. A complementation analysis of the recessive *rec* mutations reveals the existence of three genes or cistrons: *recA*, *recB*, and *recC*. *recB* and *recC* lie between *argA* and *thyA*² on the standard genetic map of *E. coli*.³ A mutation in either of these two *rec* genes leads to a similar mutant phenotype; namely, reduced but detectable recombination,⁴ increased sensitivity to ultraviolet (UV) light, and reduced breakdown of DNA following UV irradiation.⁵ *recA* mutations, on the other hand, are located between *pheA* and *cysC*⁶ on the *E. coli* map. These mutations result in a drastic reduction in recombination, high sensitivity to UV irradiation, and an increased amount of breakdown following exposure to UV. It appears from these results both *recB*⁺ and *recC*⁺ might determine a nuclease activity responsible for much of the DNA breakdown observed in wild-type cells following UV irradiation. Should the *recA*⁺ product act to reduce the breakdown catalyzed by this hypothetical *recB*⁺ *recC*⁺ nuclease, then the major part of the breakdown observed in *recA*⁻ mutants might be prevented by inactivating the nuclease. This was done by constructing double *rec*⁻ mutants.^{7, 8} Both *recA*⁻ *recB*⁻ *recC*⁺ and *recA*⁻ *recB*⁺ *recC*⁻ mutants showed lower levels of breakdown than both *recA*⁻ *recB*⁺ *recC*⁺ and wild-type *Rec*⁺ strains.

This report confirms the hypothesis that the products of the *recB*⁺ and *recC*⁺ genes are responsible for a nuclease activity associated with genetic recombination. It also establishes a biochemical basis for the similarity in phenotype of *recB*⁺ *recC*⁻ and *recB*⁻ *recC*⁺ mutants. Apparently we have independently found the ATP-dependent nuclease activity described by Buttin and Wright⁸ as "likely to be the one affected by the *rec21* mutation."

Materials and Methods. Strains: All bacterial strains used are described in Table 1.

Media: M9C medium contained 1 gm NH_4Cl , 11 gm $\text{Na}_2\text{HPO}_4\cdot\text{H}_2\text{O}$, 3 gm KH_2PO_4 , 5 gm NaCl , 4 gm glucose, 120 mg MgSO_4 , 10 mg CaCl_2 , and water to 1 liter. Enriched M9C (EM9C) consisted of M9C with 2.5 gm casamino acids and 0.1 mg thiamine added per liter.

TABLE 1. *ATP-dependent nuclease activity detected in crude lysates of various strains.*

Strains*	F ₁₅ Markers			Chromosomal Markers				Units of nuclease activity in 0.1 ml crude lysate†
	<i>thy</i>	<i>recC</i>	<i>recB</i>	<i>thy</i>	<i>recC</i>	<i>recB</i>	<i>recA</i>	
1 HF4733				—	+	+	+	13
2 JC4583‡				+	+	+	+	12
3 JC4585§				+	22	+	+	0.3
4 JC6722				+	+	21	+	0.2
5 JC6720				+	22	21	+	0.1
6 JC4588				+	+	+	56	10
7 JC2284				+	+	+	67	11
8 JC6726				+	+	+	+	8.2
9 JC6727				+	+	+	+	3.8
10 JC4594	+	22	+	+	22	+	+	0.3
11 JC4596	+	22	+	+	+	21	+	12
12 JC4590	+	22	+	+	+	+	+	12
13 JC4595	+	+	21	+	+	21	+	0.3
14 JC4589	+	+	21	+	+	+	+	12
15 JC4591	+	22	21	+	+	21	+	0.3
16 JC4597	+	+	+	+	+	+	67	20

* HF4733 was the parent of all the isogenic strains listed. It is F^- , gal^- , B^- , Sm^s and endonuclease I deficient.¹⁷ The first seven *thy*⁺ derivatives were obtained by P1 transduction using *Rec*⁺ or *Rec*[−] strains as donors.² The *thy*⁺ *recA*[−] derivatives were made by conjugation with *recA*[−] Hfr donor strains.¹ The *F'* derivatives were constructed by conjugation using the *F'* donors described by Willetts and Mount² and the *F*[−] strains derived from HF4733.

† Enzyme activity determined and crude lysates made as described in *Materials and Methods*. The lysates were made from unirradiated cells and were assayed with native *E. coli* DNA in the presence of ATP (2×10^{-4} M).

‡ The same results were obtained with JC6724 and JC2286 (independent isogenic strains).

§ The same results were obtained with JC6721 (independent isogenic strains).

