AN ENDODEOXYRIBONUCLEASE INDUCED IN E. COLI BY INFECTION WITH λPBIO TRANSDUCING PHAGES

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Summary. Infection of E. coli by λpbio transducing phages leads to the production of a DNA endonucleolytic activity. The activity has not been detected in uninfected cells, nor in cells infected with λ phages that do not carry the bio-containing, bacterial DNA segments. The endonuclease has been purified extensively. The purified enzyme catalyzes the production of double-strand breaks in DNA molecules.

Genetic recombination in bacterial viruses occurs by the breakage and rejoining of DNA molecules (1). In the case of phage λ, recombinational processes are under the control of a series of genes linked on the so-called non-essential region of the viral chromosome (reviewed by E.R. Signer (2)). In attempts to demonstrate an enzymatic breakage of DNA under the control of this region, we compared the endonucleolytic activities of extracts of E. coli infected with phage λ, and with λ particles bearing deletions in this recombination-subserving segment of the phage chromosome. The deletion mutants of λ used were the λpbio's - a set of biotin transducing λ phages in which contiguous λ genes, beginning at the prophage attachment site and proceeding to the right, are replaced by bacterial markers (3). Unexpectedly, a potent endonuclease was encountered in extracts of E. coli infected with the λpbio's rather than with λ phages that carry the genes controlling recombination. This communication describes the purification and certain properties of this enzyme.

Materials and Methods

Strain 1100 is the endonuclease I-deficient isolate of Durwald and Hoffman-Berling (4). Other E. coli K strains were obtained from Drs. C. Fuerst and B. Rolfe. Most of the λpbio phages were the gift of Dr.E.R. Signer. Phage $\lambda int_6 red_3$ was the

gift of Dr. M. Yarmolinsky (5). Other λ strains were obtained from Drs. Fuerst, M. Pearson, and M. Gold.

High yields of λ for DNA isolation were obtained by thermal induction of $\lambda cI_{857}S_7$ (6) from its prophage state in strain Y_{10-1}. Other phages were prepared by lytic infection of strain Y_{10-1}. Radioactive labeling of phage DNA was carried out during lytic growth in L.B. media. Lambda DNA, uniformly labeled with ^{14}C or ^{3}H-thymidine, was prepared by the method of Thomas and Abelson (7) and stored in the cold in 0.2 M NaCl, 0.02 M Tris pH 7.5, and 0.002 M EDTA.

Phage-infected cells were prepared by growing bacteria in L.B. to 2-3 x 10^8 cells per ml, shaking in flasks at 37°C. Magnesium sulfate was added to 0.02 M. Phage were then added at a multiplicity of infection of 5 and, after 10 min at room temperature without shaking, the flasks were agitated vigorously for another 20 min at 37°. The media were then cooled rapidly in ice-water, the infected cells harvested by centrifugation, and the cell paste frozen at -20°.

Protein fractions prepared from these cells were assayed for the production of double-strand breaks in λ DNA. Incubation mixtures contained, in 0.1 ml, 20 mμmoles of ^{14}C-labeled λ DNA (approx. 500 cpm/mμmole), Tris pH 7.0 (50 mM), $MgCl_2$ (10 mM), dithiothreitol (10 mM), and protein. In the early stages of purification, sRNA (50 μg) was added to inhibit endonuclease I (8). Incubation was at 30° for 30 min and reaction was terminated by adding EDTA (50 mM). Tritium-labeled λ DNA was then added to serve as marker. The mixtures were layered on 5 to 20% sucrose gradients in polyallomer tubes of a Beckman SW 56 rotor. The sucrose solutions contained 1.0 M NaCl, 0.05 M Tris pH 8.0, and 0.01 M EDTA (neutral sucrose). DNA was sedimented at 54,000 rpm for 180 min at 5° and the gradients were fractionated by collecting 0.14 ml samples into scintillation vials with 0.5 ml water and 5 ml Aquasol (New England Nuclear) for counting. Activities of ^{14}C and ^{3}H were calculated and plotted with the aid of a computer. The assay scores for the percent of substrate displaced to smaller molecular weight products from the distribution in the gradient of unreacted marker DNA. Under conditions where less than 25% of the substrate molecules are displaced in this manner, one unit of activity is the amount catalyzing the breakage of 10% of the

substrate molecules under standard conditions.

