Competent *Diplococcus pneumoniae* Accept Both Single- and Double-Stranded Deoxyribonucleic Acid

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Received for publication 9 October 1969

The transforming activity of fractionated complementary strands of *Diplococcus pneumoniae* deoxyribonucleic acid (DNA) bands at the position of fully denatured DNA in CsCl at pH 11.0, and is completely (>99.8%) destroyed by digestion with exonuclease-I. These results prove that pure single strands transform the normally prepared competent cells of this species. Their efficiency is about 0.5% that of native DNA of comparable size.

The usual preparations of competent *Bacillus subtilis* and *Haemophilus influenzae* do not accept single deoxyribonucleic acid (DNA) strands (1, 2, 13, 18), but in specifically altered conditions they will take up single strands in preference to helical DNA (3, 4, 16, 17). Denatured DNA is more active than the native form in the T4 phage transformation system in *Escherichia coli* spheroplasts (20).

In *Diplococcus pneumoniae* the situation was less clear. In a number of studies from our laboratory (6, 8, 14), DNA which was clearly denatured, as determined by its position in a CsCl density gradient, showed substantial biological activity. Conclusions about strand fractionation, transcription, and single-strand integration into the chromosome have, however, been independent of whether the activity resided in pure singles or in fragments with a small degree of helical structure, such as cross-linked hairpins with unequal length arms. Because the biological properties correlated with those of the physically dominant structure (titration in alkali, binding of ribopolymers, renaturation, etc.), it was clear that even when there was a short segment of complementary strand, the longer strand was the one which entered the cell.

To learn more about the requirements for DNA entry into recipient cells, we have reexamined this question. The results demonstrate that normally prepared cultures of competent pneumococci accept single strands, with an efficiency about 200-fold lower than for native DNA of comparable strand length.

**MATERIALS AND METHODS**

**Strains, media, and assay.** Bacterial strains, media, preparation of competent recipient cultures and transformation procedures are described elsewhere (15).

DNA preparations. DNA species were prepared by the method of Marmur (12). Sedimentation coefficients were calculated from boundary runs in the analytical ultracentrifuge. For native DNA, S\text{0.01} was corrected to S\text{0.0}, and molecular weight was calculated by the method of Eigner and Doty (5). The method of Studier (19) was used for alkaline runs and single-stranded DNA.

**Single-strand breaks.** DNA species were nicked with pancreatic deoxyribonuclease-I (Worthington Biochemical Corp., Freehold, N.J., 1X crystallized). Deoxyribonuclease-I (0.10 ml) at 10 \(\mu\)g/ml in 10^{-4} M NaCl and 0.1\% bovine serum albumen (BSA) was added to 9.9 ml of 10 M sodium acetate (pH 5.5)-5 M MgCl\(_2\)-0.2 M NaCl-0.05\% BSA. A 0.9-ml amount of this solution was added to 10.8 ml of KP\(_1\) (pH 8.0) and 400 \(\mu\)g DNA in approximately 0.28 ml. The reaction mixture was incubated 15 min at 20 \(^\circ\)C and stopped by the addition of 0.4 ml of 0.5 M ethylenediaminetetraacetate (EDTA), pH 8.0.

**Heat denaturation.** DNA samples were heat-denatured in 0.015 M NaCl and 0.02 M KP\(_1\), pH 10.80, by the method of Alberts and Doty (1) at concentrations in the range of 10 to 20 \(\mu\)g/ml. Samples were held for 10 min at 100 \(^\circ\)C and then quickly cooled to 0 \(^\circ\)C.

