*ENZYMATIC CLEAVAGE AND REPAIR OF TRANSFORMING DNA**

By P. J. Laipis, B. M. Olivera,† and A. T. Ganesan

LT. J. P. KENNEDY, JR. LABORATORIES FOR MOLECULAR MEDICINE, DEPARTMENT OF GENETICS AND DEPARTMENT OF BIOCHEMISTRY, STANFORD UNIVERSITY SCHOOL OF MEDICINE

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Abstract and Summary.—Single-strand nicks caused by DNase I are capable of inactivating *B. subtilis* transforming DNA. Under suitable conditions, the polynucleotide joining enzyme from *E. coli* and a similar DPN-requiring activity from *B. subtilis* can completely repair this damage, restore biological activity, and increase the single-strand molecular weight. The rates of inactivation of a single genetic marker and of a four-marker linkage group suggest that a single-strand nick is inactivating even when far from the site of genetic damage.

The genetic activity of *B. subtilis* transforming DNA is destroyed by pancreatic DNase (DNase I). The surviving activity is correlated with the single-strand length of the DNA.[1] However, these results do not prove directly that the loss of biological activity is due to simple single-strand "nicks." Enzymes (polynucleotide joining enzymes, or ligases‡) which repair single-strand nicks of the type made by DNase I have recently been purified.[2–5] The present studies show that most of the inactivation of transforming DNA caused by DNase I could be reversed by the ligases from *E. coli* and *B. subtilis*, which indicates that single-strand nicks are the inactivating damage.

Materials and Methods.—Stock solutions of DNase I (electrophoretically pure, from General Biochemicals) containing 0.1 mg/ml DNase, 0.1 mg/ml bovine serum albumin (BSA), and 0.01 *M* Tris HCl, pH 8, were divided into small aliquots and frozen in liquid nitrogen. An aliquot was used once and discarded. The enzymic purity of this preparation is not known. *E. coli* ligase and assay substrate were prepared according to Olivera and Lehman.[3] Exonuclease III was prepared according to Richardson and Kornberg.[6] T4 phage-induced DNA polymerase was a gift of Dr. Zach Hall.[7] Competent cells (SB 202, *Aro*-2, *Try*-2, *His*-2, *Tyr*-1) were prepared by the method of Stewart.[8] *His*-2 and *Tyr*-1 are believed to be single-base changes, based on their reversion frequencies ($\sim 5 \times 10^{-7}$). *Try*-2 is probably a deletion (reversion frequency $<10^{-8}$).

DNA preparation: ^{3}H-labeled DNA (12,000 dpm/μg DNA) from a *Thy*$^-$ derivative of *B. subtilis* 168 (SB 591) grown in ^{3}H-thymidine (Schwarz BioResearch) was isolated as described in reference 9.

Transformation: 0.9 ml of a competent SB 202 culture containing 1–5 $\times$ 10^8 cells/ml was added to 0.1 ml of 0.2 *M* Na citrate containing 0.01–0.2 μg/ml DNA. After 30 min incubation at 30°, transformation was terminated by the addition of 0.1 ml DNase solution (0.5 mg/ml pancreatic DNase, 10 mg/ml BSA, 0.4 *M* $MgCl_2$) and further incubated for 5 min at 30°. Aliquots were then diluted and grown on appropriate media. Controls always indicated that transformation occurred in the linear range of DNA concentration.

DNase digestion: 4.0 ml stock solution of DNA (40 μg/ml DNA; 4 mM $MgCl_2$; 0.1 mg/ml BSA; 0.015 *M* NaCl, 0.0015 *M* Na citrate (SSC/10); 0.01 *M* Tris HCl, pH 8) was incubated at 0° with 2×10^{-3} μg/ml DNase I. Aliquots were withdrawn at various times, made 0.1 *M* in ice-cold Na citrate, heated to 68° for 20 min, and then dialyzed against SSC/10, 0.01 *M* Tris HCl, pH 8. The amount of inactivation was determined by transformation assays.

Ligase repair: 2–5 units/ml of *E. coli* ligase (fraction V, 45 units/ml) were added to a solution containing 40 μg/ml DNA, SSC/10, 0.01 *M* Tris HCl, pH 8, 12 mM $MgCl_2$, 20 μM DPN, and incubated at 30° for various lengths of time. 5 μl aliquots were removed and quenched by the addition of 0.1 ml of 0.2 *M* ice-cold Na citrate. Each quenched DNA sample was used in transformation assays.

