Transforming Activity in Both Complementary Strands of Bacillus subtilis DNA

Abstract. *Under conditions favoring single-strand transformation, the two complementary strands of* Bacillus subtilis *DNA, separated by differential complexing with polyriboguanylic acid, have identical transforming activity. Moreover separated single strands, upon renaturation with unmarked (recipient) DNA, form heteroduplex molecules with similar double-strand transforming activity. These findings bear upon the mechanism of DNA integration.*

The development of a method for separating the complementary strands of DNA fragments from *Bacillus subtilis* (*1*, *2*) makes it possible to determine whether both strands are capable of generating transformants. Until recently, this question could be approached only by renaturing the separated strands with unmarked (recipient) DNA to form two kinds of heteroduplex DNA, for single strands did not appear active in *B. subtilis* transformation (*3*, *4*). We now have evidence that under modified assay conditions, single-stranded DNA exhibits substantial transforming activity (*5*, *6*). This finding allows a direct assay for transforming activity in separated complementary strands.

Evidence for transforming activity in single-stranded DNA (*6*) can be summarized briefly. The residual transforming activity in denatured DNA from *B. subtilis* has been the subject of several studies and has been ascribed to a minor fraction of native-like DNA molecules which resist denaturation (*3*, *4*) apparently because they bear covalent crosslinks (*4*). I likewise have found that native-like ("residual") DNA molecules are the source of biological activity in denatured DNA under normal assay conditions. In addition, however, I have observed substantially greater transforming activity in denatured DNA when $10^{-3}M$ ethylenediaminetetraacetic acid (EDTA) is included in the transformation medium. This concentration of EDTA inhibits the transforming activity of native and "residual" DNA approximately threefold. The major active component in the presence of EDTA is single-stranded DNA, judging by its buoyant density and its sedimentation characteristics; "residual" DNA of native-like buoyant density and different sedimentation properties is detectable as a minor active component under these conditions (*5*). While the exact role of EDTA in effecting single-strand activity is unknown, this phenomenon may be related to the enhancement by EDTA of single-strand transformation in *Hemophilus influenzae* (*7*) and to the increase in permeability of *Escherichia coli* after EDTA treatment (*8*).

I have made no attempt to develop a new competence regimen for single-strand transformation, but have used selected batches of *B. subtilis* 168 (try_2^-) bacteria prepared by a modification (*9*) of the method of Young and Spizizen (*10*). Batches vary widely in single-strand competence, which does not correlate with that for native DNA. In order to test for single-strand competence, cells are exposed to denatured DNA (0.1 μg/ml) with and without $10^{-3}M$ EDTA in the transformation medium. Cells producing several times as many transformants in the presence of EDTA have a useful degree of single-strand competence.

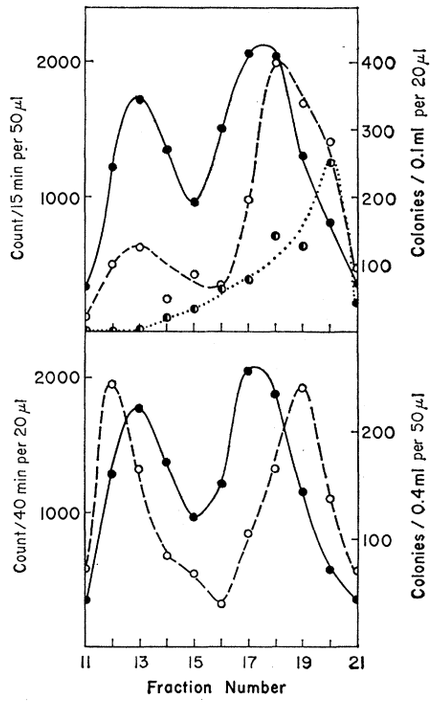

Fig. 1. Biological activity of H and L complexes separated by CsCl equilibrium density-gradient centrifugation: ——, C^{14}; - - - - - - - -, single-strand transforming activity; . . . . , "residual" DNA transforming activity. From a 5-ml gradient (Spinco SW50L rotor) containing 13.5 μg complexed DNA, 34 fractions were collected into 1 ml of distilled water. (Top) Portions were assayed directly for radioactivity and transformining activity. (Bottom) Each fraction was dialyzed successively against: $10^{-3}M$ phosphate buffer, *p*H 7, at 5°C; $0.1N$ NaOH at 37°C for 12 hours; $0.01M$ tris. *p*H 7.3, at 5°C. Transforming activity and radioactivity were again assayed. Of single-strand transforming activity, 47 percent is in the H peak and 53 percent in the L.