**Exonuclease-I treatment.** *E. coli* exonuclease-I was a gift from Worthington Biochemical Corp. Yeast transfer ribonucleic acid (tRNA; lot 6501, Schwarz Bio Research Inc., Orangeburg, N.Y.) at a concentration of 20 mg/0.1 ml in 0.01 M tris(hydroxymethyl)aminomethane (pH 7.5) and 0.02 M MgCl\(_2\) was boiled for 10 min at 80 \(^\circ\)C, diluted to 0.2 mg/ml, and added to the enzyme solution to inhibit a slight endonuclease-I activity. Dilution of enzyme and reaction media were essentially those of Lehman and Nussbaum (11). Transfer RNA was added to the diluting solution at a ratio of 1 n mole per unit enzyme. Reactions were stopped by diluting portions into cold 1 X SSC (0.15 M NaCl plus 0.015 M sodium citrate) or by quick cooling to 0 \(^\circ\)C.
Reaction with ribopolymer. The procedure of Peterson and Guild (14) was followed. Copoly G,U stock 343 with an output ratio of 1:1.1 was purchased from Miles Laboratories.

Preparative fractionation in CsCl. All preparative fractionations were done in polyallomer tubes in an SW 39 rotor with a Spinco model L preparative ultracentrifuge. Runs were carried out at approximately 20 C.

RESULTS

To test the hypothesis that covalently cross-linked structures account for all activity in denatured pneumococcal DNA, the behavior of a preparation deliberately nicked was compared to that of one essentially free of single-strand breaks (Fig. 1). In the latter case, most cross-linked structures should band in CsCl near the position of native DNA, as first observed by Alberts and Doty (1). Though there is more activity in this region in the unnicked DNA than in the nicked preparation, the bulk of the activity is still associated with structures which behave in neutral CsCl density gradients as though they were fully denatured. Note that:

(i) there is only 1 μg of denatured DNA in each gradient; (ii) the native reference DNA is not trapped under the denatured peak; and (iii) in the case of the unnicked DNA, that fraction of the activity which behaves as though it were natively is well resolved from the denatured peak. Aggregation may be ruled out, therefore.

The biological activity in the denatured region could still be due to structures cross-linked to short segments of a second strand, or to a small amount of renaturation after fractionation. To test these possibilities we employed exonuclease-I (11), which acts only on free 3'-OH ends of single-stranded DNA, and CsCl gradients at pH 11.0, which separate denatured DNA four-fold further from native DNA than do neutral gradients (21). Figure 2 shows the specificity of the exonuclease-I preparation for denatured DNA and, in addition, includes various controls. Activity in the bulk-denatured DNA was digested only to 50% (we have seen 30 to 60%). The lowest curve, however, represents fractions from the fully denatured region of a CsCl gradient at pH 11.0, and only 5% of the activity resisted the enzyme. In the latter experiments, however, progressive renaturation occurred in the gradient at pH 11. The patterns were nonreproducible and showed most of the activity in the intermediate density region.

To minimize the renaturation problem, we used...
strands prefractionated by reaction with poly G, U (14). After removing residual ribopolymer with alkali and self-annealing the DNA to maximize the renaturation of any contaminating complementary strands, the samples were fractionated according to secondary structure in CsCl at pH 11.0. Seventy percent of the activity of the light strands (Fig. 3) was at the position of fully denatured DNA, and, after digestion with exonuclease-I, none of this activity remained (0 colonies observed versus 888 prior to digestion). (In the total gradient, 16% of the activity resisted digestion, all in the intermediate density range and in good agreement with the 21% observed in bulk, prior to the final fractionation step.) The heavy strand sample showed a similar result, all the activity under the fully denatured peak being susceptible to exonuclease-I digestion.