Sucrose gradients: 1–2 μg of DNA was layered on top of a linear 5–25% neutral sucrose gradient (1 *M* NaCl, 0.01 *M* Tris HCl, pH 8) or a linear 5–20% alkaline sucrose gradient (0.8 *M* NaCl, 0.2 *M* NaOH, pH 11.8).

^{14}C λ DNA (a gift of Dr. R. Doherty) and ^{14}C T5 DNA (a gift of Mr. Thomas Broker) were used as markers. The gradients were centrifuged for 4 hr at 35,000 rpm at 4° in the Spinco model SW-39 rotor or 3 hr at 45,000 rpm at 4° in the IEC model SB-405 rotor. Fractions were collected on Whatman GF/C filters, dried, and counted in a liquid scintillation counter, with appropriate corrections.[10]

The equations of Burgi and Hershey[11] and Abelson and Thomas[12] were used to calculate molecular weight. The weight average mol wt was calculated from the expression:

$$\bar{M}_w \cong \sum_i \frac{\text{counts } (i)}{\text{total counts}} \cdot K \cdot (\text{fraction number } (i))^a$$

where $K = M_{STD}/(\text{fraction number}_{STD})^a$; $a = 2.86$ (native DNA) or 2.63 (denatured DNA); M_{STD} of λ DNA $= 33.7 \times 10^6$; and M_{STD} of T5 DNA component II $= 11.86 \times 10^6$.

Enzyme purification: Initial steps leading to the streptomycin precipitation of the lysate obtained from 15 gm of wet, packed, early log phase cells of *B. subtilis* SB19 (prototroph) were the same as in reference 13.

The supernatant (119 ml, 2.8 mg/ml protein), which contained most of the assayable ligase activity, was precipitated with 29 gm of $(NH_4)_2SO_4$ (40% fractional saturation) with stirring. After 30 min, the precipitate was discarded, and an additional 31 gm of $(NH_4)_2SO_4$ (85% fractional saturation) was added to the supernatant. After 30 min settling, the precipitate was removed and dissolved in 0.1 *M* glycyl-glycine buffer, pH 7. This fraction (AS II, 17 ml, 13 mg/ml protein) contained 3.3 units/mg protein of ligase.

Results.—Loss and restoration of biological activity: The loss of single and multiple-linked marker transforming activity of DNA from *B. subtilis* on treatment with DNase I for various lengths of time was reversed by the polynucleotide joining enzyme from *E. coli* (Fig. 1). With mildly digested DNA (less than 60% inactivated), complete restoration of biological activity was obtained.

Prolonged nuclease treatment resulted in incomplete recovery of biological activity, presumably caused by "gaps" in the DNA double helix or by double-strand breaks. Preliminary experiments indicate that our DNase I is not detectably contaminated with nonspecific phosphatase, eliminating phosphatase attack as a source of nonrepairable damage.

Exonuclease III digestion of DNase I-treated DNA (a reaction expected to produce "gaps") resulted in DNA which is not repaired by ligase or by T4 DNA polymerase alone. However, treatment of this DNA with both ligase and T4 DNA polymerase resulted in a significant recovery of biological activity (Table 1). Addition of both T4 DNA polymerase and ligase under the same conditions to an extensively degraded DNA preparation did not increase the amount of biological activity regained, an indication that "gaps" are not a frequent type of nonrepairable damage. In contrast, double-strand breaks are detectable in such extensively degraded DNA preparations.

Molecular weight measurements: Two DNA samples were degraded with DNase I until 56 and 18 per cent of their original transforming activity remained

TABLE 1. *Repair of nicked DNA.*

Repair treatment	Surviving activity (%)
None	75
Ligase	100
Exo III-treated, ligase	79
Exo III-treated, polymerase	58
Exo III-treated, polymerase and ligase	93

DNase-nicked DNA was treated with exonuclease III (0.08 unit/mg DNA) for 5 min at 37° (40 μg/ml DNA, SSC/10, 0.01 Tris HCl, pH 8, 12 mM $MgCl_2$, 20 μM DPN, 0.1 mM β-mercaptoethanol). The exonuclease was inactivated by a 10-min incubation at 68°. Ligase (2–5 units/ml) and/or T4-DNA polymerase (100 units/ml, plus 20 μM each of the four deoxyribotriphosphates) were added, and the DNA was incubated at 30° for 10 min. Samples were quenched, and the transforming ability was determined as described in *Materials and Methods*. 100% repair corresponds to 233 transformants in the 0.02-ml aliquot plated.