Materials: Carrier-free $\text{H}_3^{32}\text{PO}_4$ was obtained from Mallinkrodt Chemical Co.; thymidine-methyl- H^3 (15 Ci/mole), from International Chemical and Nuclear Corp.; bacterial alkaline phosphatase (chromatographically pure) from Worthington Chemical Co.; adenosine 5'-triphosphate disodium salt, from Schwarz BioResearch; and Brij-58, a nonionic detergent, from Atlas Chemical Industries. Other reagents were analytical grade. A sample of purified 5'-mononucleotidase was the gift of Doctor Stuart Linn.

^{32}P -labeled *E. coli* DNA was prepared by the method described by Lehman.⁹ $\text{H}_3^{32}\text{PO}_4$ was added at 1–2 mCi/liter of culture. In the purified preparations of DNA, acid-soluble radioactivity accounted for less than 0.1% of the total, and alkali-labile (presumably RNA) polymeric material accounted for less than 0.5% of the total radioactivity. Specific activity of freshly prepared ^{32}P preparations was from 15,000 to 30,000 cpm/ μmole .

Scintillation fluid was made according to the formula listed by Clark *et al.*¹⁰ Generally, 0.2 ml of a 5% trichloroacetic acid solution was added to 10 ml of this solution for radioactivity determination.

Preparation of lysates and supernatant fractions: Lysates were prepared using the method described by Godson and Sinsheimer.¹¹ Cells grown in EM9 were harvested in log phase at $3\text{--}5 \times 10^8$ bacteria per milliliter, from 400 ml of culture and resuspended in 5 ml of 0.85% saline. The bacteria were again harvested and resuspended

in 4 ml of chilled Tris-sucrose (0.01 *M* Tris, pH 8, 25% sucrose). The suspension of cells was then added to 0.42 ml lysozyme (1 mg/ml in 0.01 *M* Tris, pH 8) and 0.32 ml ethylenediaminetetraacetate (EDTA) (0.1 *M*, pH 7.5–8). After mixing well this suspension was placed on ice for 1 to 2 min. Then to this suspension were added 5 ml of cold 0.10 *M* MgSO₄ (50 mM final concentration) and 1 ml of 5% Brij-58 (in 0.01 *M* Tris, pH 7.2). After mixing well the suspension was stored at 4° with occasional shaking for 1 hr. This material was used as the crude lysate. The particulate fraction could be removed by sedimentation at 12,000 rpm for 15 min in the SS-34 rotor of the Sorvall RC2-B centrifuge, at 4°. The clear, colorless supernatant was either used directly in experiments or stored frozen at –40°.

Deoxyribonuclease assay: The reaction mixture (total volume 0.5 ml) contained ³²P-labeled native *E. coli* DNA, 30 μ moles of DNA phosphorus; 0.01 *M* Tris-HCl buffer, pH 8.0; 10 mM MgSO₄ (usually not necessary to add separately since sufficient MgSO₄ was present in the Brij lysates); adenosine triphosphate (ATP) to a final concentration of 0.2 mM; and cell lysates: usually 0.1 ml of the supernatant fraction, or of the crude lysate; water to final volume. The specific activity of added DNA was often 3000–5000 cpm/ μ mole. This assay measured the conversion of native DNA to acid-soluble products. The incubation was carried out at 37° for 10 min and then a sample was added to trichloroacetic acid. Often measurements were also made at 0 and 5 min of the incubation. Samples were taken into cold trichloroacetic acid (5% final concentration) and yeast nucleic acid was added last as carrier (500 μ g/ml final concentration). After at least 10 min on ice, the samples were centrifuged at 4° for 10 min, at 12,000 rpm in the SS-34 rotor of the Sorvall RC2-B centrifuge. Two-tenths milliliter of the supernatant solution was added to 10 ml of scintillation fluid, shaken well, and counted in a Nuclear Chicago Mark I liquid scintillation counter, at ³²P settings. The trichloroacetic acid-insoluble radioactivity was determined by resuspending the pellet and heating the solution at 95° for 30 min, centrifuging to remove cell debris, and then counting an aliquot of the supernatant solution as described above. In this way, solubilized DNA can be expressed as a percentage of total DNA nucleotide. Data obtained were corrected by running parallel control incubations lacking enzyme. A unit of nuclease activity is defined as the amount catalyzing the production of 1 μ mole of acid-soluble PO₄^{3–} in 10 min at 37°. In certain experiments we wished to measure DNA breakdown by extracts, of endogenous prelabeled ³H DNA (preparation above). In this case we added only ATP (0.5 mM final concentration) to crude Brij cell lysates, and incubated at 37° with periodic sampling and analysis as above. Alkaline phosphatase and 5'-nucleotidase sensitivities of the products of the nuclease reaction were measured by determining the amount of ³²P liberated from Norit adsorbable products.¹²