During purification, when larger numbers of protein fractions were to be assayed, ^{14}C and ^{3}H-labeled DNA's were alternated as substrate and consecutive pairs of reaction mixtures were pooled for sedimentation analysis. In this manner, the number of fractions that could be analyzed per centrifugation was doubled. The purification of the endonuclease is summarized in Table I. In studies on the action of the enzyme, the most highly purified fraction was used.

Table I

Summary of purification of endonuclease from $\lambda pbio_{10}$-infected E. coli 1100

Fraction	Total units ($\times 10^{-3}$)	Protein (mg/ml)	Specific activity (units/mg protein)
1. Alumina extract	20	30	14.8
2. Streptomycin	-	11	-
3. Ammonium sulfate	21	55	95
4. Alumina Cγ	19.8	19	200
5. DEAE concentrate	12.3	4	1025
6. Phosphocellulose concentrate	3	0.15	19800

All procedures were carried out at 0-4°C. Buffer A is 0.05 M Tris pH 7.5, 0.005 M 2-mercaptoethanol, 0.001 M EDTA. Buffer B is 0.005 M K-phosphate buffer pH 6.5 containing mercaptoethanol and EDTA as in buffer A and 20% (v/v) glycerol. Freshly frozen cells (15 g) were ground with alumina to a smooth paste and the paste was extracted with buffer A. Alumina was removed by centrifugation. The extract (45 ml, OD_{260}: 250 nm) was precipitated by the addition of 0.25 volumes of 5% streptomycin sulfate. The precipitate was discarded after centrifugation and the supernate was fractionated by precipitation with solid ammonium sulfate (AS). Virtually all the activity was precipitated between 30 -55% saturation with AS and the precipitate obtained, collected by brief centrifugation at 100,000 x g, was dissolved in 4 ml of buffer A. The AS fraction was dialyzed extensively against 200 volumes of buffer A. To 4.2 ml of this dialyzate were added 2.1 ml of alumina Cγ gel (50 mg solids per ml) and, after 10 min of gentle mixing, the suspension was centrifuged and the solids discarded. The alumina Cγ supernate was applied to a column of DEAE cellulose (2 x 9 cm) equilibrated with buffer A. The column was washed with buffer A and the activity was recovered in the wash. The active fractions were pooled (15 ml total) and concentrated by negative pressure dialysis against buffer B to a volume of 3 ml. The dialyzate was applied to a column of phosphocellulose (1.5 x 10 cm) equilibrated with buffer B. The column was washed with buffer B (100 ml) and buffer B containing, first, 0.12 M KCl(50 ml) and, then, 0.2 M KCl (40 ml). The activity was recovered in the latter fractions of the 0.2 M effluent. The active fractions were pooled (10 ml total) and concentrated to 1 ml by negative pressure dialysis against buffer A containing 50% (v/v) glycerol. The phosphocellulose concentrate was stored at -20°, retaining 50% activity after a period of 2 months.

Results and Discussion

Biological source of endonuclease. Infection of E. coli by each of the λpbio's listed in Table II resulted in appearance of the endonuclease activity in crude extracts. Extracts from uninfected cells or cells infected with λcI and λb_2 did not catalyze a significant production of double-strand breaks. Concentration of the inactive extracts and their fractionation to the alumina Cγ stage of purification did not lead to detection of activity. The endonuclease action could be detected at 5 min after phage infection.

The genetic mechanism in the expression of this endonuclease is not determined here. In particular, it is not known whether the structural gene for the enzyme is of phage or bacterial origin. If the endonuclease is encoded in the λ chromosome proper, the relevant gene must be outside the λ segment missing in $\lambda pbio_{10}$, the longest right-wise deletion tested (attachment site to cIII); and outside the region deleted in b_2 mutants, since $\lambda b_2 pbio_{11}$ induces the activity (Table II). If the genes that specify the endonuclease are bacterial, the active cistrons could reside in either the bacterial chromosome proper, or in the bacterial segments present on the chromosomes of the infecting pbio's.