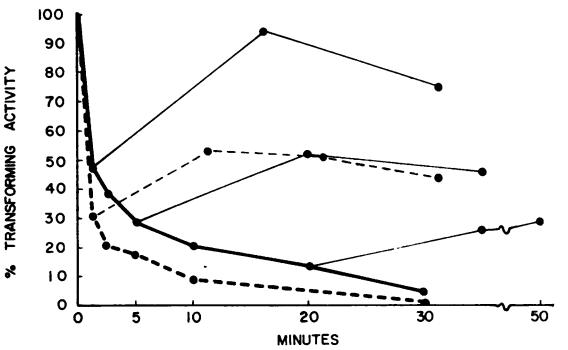

FIG. 1.—Degradation and repair of transforming DNA. SB 591 DNA was treated with DNase, repaired with ligase, and used to transform SB 202, as described in *Materials and Methods*. *His*-2$^+$ transformants (—) were scored by growth on supplemented plates, *Aro*-2$^+$ *Try*-2$^+$ *His*-2$^+$ *Tyr*-1$^+$ [linkage group$^+$] transformants (- - -) by growth on minimal plates or by determining the genotype of the *His*-2$^+$ transformants by replica plating. Both methods gave identical results. The time of DNase treatment is represented by bold lines, the additional time of ligase repair by fine lines. Each point represents the average of four experiments; maximum standard deviation between experiments for a point is ±5.8%; minimum number of colonies counted for any point in a single experiment was 132.

for a single marker, and 38 and 9 per cent of the original activity remained for a linkage group. These samples were treated with ligase. The biological activity of the mildly digested DNA was almost completely regained; the biological activity of the extensively digested DNA was not (Table 2).

Neutral and alkaline sucrose gradients were performed on the undegraded, degraded, and the repaired DNA of both samples. Neutral sucrose gradients of mildly digested DNA showed that few, if any, double-strand breaks had occurred, while the alkaline gradients show only a 20 per cent decrease in the single-strand weight average molecular weight. These single-strand breaks were almost completely repaired by ligase treatment, as judged by the increase in the single-strand molecular weight. However, neutral sucrose gradients of extensively degraded DNA (Fig. 2) show that under these conditions a reduction in the double-strand weight average molecular weight has occurred (from 27×10^6 to 22×10^6). The alkaline gradients (Fig. 3) show that the single-strand

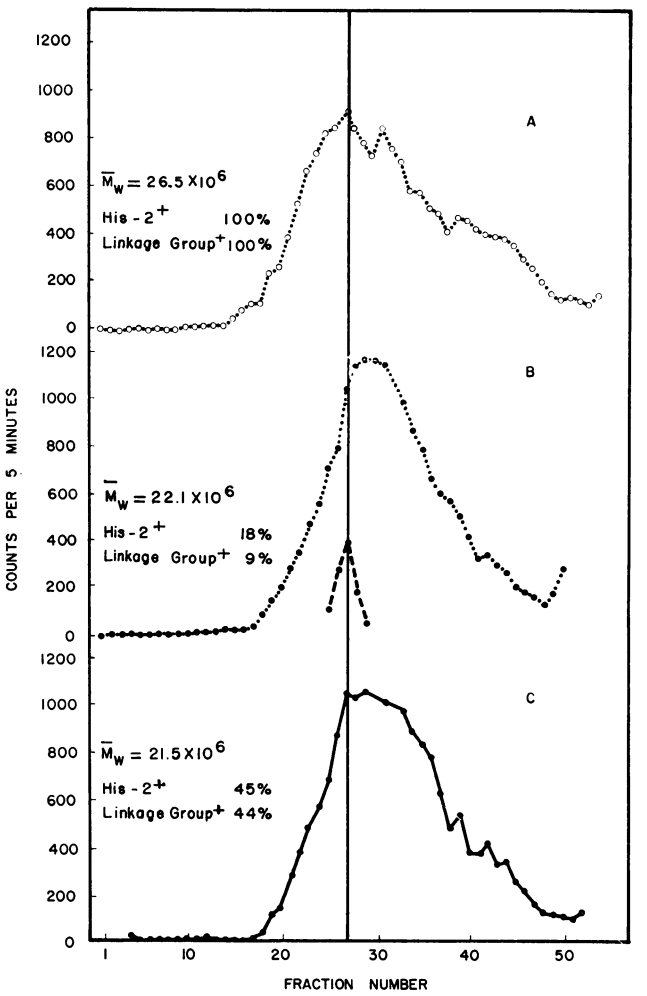

FIG. 2.—Neutral sucrose gradients of extensively degraded DNA. (*A*) No treatment; (*B*) 30 min DNase; (*C*) 10 min ligase repair; and ^{14}C λ DNA standard (- - -). Relative biological activities (from Table 2) are shown.

weight average molecular weight falls from 9.5 × 10^6 to 3.8 × 10^6. On repair with the DNA joining enzymes, the single-strand weight average molecular weight increases to 7.5 × 10^6, but the double-strand molecular weight remains the same. The data suggest that the repair of single-strand nicks was almost complete, but the single-strand molecular weight is not restored to the original value mainly because of the double-strand breaks.