Results. Endogenous DNA breakdown in lysates of recombination-deficient strains: After treatment with lysozyme, the neutral detergent Brij-58 was used to rupture cells gently.¹¹ To keep the concentration of nucleoside triphosphates at a high level, a mixture of the four ribonucleoside triphosphates was added to the lysate. Under these conditions labeled endogenous DNA was broken down as rapidly in lysates from both *recA*⁺ and *recA*[–] strains, and treatment of the cells with UV prior to lysis resulted in essentially no stimulation of the rate of breakdown in the lysate. However, lysates of *recB*[–] *recC*⁺, *recB*⁺ *recC*[–], or *recB*[–] *recC*[–] cells, under the same conditions, showed little breakdown of the endogenous-labeled DNA. Once again UV irradiation did not stimulate the rate of breakdown of the endogenous-labeled DNA. Illustrative DNA breakdown curves for *recA*⁺ *recB*⁺ *recC*⁺ and *recA*⁺ *recB*⁺ *recC*[–] lysates are presented in Figure 1. Significant DNA breakdown in these lysates was dependent upon the addition of nucleoside triphosphates. When the lysate of a labeled *recB*[–] *recC*⁺ mutant strain (see legend to Fig. 1) is mixed with a lysate from an unlabeled

wild-type *recB*⁺ *recC*⁺ and nucleoside triphosphates, the labeled DNA is degraded. This indicates that an inhibitor of degradation is not present in the mutant extracts and that the mutant DNA is subject to degradation. It also indicates that the nuclease activity acts on exogenous DNA.

An assay for the *recB*⁺ *recC*⁺ nuclease activity with exogenous DNA: Further studies were performed with an assay which measured over a short time interval (10 min) the release of trichloroacetic acid soluble material from native, purified, radioactive DNA. The digestion of labeled *E. coli* DNA and T7 DNA was quantitatively identical and most of the experiments were performed with ³²P-labeled *E. coli* DNA. Entries 1-7 in Table 1 show that *recB*⁻ and *recC*⁻ strains lack a nuclease activity which is present in *rec*⁺ or *recA*⁻ strains. These assays contained ATP at 2×10^{-4} M. The effect of *recB*⁻ and *recC*⁻ and the requirement for ATP are illustrated in Figure 2 where the rate of DNA breakdown is

FIG. 1.—Endogenous DNA breakdown in lysates. A mixture of the four ribonucleoside triphosphates each at a final concentration of 1 mM is added to the lysates at the beginning of the incubation. The dose of UV irradiation was 570 erg/mm², applied to the cells before lysis, where noted. DNA was labeled with deoxyribothymidine (methyl-³H) as previously described.¹⁰ Strains: JC2286 is *recA*⁺ *recB*⁺ *recC*⁺; JC6721 is *recA*⁺ *recB*⁺ *recC*⁻. TCA, trichloroacetic acid.

FIG. 2.—DNase activity of lysates of *Rec*⁺ and several *Rec*⁻ strains on added purified native ³²P *E. coli* DNA. 0.1 ml of each lysate is assayed with 30 μ moles of added ³²P DNA substrate in a total volume of 0.5 ml. Where ATP is present it is 2×10^{-4} M final concentration. The open circles indicate the highest levels of activity for the assays indicated. Details are in *Materials and Methods*. Strains: *rec*⁺ = JC6724; *recB*⁻ *recC*⁻ = JC6720; *recC*⁻ *recB*⁺ = JC6721; *recC*⁺ *recB*⁻ = JC6722.

measured over a 10-min interval with lysates of several strains. The lysate of the *rec*⁺ strain shows active digestion of the DNA only in the presence of added ATP. Lysates of *recB*⁻ *recC*⁺, *recB*⁺ *recC*⁻, and *recB*⁻ *recC*⁻ strains produce

very little digestion of the DNA with or without added ATP. The activity of *rec*⁺ extracts in the absence of ATP is very low (<0.5 unit). With 2×10^{-4} M ATP alone, 11 units of activity were detectable in 0.1 ml of *rec*⁺ lysate. Lower activity was detected when the ATP concentration was raised to 10^{-3} M (9.2 units per ml lysate) or when several other ribonucleotide triphosphates (CTP, UTP, and GTP or these three plus ATP and MnCl₂) were added each at a concentration of 10^{-3} M (7.2 and 5.2 units/ml lysate). We have routinely added ATP only at a final concentration of 2×10^{-4} M.

