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In Vitro Repair of Apurinic Sites in DNA

(DNA depurination/endonuclease specific for apurinic sites/DNA repair)

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ABSTRACT **Apurinic sites disappear from DNA during an incubation of the DNA with the *Escherichia coli* endonuclease specific for apurinic sites, DNA polymerase I (EC 2.7.7.7.), and T4 ligase (EC 6.5.1.1). Omission of any one of these three enzymes and, in particular, omission of the endonuclease specific for apurinic sites prevents this *in vitro* repair.**

We have shown the presence of a nuclease that cuts DNA strands near apurinic sites in *Escherichia coli* (1, 2), rat liver (3), and plants (4). The purified bacterial enzyme (2) and the purified mammalian enzyme (3) are strictly specific for apurinic sites; they do not act on intact strands in native or denatured DNA.

Apurinic sites in DNA may result from the spontaneous loss of purines (5); the rate of hydrolysis of the glycosidic linkages is, however, greatly enhanced after a treatment with alkylating agents (6) or nitrous acid (7). It has been shown that the apurinic sites have a toxic effect; in the T7 coliphage, one out of seven or eight depurinations is an inactivation hit (8, 9).

One may now wonder whether apurinic sites can be repaired and whether the nuclease specific for apurinic sites is involved in the repair process. The following experiments have been carried out to answer both questions.

MATERIALS AND METHODS

Preparation of Labeled T7 Phage DNA Containing Apurinic Sites. A thymineless mutant of *E. coli* B (A-1 thy$^-$) prepared in this laboratory (10) was first cultured at 37° in Thomas and Abelson medium (11) supplemented with tritiated thymidine (2 μg/ml; 1 mCi/μg). When the culture reached the optical density of 0.35 at 450 nm, a suspension of T7 phage (S. Luria; ATCC 11303-B_7) was added at an infection multiplicity of 0.1 and the incubation was carried out for two more hours at 37°. The phages were isolated by the method of Studier (12) and further purified on a CsCl gradient. After exhaustive dialysis at 4° against 0.15 M NaCl–0.015 M EDTA, pH 7 (NaCl–EDTA), the phage suspension was passed through a Millipore filter H.A. (0.45-μm diameter pores). From this point on, all manipulations were carried out under sterile conditions in order to reduce nuclease contamination to a minimum.

The DNA was isolated from the phage suspension by the phenol method of Mandell and Hershey (13); the aqueous phase was then dialyzed against NaCl–EDTA to completely eliminate the phenol. The specific radioactivity of the DNA was 150,000 dpm/μg. This labeled normal DNA was used to check for the absence of nonspecific endonucleolytic activities in the enzyme preparations.

To introduce a small number of apurinic sites per T7 DNA strand, acetic acid was added to reach pH 4.0 and the mixture warmed at 37° for 15 min; the solution was then brought back to neutrality with 1 M phosphate–0.01 M EDTA, pH 7.

Denaturation of DNA by NaOH or by Formamide and Estimation of the Number of Breaks and Apurinic Sites per Strand. For denaturation by NaOH, an aliquot of the solution containing 2 μg of DNA was mixed with an equal volume of 0.4 N NaOH–0.009 M EDTA. The mixture was incubated at 37° for 15 min; under these conditions, all apurinic sites give rise to single-strand breaks (2). The solution was then neutralized with 1.4 N H_3PO_4 and the volume adjusted to 1 ml with NaCl–EDTA.

For denaturation with formamide, an aliquot of the solution containing 4 μg of DNA was diluted to 2 ml with NaCl–EDTA and then dialyzed at 4° against 95% formamide [formamide freshly redistilled under reduced pressure, 0.015 M EDTA (95:5, v:v), adjusted at pH 7] with two changes. The temperature was then raised to 37° for 2 hr. To eliminate the formamide, the solution was dialyzed at 4° against 10 times diluted NaCl–EDTA with five changes.

The denatured labeled DNA (0.2 ml) was placed on a linear 5–20% (w/v) sucrose gradient in 1 M NaCl, 10 mM Tris·HCl, 1 mM EDTA, pH 7, in 5-ml cellulose nitrate tubes. These tubes were centrifuged at 35,000 rpm in the SW50.1 rotor of the Beckman L2-65B centrifuge for 90 min at 20°. The gradients were emptied with the ISCO density gradient fractionator; 40 fractions of 0.125 ml each were collected and their radioactivity was determined.