The sedimentation profiles show that there are single-strand breaks in the undegraded DNA samples. However, there is no increase in biological activity on incubation with ligase, a suggestion that these breaks are not simple 3′-OH, 5′-phosphate nicks.

DNA joining activity in B. subtilis: The enzyme fraction is relatively crude

TABLE 2. *Degradation and repair of biological activity.*

Treatment of DNA, min		Transformants/Genome Equivalent of Added DNA: *His*-2+	Linkage group+
Experiment 1: None	—	23.1×10^{-5}	6.07×10^{-5}
DNase alone	10	13.0	2.62
DNase + DPN	10 / 10	10.6	2.58
DNase + Ligase + DPN	10 / 10	22.1	5.64
Experiment 2: None	—	11.7	3.50
DNase alone	30	2.11	0.341
DNase + DPN	30 / 10	2.02	0.365
DNase + Ligase + DPN	30 / 10	5.26	1.55

^{3}H-labeled DNA from SB 591 was DNase-treated, repaired with ligase, and used to transform SB 202. *His*-2+ transformants were scored by growth on supplemented plates; linkage group+ transformants were scored by growth on minimal plates. A minimum of 112 colonies per plate were counted.

Experiment 1: 117 μg/ml of DNA was added to 3.6×10^8 viable cells/ml of SB 202.

Experiment 2: 0.17 μg/ml of DNA was added to 1.0×10^8 viable cells/ml of SB 202.

The amount of DNA per *B. subtilis* nucleus is 2.07×10^{-15} gm.[20]

and contains detectable nuclease activity when assayed by the release of acid-soluble counts from labeled DNA. Upon addition of DPN, both linked and single-marker activities are restored, while the omission of DPN leads to loss of transforming activity (Fig. 4). This suggests that a DPN linked ligase,[4, 14] as well as an endonuclease of the DNase I type, is present in this crude fraction. The requirement for DPN was confirmed using the assay of Olivera and Lehman;[3] ATP was shown to be unacceptable as a cofactor.

Discussion.—These experiments directly demonstrate that the inactivation of transforming DNA is a consequence of single-strand nicks. On treatment with DNase I, conditions can be found in which all biological inactivation is due to the single-strand nicks in the DNA duplex. Complete repair of such breaks is possible with ligase. Our experiments also show that the recovery of biological activity is correlated with an increase in single-strand molecular weight of the transforming DNA.

As the DNA preparations are more extensively cleaved, an increasing proportion of the inactivation becomes nonrepairable, probably, though perhaps not entirely, due to double-strand scissions. Our data present the weight average distributions of the untreated and DNase-treated DNA; it is thus possible to obtain only limiting ranges for the number of single-strand breaks required to inactivate a marker or cause double-strand scission.[1, 15] These ranges are broad, and, considering the uncertain validity of classical target theory for interpreting the inactivation of transforming activity,[1] we have not used them to compute corresponding "critical target sizes." However, our data indicate that between 3 and 18 single-strand nicks occur for every double-strand scission. This value is much lower than the figure of about 120 single-strand breaks per double-strand scission reported by Thomas[15] but does agree with the figure cal-

