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Adenosine Triphosphate-Dependent Deoxyribonuclease from *Diplococcus pneumoniae*: Fate of Transforming Deoxyribonucleic Acid in a Strain Deficient in the Enzymatic Activity

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The adenosine triphosphate-dependent deoxyribonuclease is required for wild-type levels of deoxyribonucleic acid (DNA) repair and genetic recombination. In an attempt to determine the physiological function of this enzyme in pneumococcal transformation, the fate of transforming DNA was followed in a wild-type strain and in a strain lacking the enzymatic activity. The qualitative and quantitative findings were closely comparable in the two strains through the step of physical association of a single strand of donor DNA with the recipient chromosome. These results are interpreted to mean that the enzyme may be involved in the subsequent hypothetical removal of excess polynucleotide sequences during conversion of the presumed hydrogen-bonded intermediate into a covalently linked recombinant structure.

In previous papers, we reported the discovery of an adenosine triphosphate (ATP)-dependent deoxyribonuclease in *Diplococcus pneumoniae* (23) and presented genetic evidence for its involvement in the process of deoxyribonucleic acid (DNA) repair and genetic recombination (24). Such a correlation between the loss of this type of enzymatic activity and the "recombinationless" character of one class of radiation-sensitive mutants was first described by Buttin and Wright in *Escherichia coli* (3, 26). Enzymes with similar biochemical properties are now known to be widely distributed among the prokaryotes (1, 2, 8, 21, 25).

In the present report, we describe the results of investigations on the fate of transforming DNA in two isogenic strains differing only in the recombination and DNA repair phenotype associated with the ATP-dependent deoxyribonuclease. Our conclusion is that the enzyme is not necessary for the steps leading to the formation of the presumed primary recombinant structure, a heteroduplex containing one donor strand and one recipient strand of DNA (6). Rather, we believe that the enzyme may be involved in the trimming of excess polynucleotides from the donor or recipient strands of such a structure, or both, after its formation.

MATERIALS AND METHODS

Strains. R1-26, the strain currently used as recipient for transformation assays in the laboratory of R. D. Hotchkiss (9), was transformed to streptomycin resistance with the *str-r*41 marker (5, 18). The resulting strain, henceforth referred to as *rec*$^+$, was the recipient strain into which the recombination-deficient marker of strain A5-5-9A (24) was introduced by transformation. The resulting *rec*$^-$ strain was isolated by selecting γ-radiation sensitive transformants as previously described (24). The agar plate method for obtaining γ-radiation survival curves, the method of determining transformation frequency as a function of irreversibly bound DNA, and the ATP-dependent deoxyribonuclease assay have been described (23, 24). D. Morrison very generously supplied the strain used for labeling DNA with tritiated thymidine: erythromycin resistant, *ery-r*2 (16); aminopterin resistant; thymidine requiring, *thy*$^-$.

Transforming DNA. The *thy*$^-$ strain was grown in NS medium (19) containing an excess of glucose and supplemented with thymidine and thymidine-*methyl*-^3H (17-20 Ci/mmole, Schwarz/Mann) at a concentration of about 44 μCi/1.25 μg of thymidine per ml of medium. Dilute NaOH was added during growth whenever needed to maintain neutrality. The

cells were harvested in late logarithmic phase, and the DNA was isolated essentially by the method described by Hotchkiss (12). However, the ribonuclease step consisted of treatment not only with boiled ribonuclease A (Worthington) but also with ribonuclease T_1 (Calbiochem): 125 units/ml for 15 min at 37 C followed by an additional 125 units/ml for 15 min at 37 C. The ^{3}H-DNA in the stock solution was more than 99.9% acid insoluble and contained approximately 0.3% alkali-labile material as measured by treatment with 1 N NaOH for 20 hr at room temperature. The DNA concentration was estimated by absorbance (23). The specific activity was 6×10^5 to 8×10^5 counts per min per μg of DNA as determined by counting a diluted sample on glass fiber paper (GF/A, Whatman) in BBOT scintillation fluid (23) with the ^{3}H preset channel in the Intertechnique liquid scintillation spectrometer model SL 36. To minimize the extent of radiochemical damage, the ^{3}H-DNA was used within 7 days of its isolation.