The mean number of breaks per DNA strand has been calculated by a modification of the FORTRAN program of Gillespie *et al.* (14)*. The program simulates the random breakage of the T7 DNA strands by a Monte Carlo experiment and compares the theoretical sedimentation profile calculated by the computer with the profile given experimentally until a best fit is obtained. The original program has been adapted to centrifugation in neutral sucrose gradients and now gives directly the average number of breaks per strand. Repeated determinations, on aliquots from the same DNA solutions, have shown that the standard error remains under 0.15 when the number of breaks per T7 DNA is below 4.0.

Abbreviations: NaCl–EDTA, 0.15 M NaCl–0.015 M EDTA, pH 7; Tris–Mg buffer, 50 mM Tris·HCl–5 mM $MgCl_2$, pH 7.5.

* We are grateful to Dr. C. J. Gillespie who sent us his detailed computer program.

TABLE 1. *Average number of breaks and apurinic sites per strand in depurinated T7 DNA incubated with various combinations of endonuclease specific for apurinic sites, DNA polymerase I, and ligase*

3-hr incubation at 37°	Enzymes	Average no. of breaks per T7 DNA strand		Average no. of apurinic sites per T7 DNA strand
		NaOH	form-amide	
−		3.2	1.1	2.1
+	None	3.1	1.5	1.6
+	Endonuclease, polymerase, ligase	0.8	0.8	0.0
+	Polymerase, ligase	2.7	1.5	1.2
−		2.4	0.5	1.9
+	None	2.4	0.8	1.6
+	Endonuclease, polymerase, ligase	0.5	*	
+	Endonuclease, polymerase	2.5	2.8	0.0
+	Endonuclease, ligase	2.0	2.2	0.0
+	Endonuclease	2.8	2.8	0.0

Freshly prepared T7 phage DNA containing an average of 0.5 apurinic site and break per strand was submitted to a limited depurination in the conditions given in the *text;* two preparations were made and each was used in a different set of experiments. The partially depurinated DNA was incubated with various combinations of the three enzymes: endonuclease specific for apurinic sites, DNA polymerase I, and ligase. The DNA was then denatured by NaOH or formamide before being centrifuged on a neutral, linear 5–20% sucrose gradient. In each case, the average number of breaks per DNA strand has been calculated from the sedimentation profile by the computer method of Gillespie *et al.* (14). Alkaline treatment hydrolyzes a phosphodiester bond near each apurinic site (16), while formamide treatment leaves them intact (15). Consequently, centrifugation after formamide denaturation yields the average number of breaks per strand; centrifugation after NaOH denaturation yields the sum of breaks and apurinic sites; the difference between the values from the two methods gives the average number of apurinic sites per DNA strand.

* This sample has been lost, but, because the endonuclease was present, there was very likely no apurinic site left. Even if the 0.5 break per strand found after NaOH denaturation were derived from apurinic sites, the conclusion would be that a minimum of 1.1 apurinic sites per strand has been repaired by the cooperative action of the three enzymes (0.5 apurinic site left against 1.6 in the control without enzyme).

Denaturation of DNA with formamide at pH 7 leaves the apurinic sites intact (15); the breaks, whose number is estimated from the sedimentation profile on sucrose gradients, were present in the DNA before denaturation. Denaturation with NaOH also hydrolyzes a phosphodiester bond near each apurinic site (16); the number of breaks, found by sedimentation on a sucrose gradient, is thus the sum of the breaks and apurinic sites present in the DNA before denaturation. The number of apurinic sites is obtained by subtracting the number of breaks after formamide treatment from the number of breaks after NaOH treatment.