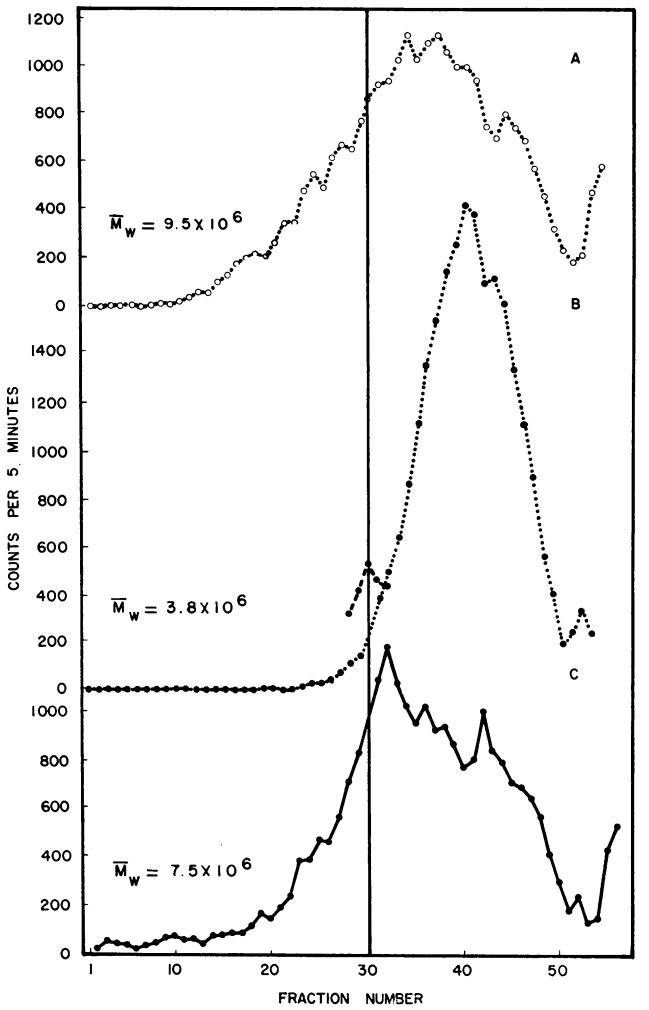

FIG. 3.—Alkaline sucrose gradients of extensively digested DNA. (*A*) No treatment; (*B*) 30 min DNase; (*C*) 10 min ligase repair; and ^{14}C T5 component II DNA standard (- - -).

culated from Bodmer's data.[1] The reason for this unusual rate of double-strand scission is not known; we cannot rule out enzymic impurities in our DNase, or perhaps the nonrandom action of our DNase under the conditions of our experiments.

Our experiments are in agreement with those of Bautz,[16] who showed that ligase could repair DNase-induced breaks in T4 DNA, as measured by the recovery of r_{II} function in a spheroplast assay using urea-treated T4 helper phage. In addition, Takagi *et al.*[17] have reported that the biological activity of transforming DNA nicked with DNase I or DNase K2 could be restored, using T4 ligase.

The loss of transforming activity of the linkage group is only twice as great as

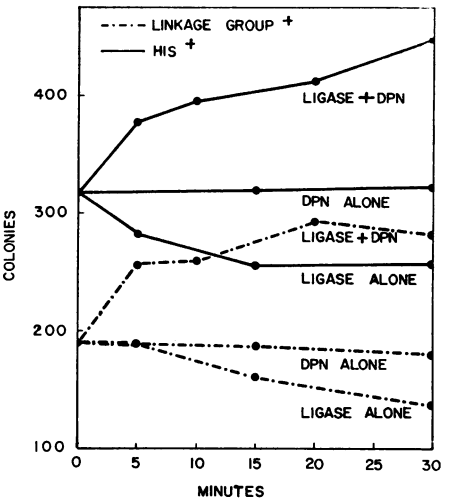

FIG. 4.—Repair with the *B. subtilis* ligase. 5 μl (44 units/ml) of *B. subtilis* ammonium sulfate fraction AS II (see *Materials and Methods*) were added to 50 μl of mildly digested DNA, with and without 20 μM DPN. The reaction mixture was incubated at 30° and 5-μl aliquots were removed at various times and used to transform SB 202. *His*-2$^+$ colonies per 0.02 ml and linkage group$^+$ colonies per 0.05 ml are shown; 100% repair would be 436 and 328 colonies, respectively.

the fall in transforming activity of a single marker. Much of this inactivation is caused by single-strand nicks, since it is reversed by treatment with ligase. The unusually high inactivation rate of a single marker compared to the inactivation rate of the linkage group is in contrast to the results obtained in Bautz's T4 experiments.

Possible mechanisms for this phenomenon are (1) the involvement of a step during synapsis or integration very sensitive to single-strand length or, perhaps, (2) a requirement by the DNA uptake mechanism for intact single strands in the transforming DNA. Experimental evidence consistent with either of these possibilities exists.[18, 19]

Our data cannot distinguish between these or any other mechanism which supposes that a single-strand break can inactivate a marker as much as 10,000 bases away from it. However, it is clear from the differences between our data and those of Bautz that the inactivation by single-strand nicks of single-marker activity in *B. subtilis* and T4 transformation proceeds with vastly different efficiencies.

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Abbreviations used: BSA, bovine serum albumin; SSC/10, 0.015 *M* NaCl, 0.0015 *M* Na citrate.

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† Postdoctoral fellow of the Damon Runyon Memorial Fund for Cancer Research. Present address: Department of Biochemistry, College of Medicine, University of the Philippines, Manila, Philippines.

‡ In this paper the terms ligase and polynucleotide joining enzyme will be used interchangeably.

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