DNA uptake. Cells were grown for several generations in the transformation medium used in this laboratory, essentially the glucose medium described in detail by Lacks and Hotchkiss (15), to a light turbidity (approximately 10^7 colony-forming units per ml). Glycerol was added to 10% (v/v), and the culture was divided into samples and stored at −70 C. To prepare competent cells, a sample of these precultures was used to inoculate 300-ml volumes of fresh medium. The cells were grown to that density which a previous experiment had shown to be ideal for maximal competence, chilled in an ice-water bath for 5 min, and incubated at 30 C for 20 to 35 min. The ^{3}H-DNA was added to a final concentration of 0.2 to 0.4 μg/ml. Two min later, 20 μg of thymidine per ml was added. The DNA exposure, limited to 5 min, was terminated by 3 to 8 μg of deoxyribonuclease I (Worthington) per ml. Samples of 25 or 50 ml were withdrawn to chilled tubes which were swirled in ice-water for 5 min. The viable count, the number of erythromycin transformants, and the average chain length (microscope examination) were determined. The cells were washed twice with solution D (7), twice with SC (0.15 M sodium chloride 0.1 M sodium citrate), and lysed with 5 μliters of 5% deoxycholate in the presence of 50 μliters of 5% Sarkosyl (Geigy Industrial Chemicals) while suspended in 0.8 ml of SC per 50-ml culture sample. The suspension was kept at room temperature until it completely cleared (7–15 min) and stored on ice for several hours before being analyzed in CsCl or on sucrose gradients.

CsCl and sucrose gradients. Alkaline CsCl solutions (*p*H 11) were prepared by combining 0.6 ml of the crude lysate with 2.4 ml of a stock CsCl solution (14) and by overlayering with 2 ml of mineral oil. The mixtures, in polyallomer tubes, were centrifuged at 37,000 rev/min for 45 to 60 hr in an SW50 rotor (Beckman) at 13 C. Four-drop fractions were collected from a Buchler tube piercing unit onto 2.4-cm Whatman 3MM filter discs. About 90 fractions were obtained where the drops in the last 20 or so fractions appeared to be smaller in volume. The filter discs were washed in acid, air dried, and counted (23). To prepare neutral sucrose gradients, 0.3 ml of a 60% (w/v) sucrose solution was used as a shelf over which was layered 4.7 ml of a 5 to 20% linear sucrose gradient. The sucrose was prepared in 1 M NaCl, 1 mM ethylenediaminetetraacetic acid, and 10 mM Tris-HCl, *p*H 7.6. The gradients, in polyallomer tubes, were overlayered with 0.1 ml of the crude lysate and centrifuged at 35,000 rev/min for 120 min in the SW50 rotor at 6 C. Twenty-drop fractions were collected and treated as above.

Native and alkali-denatured DNA analysis in CsCl. To denature the DNA, 10 μliters of ^{3}H-DNA (79 μg/ml) was mixed with 0.79 ml of 0.85% NaCl and 0.1 ml of 0.9 N NaOH. After 10 min at room temperature, the solution was neutralized by the addition of 0.1 ml of 0.9 N HCl. Then 10 μliters of the denatured DNA and 10 μliters of a 100-fold dilution of the native ^{3}H-DNA stock solution were added to a 50-ml culture sample that had been previously exposed to deoxyribonuclease and washed twice with both solution D and SC. The cells were immediately lysed, and the lysate was analyzed in alkaline CsCl as described above.

RESULTS

The rec⁻ transformant. The *rec*⁺ strain was transformed with DNA from the *rec*⁻ strain A5-5-9A. The transformant obtained, selected by its increased sensitivity to γ-radiation (Fig. 1), lacks the ATP-dependent deoxyribonuclease activity in addition to being

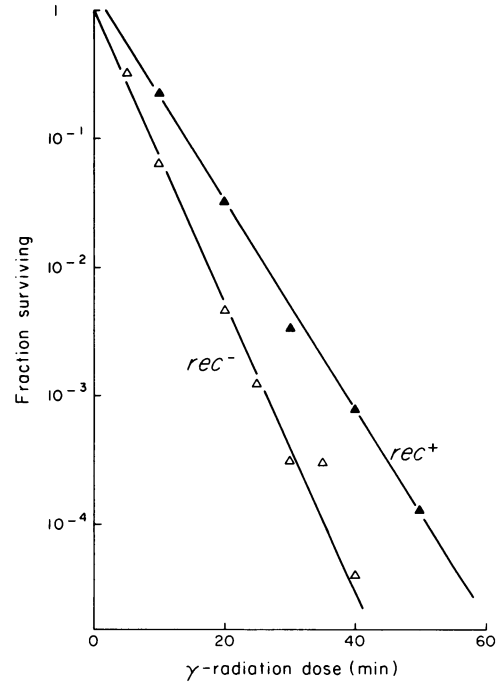

FIG. 1. *The fraction of colony-forming units surviving as a function of γ-radiation dose. The irradiation was carried out as previously described (24) with ^{60}Co as the source of the γ-radiation. The dose rate was approximately 1,500 R/min.*