The ATP-dependent DNase also requires magnesium for activity which reaches a maximum at 20 mM of magnesium sulfate. Above this concentration additional magnesium inhibits the reaction; no activity remains above 200 mM magnesium sulfate.

Nature of the ATP-dependent nuclease activity: The data in Table 2 indicate that the nuclease activity on denatured DNA of a lysate from a *recB*⁺ *recC*⁺ strain was not stimulated by ATP and there was little difference between lysates from *recB*⁺ *recC*⁺ and *recB*⁻ *recC*⁻ strains. ATP stimulation and the difference in nuclease activity between these *rec*⁺ and *rec*⁻ strains were observed only on native DNA substrate. The nature of the trichloroacetic acid-soluble products of the over-all ATP-dependent nuclease reaction on native DNA was examined by measuring their sensitivity to several enzymes. Virtually all of the trichloroacetic acid-soluble products were found to be Norit adsorbable. After alkaline phosphatase treatment, 80 per cent and, after 5'-nucleotidase treatment, 70 per cent of the acid-soluble ³²P-labeled nuclease products were converted to Norit nonadsorbable ³²P. This occurred similarly in samples taken after one, two, and five minutes of nuclease digestion. It appears from these data that the over-all ATP-dependent activity in the lysate is primarily exonucleolytic, acting on double-stranded DNA, and producing primarily 5'-mononucleotides.

TABLE 2. Nuclease activity in crude extracts on native and denatured DNA.

	Units of Activity—Standard Assay—			
	Denatured DNA*		Native DNA	
	-ATP	+ATP	-ATP	+ATP
JC6724 (<i>recB</i> ⁺ <i>recC</i> ⁺)	13	12	0.3	10
JC6720 (<i>recB</i> ⁻ <i>recC</i> ⁻)	11	10	0.3	0.1

* Denatured DNA was prepared by heating a sample of native ³²P *E. coli* DNA (in 0.01 M Tris, pH 8, containing 10^{-3} M EDTA) at 100° for 10 min and then rapidly chilling the sample in an ice bath.

Identity of *recB*⁺ and *recC*⁺ genes as the determinants of the nuclease activity: To prove that those strains which are Rec⁻ because of mutations in the *recB* or *recC* genes are nuclease deficient for the same reason requires some genetic test. First we isolated a Thy⁻ (thymine-requiring) derivative of a *recC*⁻ strain (JC6721) and then cotransduced *thy*⁺ and *recC*⁺ from an appropriate donor. Two recombinants inheriting both donor markers both contain significant levels of ATP-dependent nuclease activity in lysates (Table 1, entries 8 and 9).

Merodiploid strains carrying *recB* and *recC* mutations in the *trans* position can be constructed in order to test for complementation between the mutations.

Willetts and Mount² have shown that strains with the genotype F *recB*⁺ *recC22*/*recB21 recC*⁺ or F *recB21 recC*⁺/*recB*⁺ *recC22* are phenotypically recombination proficient and resistant to UV irradiation. Willetts and Mount also have shown that the *recB21* and *recC22* mutations are recessive to their respective wild-type alleles, and that homozygotes for each mutation are recombination deficient and sensitive to UV irradiation. All of these properties must be reflected in the ATP-dependent nuclease content of these strains if *recB* and *recC* determine this nuclease activity. We have constructed several counterparts of the strains tested by Willetts and Mount (Table 1, entries 10–15). It is seen that F' strains homozygous for *recB21* or *recC22* have negligible ATP-dependent nuclease activity (Table 1, entries 13, 15, and 10). Those F' strains heterozygous for either *recB21* or *recC22* have wild-type levels of ATP-dependent nuclease activity as does that strain heterozygous for both mutations (Table 1, entries 14, 12, and 11, respectively). These results are consistent with the recessivity of *recB21* and *recC22* and the determination by *recB*⁺ and *recC*⁺ of the ATP-dependent nuclease activity measured here. Gene dosage of the nuclease in a strain diploid for *recB*⁺ *recC*⁺ is also shown in Table 1 (entries 7 and 16).

Absence of complementation *in vitro*: The previous data indicate that complementation between the *recB*⁺ and *recC*⁺ genes occurs *in vivo*. We tested whether the same interaction would occur *in vitro*. A mixture of the lysates from the *recB*[−] *recC*⁺ and *recB*⁺ *recC*[−] strains (JC6722 and JC6721, respectively) showed no increase in nuclease activity over that exhibited by each extract alone. Thus far we have not seen *in vitro* complementation between *recB21 recC*⁺ and *recB*⁺ *recC22* extracts.