Donor C^{14}-DNA (3400 counts min^{-1} μg^{-1}) was isolated by the method of Marmur (*11*) from cells (in the late log phase of growth) of *B. subtilis* W23 labeled with uracil-2-C^{14} (0.5 μg/ml, 30 mc/mmole, New England Nuclear) for one generation prior to harvesting. Unlabeled recipient DNA was similarly isolated from cells of strain 168 in the maximum stationary phase of growth.

For separation of complementary strands, DNA-polyriboguanylic acid (polyG) complexes were formed by a modification (*12*) of the method of Kubinski *et al.* (*2*) and separated by preparative CsCl equilibrium density-gradient centrifugation. As shown in Fig. 1, about 37 percent of the C^{14} appears in the heavy (H) peak and 63 percent in the light (L). Strand resolution is apparently imperfect, but, as shown below, the strands of DNA fragments containing the try_2 locus are separated in the CsCl gradient more effectively than average DNA strands (C^{14} profile).

When gradient fractions are assayed for transforming activity without any prior treatment to free them from polyG, less single-strand transforming activity occurs in the H peak than in the L (Fig. 1, upper). Considerable activity can be detected (without EDTA) in "residual" DNA of still lighter density. Alkali treatment to eliminate polyG resulted in two nearly equal peaks of single-strand transforming activity (Fig. 1, lower). "Residual" DNA activity was selectively lost during alkali treatment and was negligible in these samples. Survival of single-strand activity was also low (about 10 percent), but alkali degradation presumably is not strand specific; the low recovery therefore should not alter the shape of the profile. It thus appears that, if the two strands of DNA fragments containing try_2^+ (try_2^+-DNA) have been separated by this method, they have equal ability to transform.

In order to assess the extent of strand resolution for try_2^+-DNA, the

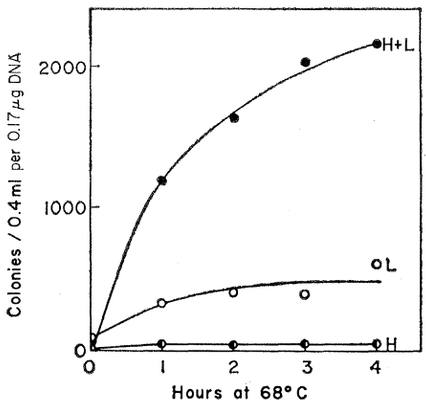

Fig. 2. Renaturation kinetics for DNA in the H and L peaks and for a mixture of H and L DNA (1:1). Double-strand transforming activity is plotted against annealing time. Alkali-treated fractions pooled from the gradient of Fig. 1 (fractions 11 to 14 for H, 17 to 21 for L) were concentrated to 10 μg/ml by evaporation at 37°C in an air stream. For renaturation, 0.1 ml of DNA solution [H, L, or H + L (1:1)] and 20 μl of 3*M* KCl were mixed, and 20-μl portions were sealed into capillaries for incubation at 68°C. Double-strand transforming activity was assayed without EDTA.

ability of H and L DNA to renature separately and in 1 : 1 mixture was measured by appearance of double-strand try_2^+ transforming activity (Fig. 2). The fact that H DNA fails to renature alone, although it does so readily when mixed with L, shows that H DNA contains only one of the complementary strands of try_2^+-DNA. Light DNA alone does renature slowly, indicating slight contamination by the opposite complementary strand. If it is assumed that DNA renaturation is bimolecular, one can calculate the extent of this contamination. In that DNA from the L peak renatures at about one-fifth the rate of a 1 : 1 mixture of H and L DNA and contains twice as much L, it must contain one-tenth as much H, or 5 percent. Thus DNA in the L peak is about 95 percent pure.

The distribution of try_2^+-DNA is therefore approximately 48 percent in the H peak and 52 percent in the L peak. This correlates closely with the observed distribution of single-strand transforming activity (Fig. 1): 47 percent in the H peak and 53 percent in L. Taken together, these results indicate that both H and L complementary strands have transforming activity and that they are equally effective in single-strand transformation.