deficient in genetic recombination (Table 1). The γ-radiation sensitivity of strain A5-5-9A and the *rec*$^-$ transformant is the same. These three phenotypic characters (increased sensitivity to γ-radiation, deficiency in recombination, and loss of the ATP-dependent deoxyribonuclease activity) along with an increased sensitivity to ultraviolet radiation have already been shown to transfer as a single marker in genetic transformation experiments with strain A5-5-9A (24). Thus, the defect in the enzymatic activity is presumably responsible for the increased radiation sensitivity and the decreased recombination proficiency.

Alkaline CsCl density-gradient analyses. In an attempt to determine the physiological function of this enzyme in the recombination process, the fate of transforming DNA was studied in the *rec*$^-$ transformant with alkaline CsCl density-gradient centrifugation techniques. Lacks et al. (14) reported that the density-gradient patterns show similar relations between the components with either neutral or alkaline (*p*H 11) CsCl analyses of donor DNA from recipient pneumococcal cells. However, under alkaline conditions, single-stranded DNA is more completely resolved from double-stranded DNA as Vinograd and his co-workers originally reported (22). The density-gradient pattern observed under *p*H 11 conditions shows essentially quantitative resolution of single-stranded from double-stranded DNA even in the presence of relatively large amounts of native, recipient DNA, i.e., about 1 mg (Fig. 2).

With DNA uptake being limited to 5 min, the distribution of donor DNA in an alkaline CsCl gradient was followed as a function of time incubated at 30 C in the two isogenic strains which presumably differ only in a single mutation affecting recombination proficiency. A typical set of density-gradient patterns so obtained is shown in Fig. 3. The *rec*$^+$ and *rec*$^-$ patterns not only are remarkably similar, but they also closely resemble the observations reported earlier by Lacks and his co-workers (14) with a wild-type pneumococcal strain as recipient. Only two bands of acid-insoluble material are found in the gradient: one coinciding with the single-stranded DNA and the other with the double-stranded DNA. Initially, the majority of the acid-insoluble donor radioactivity is found in the former, while the remainder bands with the latter. With increasing time, the amount of tritium in the native position increases as that in the single-stranded region decreases.

Figure 4 summarizes the results from three such analyses. There appears to be no significant difference between the *rec*$^+$ and *rec*$^-$ strains in: (i) the donor radioactivity found in the single-stranded (about 80%) and double-stranded (about 20%) regions at t_0; (ii) the rate of disappearance of the former (half-life of

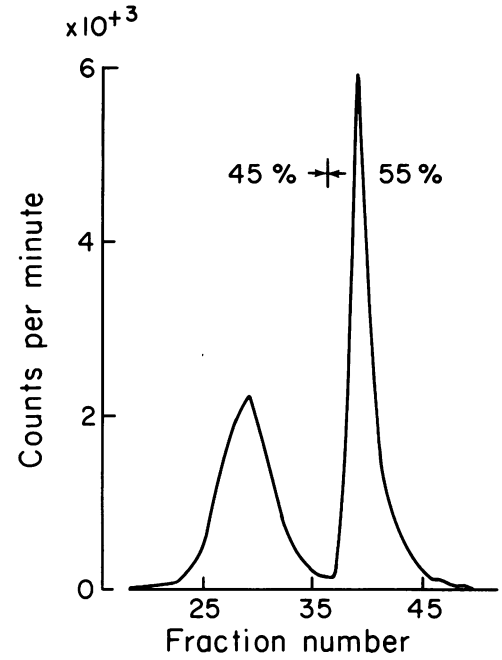

FIG. 2. *CsCl density-gradient analysis of native and alkaline-denatured DNA at pH 11. An equal number of counts per minute of* ^{3}*H-labeled native and alkaline-denatured DNA in a cell lysate was centrifuged in CsCl. The percent of the radioactivity recovered as single-stranded (left) and double-stranded material (right) is indicated.*

TABLE 1. *Characteristics of bacterial strains*

Strain	ATP-dependent deoxyribonuclease activity (units/mg of protein)[a]	Normalized recombination frequency[b]
rec$^+$	0.53	1.00
rec$^-$	<0.01	0.18[c]
A5-5-9A	<0.01	0.15

[a] One unit of enzymatic activity is that amount of enzyme which hydrolyzes 1 nmole of DNA to acid-soluble fragments per min under standard reaction conditions (23).