The λpbio's have the following two general properties in common. First, adjacer λ genes proximal and to the right of the prophage attachment site are deleted. Secondly the deleted phage DNA segments are replaced by specific stretches of bacterial DNA, incorporating bacterial genes adjacent and to the right of the λ attachment site on the host chromosome. Thus, one possible mechanism for the induction of the endonuclease activity may depend primarily on the deficit of λ genes commonly deleted in all the pbio tested. Alternatively, the induction may depend in a positive way on the presence in the phage chromosomes of an attachment site-proximal bacterial DNA segment. Attempt to supplement a presumptive deficit of λ genes in trans by simultaneous infection with $\lambda pbio_{10}$ and λb_2 (or λcI) had no effect on the appearance of this activity (Table II A simple interpretation, however - that the structural gene for the endonuclease is a bacterial gene, located at the left extremity of the biotin operon, carried by all the pbio's tested, and highly amplified by phage infection - is not established by our results.

Table II

Endonuclease activity of E. coli 1100

Infecting phage	Specific activity of alumina extracts (units/mg protein)
1. none	<0.5
2. λcI	<0.5
3. λb_2	<0.5
4. $\lambda int_6 red_3$	<1
5. $\lambda pbio_{10}$	12
6. $\lambda pbio_{11}$	11
7. $\lambda pbio_{7-20}$	16
8. $\lambda b_2 pbio_{11}$	15
9. mixedly infect λcI or λb_2 with $\lambda pbio_{10}$	10-14

E. coli 1100 cells were infected with each of the given strains of λ phage and, after 20 min, cell extracts were prepared and assayed as described in Materials and Methods.

Action of the endonuclease. Breakage of DNA by the purified enzyme is strictly dependent on the presence of magnesium. Manganese is not an effective substitute; less than 5% maximum activity is seen with the latter cation. The pH optimum for reaction is between 6.5 and 7.5 in Tris. Cl^-. The reaction proceeds equally well in phosphate buffer. High concentrations of sRNA have no effect on either the rate or extent of DNA breakage, the activity thus being distinguished from that of endonuclease I (8).

Fig.1A shows the progress of reaction with time. With increasing time, an increasing proportion of λ DNA molecules receive one or more double-strand breaks as revealed by sedimentation velocity analysis in neutral sucrose gradients. Initially, a peak appears sedimenting 0.82 ± 0.01 times as fast as intact marker molecules, and containing a shoulder with a relative sedimentation constant of 0.68 ± 0.01. As the fraction of intact molecules surviving decreases, the shoulder becomes more and more

prominent and, eventually, the profiles become most dense in the region specifying a sedimentation velocity 0.60 - 0.70 that of intact λ DNA. Sedimentation through neutral gradients after alkaline denaturation and reneutralization of reacted substrate DNA is more effective in resolving the product fragments. Under these conditions, at least two peaks, representing λ DNA fragments, can be detected readily. The sedimentation profiles of the products of this reaction do not resemble patterns obtained when nucleolytic events are fully random in respect to position (9). It