The possibility remains that, in transformation by double-stranded DNA, there could be a strand-selective step which is circumvented by single-strand transformation. There is considerable evidence that in double-strand transformation of *B. subtilis*, only one of the two DNA strands taken up by a given bacterium transmits its genetic information to progeny (*13*, *14*). It is of interest to determine whether either complementary strand, or only one, can be the genetically effective one in a native donor DNA molecule. This can be determined by renaturation of separated single strands with unmarked (recipient) DNA and comparison of the double-strand transforming efficiency of the two kinds of heteroduplex DNA produced.

The source of separated single strands for this experiment was a sample of the same DNA-polyG complex described (Fig. 1), fractionated in a separate CsCl gradient, but treated identically. The profile of single-strand transforming activity after alkali treatment (Fig. 3, right) was similar to that in Fig. 1, except that slightly higher activity was found in the L peak (58 rather than 53 percent). Individual alkali-treated fractions were concentrated and mixed with denatured recipient DNA, and renaturation rates were measured by appearance of double-strand transforming activity. The initial slope of the renaturation curves (see Fig. 3, left, for typical kinetics) was taken as the relative renaturation rate for each fraction (Fig. 3, right).

If one assumes that bimolecular renaturation kinetics are applicable, one would expect the rate of appearance of try_2^+ double-strand transforming activity to be proportional to the concentration of try_2^+ single strands and to that of try_2^- complementary strands (constant for all samples). An additional factor in the relative rate might be an efficiency coefficient reflecting any difference in transforming efficiency (E_H and E_L) for the heteroduplex molecules:

$$\frac{H\ +}{L\ -} \quad \text{and} \quad \frac{H\ -}{L\ +}$$

The ratio of renaturation rate to concentration of try_2^+ strands (as measured by single-strand transforming activity) for each fraction (Fig. 3, right) is an estimate of the relative transforming efficiency, which is uniform across each peak but appears to differ between the two (Fig. 3, right), E_H/E_L being approximately 1.5.

While the significance of this apparent small difference in transforming efficiency is questionable, qualitatively it is clear that each strand of duplex DNA can act as donor of genetic information. The high purity of try_2^+ H strands demonstrated earlier leaves little doubt that the H^+/L^- heteroduplex molecules formed from them are biologically active. It is also apparent that H^-/L^+ molecules are active since most of the heteroduplex transforming activity is generated by L DNA. Thus strand selection bias in native DNA transformation, as in single-strand transformation, appears small or nonexistent.

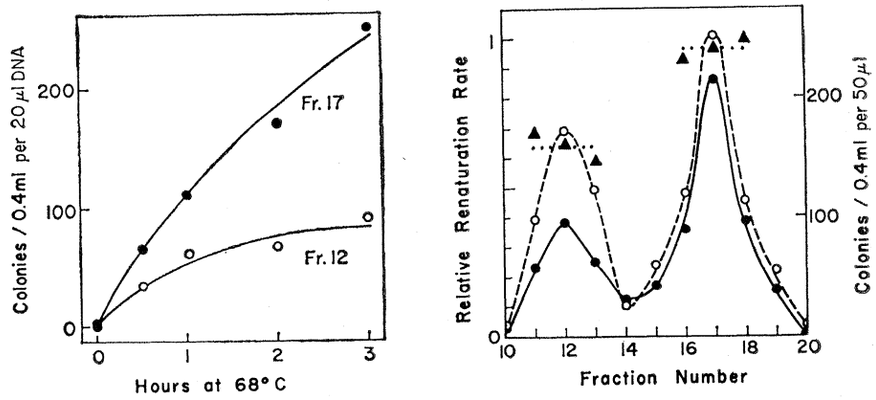

Fig. 3. Double-strand transforming activity of two kinds of heteroduplex DNA formed by renaturation of H and L fractions with recipient DNA. Single-strand transforming activity of alkali-treated fractions is shown at right (.....); the fractions were prepared as described for Fig. 1, and concentrated fivefold; 50 μl (<0.4 μg DNA) was mixed with 50 μl of recipient DNA (20 μg/ml, denatured at 100°C for 5 minutes) and 20 μl of 3*M* KCl. Renaturation kinetics for each fraction were measured as described for Fig. 2; the kinetics for the two peak fractions are shown at left. The relative rate of renaturation (initial slope) for each fraction is plotted at right (——). Relative transforming efficiency of renatured DNA (ratio of renaturation rate to single-strand transforming activity) is indicated for the peak fractions (. . . .).