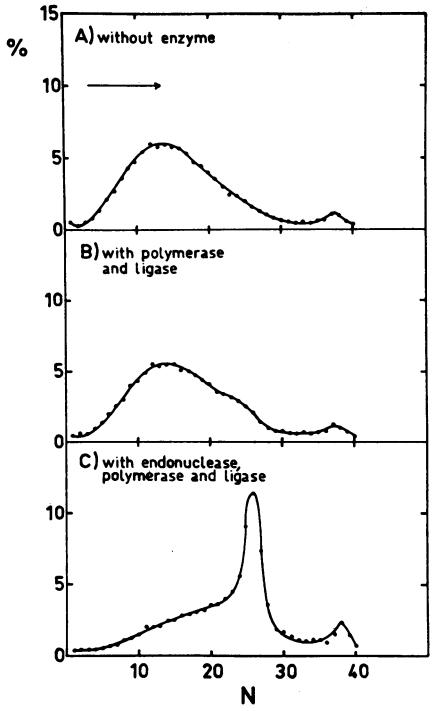

FIG. 1. T7 phage DNA (3 μg) containing apurinic sites and labeled with tritium (150,000 dpm/μg) was incubated, as described in the *text,* without enzyme (*A*); with DNA polymerase I and ligase (*B*); with the endonuclease specific for apurinic sites, DNA polymerase I, and ligase (*C*). After a 3-hr incubation at 37°, the 100-μl solution was brought to 1 ml with NaCl–EDTA and dialyzed against the same solvent. To 0.2 ml of the dialyzed solution, NaOH was added to a final concentration of 0.2 M, and after an incubation of 15 min at 37°, the pH was adjusted to 7 with 1.4 M H_3PO_4. An aliquot (0.2 ml) of the neutralized denatured DNA solution was then layered on top of a neutral linear 5–20% sucrose gradient in 1 M NaCl–10 mM Tris·HCl–1 mM EDTA, and centrifuged for 90 min at 35,000 rpm in the SW-50 rotor of a Beckman L2-65B centrifuge at 20°. Fractions of 0.125 ml were then collected, and their radioactivity was measured in a Triton–toluene scintillation mixture. The sedimentation profile gives the percentage of the total recovered radioactivity (%) found in each fraction (*N* = fraction number); recovery was above 90%. The *arrow* indicates the direction of sedimentation.

The Enzymes. The endonuclease for apurinic sites had been purified from *E. coli* B41 by Paquette *et al.* (2); the enzyme was kept frozen in 0.15 M NaCl–15 mM Na citrate–10 mM $MgCl_2$, pH 7. DNA polymerase I (EC 2.7.7.7; DNA nucleotidyl transferase), corresponding to fraction 7 of the purification method described by Richardson *et al.* (17), was a gift of Dr. J. Slater, Department of Biology, University of Montreal; it was kept frozen in 20% glycerol, 20 mM Tris·HCl, 1 mM reduced glutathione, 0.2 mM EDTA, pH 7.5, containing 1 mg of bovine-serum albumin per ml; its activity was determined (18) and found to be 4.2 units/ml. The polynucleotide ligase [EC 6.5.1.1; polynucleotide synthetase (ATP)] was a gift from Drs. A. Kato and M. J. Fraser, Department of Biochemistry, McGill University, who prepared it from *E. coli* infected with T4 phage; the enzyme was kept in 50% glycerol, 0.5 M KCl, 0.01 M K phosphate, pH 7.6, and the

preparation had an activity of 3 units/ml. After incubation for 1 hr at 37° with labeled T7 phage DNA taken before the depurination step, each of the three enzymes was found to be devoid of nonspecific endonucleolytic activities by the centrifugation technique after denaturation with NaOH.

RESULTS AND DISCUSSION

Two preparations of T7 phase DNA with a few apurinic sites per strand were made. As shown in Table 1, there was an average of 2.1 apurinic sites and 1.1 breaks per T7 DNA strand in one preparation and an average of 1.9 apurinic sites and 0.5 break per T7 DNA strand in the other. Each solution was dialyzed against 50 mM Tris·HCl–5 mM $MgCl_2$, pH 7.5 (Tris–Mg buffer), with three changes, and used in a different set of experiments (Table 1).

To 50 μl of the labeled depurinated DNA (3 μg) in Tris–Mg buffer were added 20 μl of Tris–Mg buffer containing a mixture of nucleoside triphosphates (1 mM ATP, 1 mM dATP, 1 mM dGTP, 1 mM dCTP, and 1 mM dTTP), 10 μl of the endonuclease specific for apurinic sites, 10 μl of DNA polymerase I (0.04 unit), and 10 μl of the T4 ligase (0.03 unit). The mixture was incubated for 3 hr at 37°. Then, NaCl–EDTA was added to reach a volume of 1 ml before the mixture was dialyzed against NaCl–EDTA at 0°. After denaturation with NaOH or formamide, aliquots were centrifuged on neutral sucrose gradients to determine the average number of apurinic sites and breaks per T7 phage DNA strand. Controls were carried out omitting one, two, or the three enzymes; in each case, the total volume was brought to 100 μl with Tris–Mg buffer. The results are summarized in Table 1.