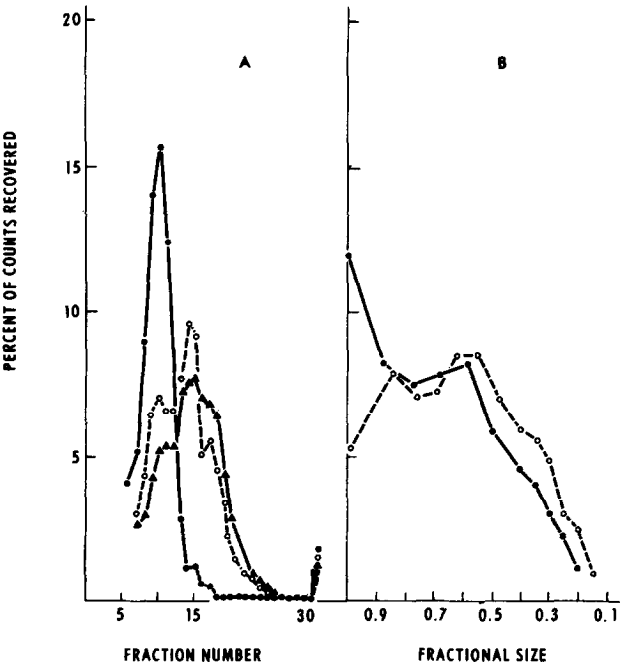

Fig. 1. (A) Sedimentation profiles of λ DNA reacted with 30 units of endonuclease for varying periods of time. Reaction was conducted as described in Materials and Methods and samples were taken for analysis in neutral sucrose gradients at 0 min (●——●), 10 min (o----o), and 30 min (▲——▲). Sedimentation is from right to left. (B) Molecular size distribution of reaction products in neutral (●——●) and alkaline (o---o) gradients. Reaction was allowed to continue until approximately 30% of substrate molecules had received at least one double-strand break. Samples of the reaction mix were then analyzed by sedimentation in neutral and alkaline gradients. 5 to 20% sucrose alkaline gradients contained 0.1 M NaOH, 0.9 M NaCl, 0.01 M EDTA. Sodium hydroxide (0.1 M final concentration) was added to samples prior to layering on alkaline gradients. Calculations for this type of analysis were according to Meselson and Yuan (10). The ordinate is arithmetic and units are arbitrary. The abcissa represents the ratio of the molecular sizes of DNA products to that of unreacted marker DNA taken as unity. Molecular sizes were calculated from sedimentation constants using Studier's equations (12).

has been difficult to generate fragments of molecular weight less than 20% that of intact λ DNA in "pushing" the reaction with high concentrations of enzyme.

To compare the number of double and single-strand breaks produced, the frequencies of fragment sizes in neutral and alkaline sucrose gradients were plotted. Fig. 1B represents such an analysis for the case where 30-40% of the substrate duplexes have received one or more double-strand breaks. At this level of reaction, approximately one half of the molecules that appear intact in neutral gradients have suffered one or more single-strand breaks, giving a greater density of smaller molecular weight fragments in alkaline gradients. The results of Fig. 1B thus reveal a moderate excess of single over double-strand cuts. At lower levels of reaction (more than 90% survival of intact λ duplexes), this excess of single over double-strand breaks is even more marked. However, when reaction is extensive (all substrate duplexes broken), pairs of curves of the type shown in Fig.1B are virtually indistinguishable. Taken together, the results are compatible with a mechanism of enzyme action in which single-strand incisions precede and are converted to double-strand breaks during the course of reaction (10,11).

Neither the role of this nuclease *in vivo*, nor the genetics of its expression have been determined. Endonucleases previously studied that catalyze double-strand breaks in DNA and for which biological roles have been implicated (10,13) appear to mediate degradative functions. Studies with λ*pbio* mutants, conditionally defective in the endonuclease described here, might provide both the genetic definition and a physiological reason for this enzyme.

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References

1. Meselson, M., J. Mol. Biol., 9, 734 (1964).
2. Signer, E. R., Ann. Rev. Microbiol., 22, 451 (1968).
3. Manly, K. F., Signer, E. R., and Radding, C. M., Virology, 37, 177 (1969).
4. Durwald, H., and Hoffman-Berling, H., J. Mol. Biol., 34, 331 (1968).

5. Gottesman, M. E., and Yarmolinsky, M. B., Cold Spring Harb. Symp. Quant. Biol., 33, 735 (1968).
6. Goldberg, A. R., and Howe, M., Virology, 38, 200 (1969).
7. Thomas, C. A., Jr., and Abelson, J., in "Procedures in Nucleic Acid Research" (G. L. Cantoni and D. R. Davies, eds.) p.553, Harper and Row, 1966.
8. Lehman, I. R., Roussos, G. G., and Pratt, E. A., J. Biol. Chem., 327, 819 (1962).
9. Litwin, S., Shahn, E., and Kozinski, A. W., J. Virology, 4, 24 (1969).
10. Meselson, M., and Yuan, R., Nature, 217, 1110 (1968).
11. Bernardi, G., and Cordonnier, C., J. Mol. Biol., 11, 141 (1965).
12. Studier, F. W., J. Mol. Biol., 11, 373 (1965).
13. Center, M. S., Studier, F. W., and Richardson, C. C., Proc. Natl. Acad. Sci. U. S., 65, 242 (1970).