[b] As defined by Vovis and Buttin (24) except that ^{3}H-DNA from pneumococcus was used here to measure both DNA uptake and genetic recombination.

[c] Average of eight separate experiments.

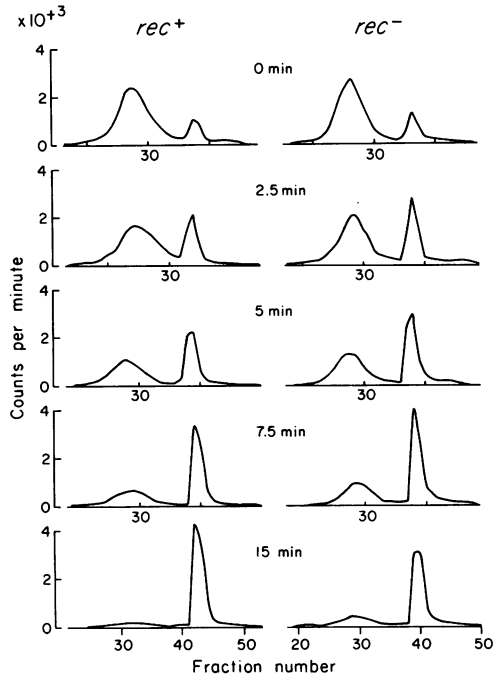

FIG. 3. *Fate of transforming DNA in rec⁺ and rec⁻ cells. Cells, incubated for the indicated times at 30 C after 5 min of exposure to ³H-labeled pneumococcal DNA, were lysed, and the crude lysates were analyzed in alkaline CsCl (pH 11). The gradient fractions, collected on paper discs, were acid washed. Patterns have been aligned with respect to the native DNA peak (right).*

approximately 6 min) or of appearance of the latter; (iii) the extent of the initial, irreversibly bound radioactivity (radioactivity removable neither by extensive washing nor by deoxyribonuclease treatment) converted to double-stranded material.

Neutral sucrose analyses. The donor DNA in the *rec⁺* and *rec⁻* recipient strains was also sedimented immediately after termination of DNA exposure and 30 min later on preformed neutral sucrose gradients to estimate the relative size of the material with which it was associated (Fig. 5). Again, there is no significant difference between the results observed with the two strains. At t_0, roughly 20% of the acid-insoluble tritium is associated with rapidly sedimenting material, whereas, 30 min later, at a time when all the acid-insoluble donor label is found in the native DNA region of a CsCl gradient (Fig. 4), the vast majority of the acid-insoluble label sediments rapidly. Presumably, the amount of rapidly sedimenting material is an indication of the donor label physically associated with chromosomal DNA.

DISCUSSION

The present study was undertaken in an attempt to elucidate the role the ATP-dependent deoxyribonuclease plays in the transformation process. Greatly simplified, transformation in pneumococcus is thought to involve the destruction of one of the strands of transforming DNA during or very shortly after entry of the donor DNA molecule into the recipient cell and the physical insertion of the surviving strand into the recipient chromosome to yield a DNA molecule that is physically and genetically hybrid. The single-strandedness of donor DNA immediately after uptake can be demonstrated by neutral or alkaline CsCl density-gradient analysis (reference 14; Fig. 3) and by methylated albumin-coated Kieselguhr columns (13). The physical destruction of the one donor strand may, however, be preceded by the intervention of DNA repair processes, for a single, heteroduplex DNA molecule containing two markers *trans* to each