Physical studies have thus far failed to show conclusively whether one or both strands of native donor DNA are physically integrated into the *Bacillus subtilis* recipient genome (*15*). The fact that only one donor strand is genetically effective in a given cell (*13*, *14*) argues against double-strand integration; but it does not rule out the possibility that two strands are integrated and one is enzymatically repaired to match the other. This double-strand integration model is, however, difficult to reconcile with the finding that either strand can be the genetically effective one. The putative repair enzyme would have to correct exclusively the H strand in one cell and exclusively the L strand in another over a region of several genes (*13*). The need for this unlikely repair specificity makes the double-strand integration model less attractive. A single-strand integration model is more consonant with the findings reported here, which would then imply that either strand can be integrated.

Both complementary DNA strands have been found effective in transformation of *Diplococcus pneumoniae* and *Hemophilus influenzae* (*16*) as well as of *Bacillus subtilis*. All three likewise have been found susceptible to transformation by single-stranded DNA (*5*, *7*, *17*). Physical studies have shown single-strand integration in *D. pneumoniae* and *H. influenzae* (*18*), and segregation studies suggest the same mechanism for *B. subtilis* (*13*, *14*). It thus appears that there are extensive similarities underlying the seeming diversity of these three transformation systems.

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Antibody Formation: Stimulation by Polyadenylic and Polycytidylic Acids

Abstract. *Complexes of polyadenylic and polyuridylic acids, or of polycytidylic acid and methylated bovine serum albumin, enhance the early rate of increase in numbers of antibody-forming spleen cells in mice immunized with sheep red blood cells or other particulate antigens. Polyadenylic and polycytidylic acids, respectively, appear to be the source of the critical stimulators which, as demonstrated by others in bacteria, may act by influencing nucleotide kinase activity. The stimulated antibody response, but not the normal response, is antagonized by kinetin riboside and by an adenosine derivative occurring in sRNA.*

Oligodeoxyribonucleotides, such as those obtained after digestion of calf-thymus DNA by pancreatic deoxyribonuclease, enhance the early rate of increase in numbers of antibody-forming spleen cells (*1*), as determined after immunization of mice with sheep red blood cells. The active oligomers also enhance cell multiplication and rates of DNA synthesis in Gram-positive bacteria (*2*, *3*). In bacteria, these stimulatory effects are produced by di- to hexanucleotides (*4*), and tests with pneumococci have indicated that the effects on DNA synthesis are the consequence of stimulated nucleotide kinase activity (*3*). In studies on antibody-forming cell populations of mice, we have observed that stimulatory effects, equal to those observed after oligodeoxyribonucleotide administration, can also be obtained with the aid of homopolymers of certain ribonucleotides. Thus, homopolymers of adenylic acid (polyA) or cytidylic acid (polyC) injected in appropriate form—simultaneously with, shortly before, or shortly after—the injection of antigen enhance antibody formation. Firshein subsequently demonstrated that these homopolymers also stimulate DNA synthesis and nucleotide kinase activity in pneumococci (*5*).

CF-1 or AKR mice, weighing approximately 20 g, were injected in the tail vein with 10^8 or 10^9 sheep erythrocytes per animal; at the same time, polyA, polyC, or polyU (polyuridylic acid) was injected intravenously in quantities ranging from 100 to 1000 μg per animal. Forty-eight hours later, the animals were killed, and the number of spleen cells forming or releasing antibody to sheep red blood cells was determined (*6*); groups of five animals each were used in all of these assays. No stimulated responses were obtained when any of the three homopolymers were injected at time of antigen administration. However, a mixture of all three (750 μg each of polyA, polyC, and polyU per mouse) produced a significant increase in the number of hemolysin-forming spleen cells (per 10^8 spleen cells) 48 hours later (an average of 854.4 in contrast to 246.8 in the controls receiving antigen only). Testing of the homopolymers in pairs revealed that the combined administration of polyA and polyU was responsible for the stimulation (Table 1). When polyA and polyU were injected separately into different sites (one intraperitoneally and the other intravenously) no stimulation occurred; a degree of stimulation did occur when they were administered consecutively into the same site at 30-minute intervals, but not at 3-hour intervals (Table 1). These observations suggested that a double-stranded polymer of polyA and polyU, readily