Defective Bacteriophage PBSH in *Bacillus subtilis*

III. Properties of *Adenine-16* Marker in Purified Bacteriophage Deoxyribonucleic Acid

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Received for publication 1 August 1969

The *adenine-16* (*ade-16*) marker (the marker nearest the chromosomal origin of *Bacillus subtilis*) in purified PBSH deoxyribonucleic acid (DNA) renatured more rapidly and to a greater extent than any other marker in the phage DNA, and more rapidly and to a greater extent than all markers, including *ade-16*, in bacterial DNA. The renaturation of the phage DNA *ade-16* marker followed a first-order reaction, whereas renaturation of bacterial markers was initially a second-order reaction. No cross-linkages were detected in DNA molecules containing the *ade-16* marker. Buoyant density measurements and inactivation by heat and micrococcal deoxyribonuclease of the *ade-16* marker did not reveal large segments of clusters of the individual bases in these molecules. Alternative mechanisms for the unique renaturation behavior of the *ade-16* marker are discussed.

Induced PBSH phages of *Bacillus subtilis* contain exclusively bacterial deoxyribonucleic acid (DNA; 6, 10, 11). DNA extracted from PBSH particles induced from the mitomycin C-sensitive strain MY2Y1U5 is 10- to 20-fold richer in the origin-region marker [*adenine-16*(*ade-16*)] compared to host cell DNA. This DNA is of homogeneous molecular weight and small (about 12×10^6 daltons) and thus offers an opportunity for studying biophysical and biochemical properties of the initiation region of the bacterial chromosome. To elucidate the physical and biochemical nature of the initiation (origin) region of the bacterial chromosome, this paper describes the marker density distribution of PBSH DNA, its denaturation and renaturation properties, the differential heat-inactivation of various markers, and enzymatic marker-inactivation experiments.

It was observed that the renaturation properties of the *ade-16* marker of the phage DNA differed from other markers of the phage DNA and from all markers, including *ade-16*, of bacterial DNA. The cause of this unusual behavior of the *ade-16* marker will be discussed.

MATERIALS AND METHODS

Preparation of phage, phage DNA, and bacterial DNA, sucrose gradients, strains used, and transfor-

mation procedures have been described previously (5, 6).

Denaturation and renaturation. Phage and bacterial DNA in SSC (0.15 M NaCl plus 0.015 M sodium citrate) were denatured in tightly-closed glass or polycarbonate tubes, or both. Heat-denaturation was carried out in a boiling water bath for 12 min in glass tubes, 20 or 30 min in polycarbonate tubes. The temperature in the polycarbonate tubes rose very slowly and approached 100 C after immersion for 15 min. After heating, tubes were put into crushed ice. In spite of the slow cooling in the polycarbonate tubes, this procedure assured denaturation of the DNA. Tubes containing the samples to be renatured were transferred to a 65 C bath, both with and without prior cooling at 0 C. Essentially no difference was observed between denaturation in the two kinds of tubes, although it was originally suspected that much denatured DNA would remain stuck to the glass tubes. Samples (0.25 ml) were withdrawn with a plastic pipette from the renaturing tube and put into precooled tubes in ice. Alkali-denaturation, when used, was done by adding 1 part of 1 M NaOH to 20 parts of a solution of native DNA. After 10 min at room temperature, solutions were brought to pH 6 to 8 by addition of N HCl in 0.2 M tris(hydroxymethyl)aminomethane (Tris).

CsCl density gradient centrifugation. High resolution CsCl density gradients were run for 60 hr in a Spinco no. 50 fixed-angle rotor at 40,000 rev/min at 24 C. Samples (3 ml) were placed in 4-ml tubes (Spinco no. 303404) in a special adaptor (Spinco no. 303957). Gradients were collected through the bottom of the tubes.

Marker inactivation. Heat-inactivation of markers

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was carried out by heating DNA samples at a concentration of 4.5 μ g/ml in tightly sealed polycarbonate tubes in a thermostatically controlled water bath for 30 min. Regulation was within 0.1 C. After heating, the samples were cooled in ice water.