Fractionation of *rec*⁺ strain lysate: We have noted that the ATP-nuclease activity is found in the supernatant fraction of the lysate. Separation from the lysate of the cell envelopes with their contents of protein and high molecular weight nucleic acid was performed by centrifugation at 12,000 × *g* for 15 minutes¹¹ and resulted in a threefold purification of the activity. The activity in the supernatant fraction is destroyed by heating at 50° for 15 minutes; 86 per cent is lost by this treatment. The dependence of activity (in 10 min) upon supernatant extract concentration is linear over the range of 0.1 to 0.4 ml extract.

Discussion. The enzymology of genetic recombination has received much attention in recent years mainly from the point of view of ascertaining whether known enzymes of DNA metabolism are present in recombination-deficient mutants.^{10, 13, 14} In the case of cells infected with lambda bacteriophage mutants this method has led to the identification of the lambda exonuclease as one of the proteins involved in the recombination of vegetative λ DNA.¹⁵

Renewed efforts to determine the enzymes involved in *E. coli* recombination have led to the discovery of new nucleases, enzymes including "the 'nucleoside-di- or triphosphates-dependent DNase' which appears to represent in *E. coli* extracts the major activity stimulatable by XTP's (and) is very likely to be the one affected by the *rec21*[−] mutation."⁸ Working on an independent but similar line of research we have confirmed this speculation, as have Buttin and Wright themselves.¹⁶ In addition we have shown that each of the *recB21* and *recC22* mutations results in the same loss in ATP-dependent nuclease activity and examina-

tion of the double mutant (*recB21 recC22*) shows no additional decrease in activity. The possibility that *recB21* and *recC22* are mutations in one gene has been discussed and rejected by Willetts and Mount² largely on the basis of apparent distance of each from *thyA*. It appears that the two genes cooperate to determine the single nuclease activity since no complementation *in vitro* was observed. Hence the hypothesis that the nuclease activity is a multisubunit protein consisting of nonidentical polypeptides is reasonable.

Note added in proof: After this paper was submitted, a paper by M. Oishi (these PROCEEDINGS, 64, 1292 (1969)) has reported partial purification and studies of the same enzyme. Where overlap occurred, his conclusions were similar to our own.

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¹ Clark, A. J., *J. Cell. Physiol.*, **70**, Suppl. 1, 165 (1967).

² Willetts, N. S., and D. W. Mount, *J. Bacteriol.*, **100**, 923 (1969).

³ Taylor, A. L., and C. D. Trotter, *Bacteriol. Rev.*, **31**, 332 (1967).

⁴ Low, B., these PROCEEDINGS, **60**, 160 (1968).

⁵ Howard-Flanders, P., and R. P. Boyce, *Radiation Res.*, Suppl., **6**, 156 (1966).

⁶ Willetts, N. S., A. J. Clark, and B. Low, *J. Bacteriol.*, **97**, 244 (1969).

⁷ Willetts, N. S., and A. J. Clark, *J. Bacteriol.*, **100**, 231 (1969).

⁸ Buttin, G., and M. Wright, *Cold Spring Harbor Symposia on Quantitative Biology*, vol. 33 (1968), p. 259.

⁹ Lehman, I. R., *J. Biol. Chem.*, **235**, 1479 (1960).

¹⁰ Clark, A. J., M. Chamberlin, R. P. Boyce, and P. Howard-Flanders, *J. Mol. Biol.*, **19**, 442 (1966).

¹¹ Godson, G. N., and R. L. Sinsheimer, *Biochim. Biophys. Acta*, **149**, 476 (1967).

¹² Lehman, I. R., G. G. Roussos, and E. A. Pratt, *J. Biol. Chem.*, **237**, 819 (1962).

¹³ Gellert, M., these PROCEEDINGS, **57**, 148 (1967).

¹⁴ Gefter, M. L., A. Becker, and J. Hurwitz, these PROCEEDINGS, **58**, 240 (1967).

¹⁵ Signer, E., H. Echols, J. Weil, C. Radding, M. Shulman, L. Moore, and K. Manly, *Cold Spring Harbor Symposia on Quantitative Biology*, vol. 33 (1968), p. 711.

¹⁶ Wright, M., and G. Buttin, *Bull. Soc. Chem. Biol.*, in press.

¹⁷ Dürwald, H., and H. Hoffmann-Berling, *J. Mol. Biol.*, **34**, 331 (1968).