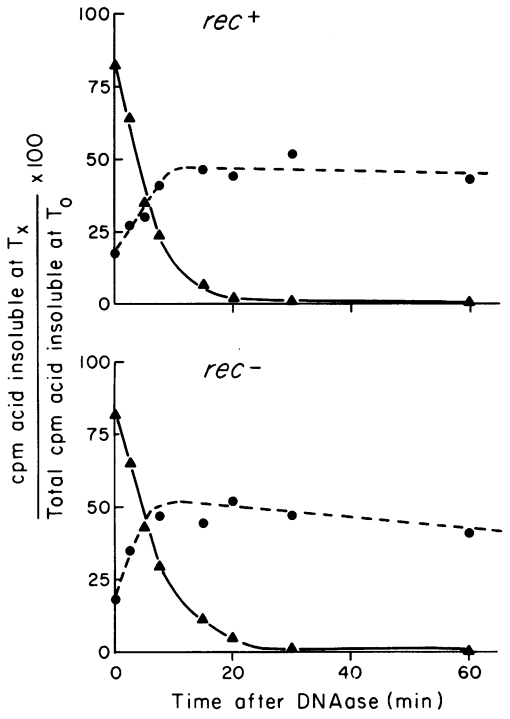

FIG. 4. *Fate of transforming DNA in rec⁺ and rec⁻ cells. Experimental details were as described for Fig. 3. Values shown are the averages from three experiments, each one using a different ³H-DNA preparation. Acid-insoluble counts per minute banding in alkaline CsCl (pH 11) in the single-stranded DNA region (▲) and in the double-stranded DNA region (●).*

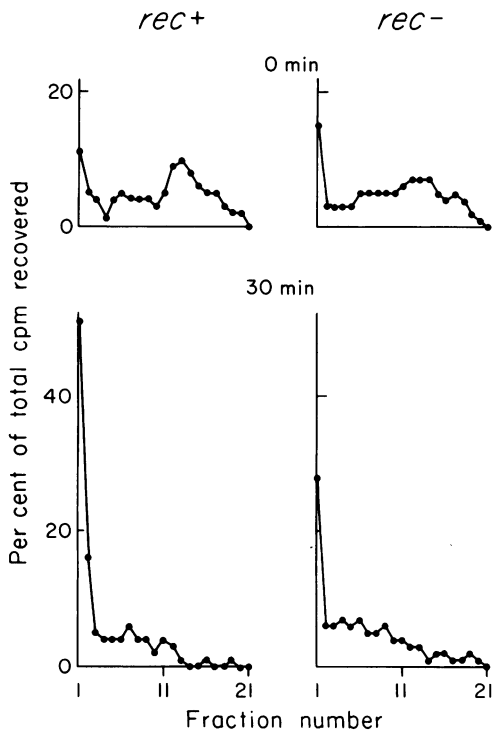

FIG. 5. *Neutral sucrose fractionation of donor DNA from a recipient cell lysate. Lysates were prepared from cells that had been incubated at 30 C for the indicated times after 5 min of exposure to ^{3}H-DNA. The crude lysates were analyzed in preformed, neutral sucrose gradients. The fractions were collected on paper discs and acid washed. Sedimentation was to the left.*

other can give rise to a transformant containing both markers (17). The appearance of donor label in native DNA at the expense of the single-stranded material (references 13, 14; Fig. 3) argues for the temporal sequence: double-stranded → single-stranded → double-stranded. Such a single-strand insertion into the recipient chromosome was demonstrated by the identification of a hybrid containing large stretches of donor atoms in one strand (6). Furthermore, the fact that individual transformed cells give rise to mixed clones suggests the existence of a genetic hybrid in the original transformed cell (10, 11).

The enzyme-deficient *rec*$^-$ strain is approximately six times less efficient in transformation than its *rec*$^+$ parent (Table 1), yet there does not appear to be any difference between the *rec*$^+$ and *rec*$^-$ strain in DNA uptake (Vovis, *unpublished data*) or in the qualitative or quantitative fate of such irreversibly bound donor DNA through the stage where a single strand of donor DNA is physically associated with the recipient DNA (Fig. 3, 4, 5). Thus, the ATP-dependent deoxyribonuclease does not appear to be necessary for any of the steps leading to this stage in transformation; rather it is probably required in a later step.

Presumably, the inserted single strand becomes physically associated with the recipient chromosome first through hydrogen bonding and then through covalent linkage, i.e., joint molecules precede recombinant molecules (20). The transition from one structure to the other probably requires the action of nuclease(s) to trim, polymerase(s) to extend, and ligase(s) to link polynucleotide sequences. The ATP-dependent deoxyribonuclease might be the hypothetical nuclease required in this transition reaction, i.e., it may trim overlapping polynucleotides from the recipient or donor strand, or both. Such a physiological function would be similar in a general way to that proposed by Radding (4) for the λ exonuclease and is not inconsistent with what is presently known of the in vitro activity of the pneumococcal enzyme (Vovis, *unpublished data*). Experiments are planned to test this supposition.

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