The specific activity (transformants per microgram of DNA in solution) of the single strands in the light sample is shown in Table 1 to be about 0.4% of that of a native DNA, at concentrations at which DNA is limiting. The median size of the fractionated-strand preparations, after the manipulations, was 2.1×10⁶ daltons, whereas that of the native control was 7×10⁶. After correction for the dependence of activity on length (9),

Table 1. Relative efficiency of single-strand DNA, corrected for double-strand contamination

<table>
<thead>
<tr>
<th>Expt</th>
<th>DNA conc*</th>
<th>Native DNA²</th>
<th>Strands³</th>
<th>Percent efficiency⁴</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Before</td>
<td>After</td>
<td>Corrected to pure single</td>
</tr>
<tr>
<td>A</td>
<td>0.001</td>
<td>39,000</td>
<td>640</td>
<td>230</td>
</tr>
<tr>
<td></td>
<td>0.01</td>
<td>480,000</td>
<td>5,400</td>
<td>1,600</td>
</tr>
<tr>
<td>B</td>
<td>0.001</td>
<td>55,000</td>
<td>550</td>
<td>120</td>
</tr>
<tr>
<td></td>
<td>0.01</td>
<td>450,000</td>
<td>3,000</td>
<td>800</td>
</tr>
</tbody>
</table>

* By absorbance at 258 nm; values expressed as micrograms per milliliter.
² Median single-strand molecular weight = 7×10⁶; data indicate transformants to streptomycin resistance per milliliter of cells.
³ Light-strand fraction from a poly G, U fractionation after digestion of residual ribopolymer with alkali, dialysis, and annealing at 65°C for 5 hr in 2×SSC. Median single-strand molecular weight = 2.1×10⁶. See Fig. 3 for the distribution of this preparation in CsCl, pH 11.
⁴ Ratio of single to native; mean, 0.45%.

Twice the residual activity "after" subtracted from the "before" figure, on the assumption that half of all partially double-strand structures are inactivated by exonuclease-I. This is the most conservative estimate; the true efficiency of the strands is probably higher.

The relative efficiency of single strands would be 0.5 to 0.6% that of native DNA of comparable strand length. We have not been able so far to find conditions where only denatured DNA is active.

DISCUSSION

In D. pneumoniae, in contrast to B. subtilis and H. influenzae, the bulk of the residual activity in denatured DNA is associated with the fully denatured fraction in neutral (Fig. 1) or pH 11.0 (Fig. 3) CsCl density gradients. It also runs close to the absorbance pattern in alkaline sedimentation velocity experiments and comes off hydroxyapatite columns in the denatured region (Miao, unpublished data). These results differ from those of Mulder and Fox, cited in Mulder and Doty (13), in which it was stated that the pneumococcal system showed results like those of H. influenzae. Even though the activity is susceptible to exonuclease-I digestion, a criterion usually accepted as proof of single strand character, it could be argued that complements associate temporarily in solution or on the cell surface and thus are aided in entry. By removing

Fig. 3. Distribution in CsCl, pH 11.0, of transforming activity of purified light DNA strands. DNA was separated into complementary-strand fractions by reaction with poly G, U in CsCl. The light-strand fractions were pooled, incubated in 0.2 N KOH, dialyzed overnight to remove ribopolymer, and fractionated in pH 11.0 CsCl with a native DNA reference (X). Fractions were assayed before (●) and after treatment with exo-I for 60 min (○). Total DNA was 1 μg of each species. Run at 28,500 rev/min, 72 hr. The density difference between peak fractions is about 0.06 g/ml under these conditions.
one of the complements entirely, however, we have eliminated this possibility. The sole remaining argument is that cross-links occur in such a way that the long arm of an asymmetric hairpin structure is always terminated by 3' -OH. The burden of proof of such an argument may be left to the proponent.

We conclude that single strands of DNA transform normally prepared competent cultures of *D. pneumoniae* with an efficiency about 0.5% that of native DNA of comparable strand size. From the work of Lerman and Tolmach (10), it is evident that the lower efficiency is largely or entirely at the entry step.

ACKNOWLEDGMENTS

This investigation was supported by Public Health Service grant GM-10965 from the National Institute of General Medical Sciences, by AEC Contract AT-(40-1)-3941, and by a grant from the United Medical Research Fund.

We thank Verane L. Lee for technical assistance.

LITERATURE CITED