Incubations in Tris–Mg buffer without any enzyme lead to the hydrolysis of only a few apurinic sites (0.5 and 0.3 per strand in two different experiments), while incubation with the endonuclease alone completely converted the apurinic sites into breaks. The results from the first set of experiments indicate that, when the three enzymes were present (endonuclease specific for apurinic sites, DNA polymerase I, and ligase), not only did the apurinic sites disappear by the end of the incubation, but also the number of breaks decreased: hence the apurinic sites were not simply hydrolyzed; nearly all of them were repaired. In spite of the loss of a formamide-denatured sample, the same conclusion can be drawn from the second set of experiments. Omission of any one of the three enzymes prevents the repair of the apurinic sites. This is particularly true when the nuclease specific for apurinic sites is absent (first set of experiments) and is illustrated in Fig. 1, which compares the sedimentation profiles after NaOH denaturation. When the depurinated DNA was incubated with the three enzymes (*C*), a peak of intact T7 phage DNA strands ($N = 26$) can be observed that is absent in the control (*A*) or when the nuclease for apurinic sites had been omitted (*B*).

Taken together, the above data show that apurinic sites can be repaired *in vitro*, and that the participation of the endonuclease specific for apurinic sites is essential. The three enzymes used have been prepared from *E. coli* (or *E. coli* infected with T4 phage) so that it is likely that the repair of apurinic sites proceeds *in vivo* within the bacterial cell.

We have previously postulated (4) that the spontaneous depurination of DNA calls for a cellular system for maintenance of the genetic information encoded in DNA molecules. The fact that we have found an endonuclease for apurinic sites in organisms of three different kingdoms (animal, plant, and bacteria) (4) and that, here, we have clearly demonstrated the participation of this enzyme in the repair of apurinic sites *in vitro*, suggests that this nuclease is a repair enzyme involved in the maintenance of DNA in all cells.

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1. Verly, W. G. & Paquette, Y. (1972) *Can. J. Biochem.* **50,** 217–224.
2. Paquette, Y., Crine, P. & Verly, W. G. (1972) *Can. J. Biochem.* **50,** 1199–1209.
3. Verly, W. G. & Paquette, Y. (1973) *Can. J. Biochem.* **51,** 1003–1009.
4. Verly, W. G., Paquette, Y. & Thibodeau, L. (1973) *Nature New Biol.* **244,** 67–68.
5. Lindahl, T. & Nyberg, B. (1972) *Biochemistry* **11,** 3610–3618.
6. Lawley, P. D. & Brookes, P. (1963) *Biochem. J.* **89,** 127–138.
7. Schuster, H. (1960) *Z. Naturforsch. B* **15,** 298–304.
8. Lawley, P. D., Lethbridge, J. H., Edwards, P. A. & Shooter, K. V. (1969) *J. Mol. Biol.* **39,** 181–198.
9. Brakier, L. & Verly, W. G. (1970) *Biochim. Biophys. Acta* **213,** 296–311.
10. Verly, W. G., Crine, P., Bannon, P. & Forget, A. (1974) *Biochim. Biophys. Acta,* in press.
11. Thomas, C. A. & Abelson, J. (1966) in *Procedures in Nucleic Acid Research,* eds. Cantoni, G. L. & Davies, D. R. (Harper and Row, New York and London) p. 553.
12. Studier, F. W. (1969) *Virology* **39,** 562–574.
13. Mandell, J. & Hershey, A. (1960) *Anal. Biochem.* **1,** 66–77.
14. Gillespie, C. J., Gislason, G. S., Dugle, D. L. & Chapman, J. D. (1972) *Radiat. Res.* **21,** 272–279.
15. Strauss, B. S. & Robbins, M. (1968) *Biochim. Biophys. Acta* **161,** 68–75.
16. Tamm, C., Shapiro, H. S., Lipshitz, R. & Chargaff, E. (1953) *J. Biol. Chem.* **203,** 673–688.
17. Richardson, C. C., Schildkraut, C., Aposhian, H. V. & Kornberg, A. (1964) *J. Biol. Chem.* **239,** 222–232.
18. Aposhian, H. V. & Kornberg, A. (1962) *J. Biol. Chem.* **237,** 519–525.