The micrococcal nuclease (Worthington Biochemical Corp., Freehold, N. J.) reaction was done at 10 C in siliconized glass tubes. All samples were in duplicate. The reaction mixture (1.65 ml) contained 0.03 μ g of enzyme, 2 optical density (OD) units (at 260 nm) of DNA in a solution containing 0.01 M NaCl, 0.02 M borate buffer (pH 8.8), and 0.04 M CaCl₂. The reaction was terminated by pipetting 0.1 ml of the reaction mixture into a tube containing 0.02 ml of 0.8 M MgCl₂ in ice; 0.05 ml of each sample was used in the transformation assay.

RESULTS

Renaturation of PBSH-extracted DNA. Physical separation of phages containing the *ade-16* marker from the rest of the phage population was unsuccessful with either sucrose gradient velocity sedimentation or CsCl density sedimentation, and neither could *ade-16*-transforming DNA be separated from the bulk of the phage DNA by either method. We studied the denaturation-renaturation properties of the origin-derived DNA fragments by following the transformation activity of phage DNA during the denaturation-renaturation cycle. If there were differences in conformation or composition between the *ade-16* marker (present in molecular fragments containing the chromosomal origin) and the bulk of the bacterial DNA in phage particles (differences which were not made evident by physical separation methods), they might show up in renaturation experiments. Purified phage DNA in SSC was denatured by heat (or alkali) treatment for 12 to 30 min (see above). Denaturation and renaturation were followed by means of transformation. Figure 1 shows a typical result. Under conditions where the methionine (*met*) and other markers showed little recovery of biological activity, the activity of the *ade-16* marker was completely recovered. A value of more than 100% *ade-16* activity was found to be recovered in this experiment. Since the transformation assay was carried out with a nearly saturating DNA concentration, all but that portion of the transforming DNA containing *ade-16* markers was in the denatured state. Because double-stranded DNA, but not single-stranded DNA, is known to compete for sites in the competent cells (12), the lack of competition during the renaturation period caused the apparent recovery of more than 100% *ade-16*-transforming activity. In additional experiments, when renaturation was performed at a lower concentration (4.5 μ g/ml), 70 to 80% *ade-16* activity was commonly recovered.

Figure 2 shows a control experiment. DNA extracted from spores of the host cell was denatured and renatured, and the renaturation was again followed by transformation of suitable recipient cells. In these experiments 20 to 25% renaturation of biological activity was regularly obtained.

Renaturation of fully denatured (e.g., strand-separated) DNA is approximately second-order as a function of time, whether measured as a decrease in OD at 260 nm (13) or as an increase in transforming activity (J. Lanyi, Thesis, Harvard University, Cambridge, Mass., 1963). The kinetic order of renaturation of denatured DNA is not affected by the DNA concentration in the reaction mixture in the range of 1.7 to 77 μ g/ml or by

FIG. 1. Assay by transformation of renaturation of PBSH DNA at 65 C in SSC (0.15 M NaCl plus 0.015 M sodium citrate, pH 7.0). Phage DNA (18 μ g/ml in SSC) was heated for 12 min at 100 C. One sample [native (N)] was taken just before denaturation by heat; another (D) was quenched in ice-water immediately after heating. The tube with DNA to be renatured was transferred to a bath at 65 C after heating. Samples were used to transform strain LMA-16 (*leu*⁻, *met*⁻, *ade-16*⁻, MU8U5U16).

FIG. 2. Renaturation of bacterial DNA (4.5 μ g/ml in SSC) at 65 C. After heating the DNA at 100 C for 12 min, the samples were allowed to renature for various times. At the indicated times, renaturation was stopped by quenching in ice-water. Samples were used to transform strain LMA-16.

ionic strengths of the reaction buffer of between 0.02 to 1.0 M in salts like NaCl (13). The order of a renaturation reaction may be determined by plotting the reciprocal concentration of the reacting species versus time. In a semilog plot, a first-order reaction yields a straight line. In a linear plot, deviations from a second-order reaction will show as deviations from a straight line. The renaturation reaction consists of two steps:

Two species of denatured molecules (the complementary strands) combine with second-order kinetics to form a "nucleated" intermediate. The reaction occurs by diffusion, and results in a species of double-stranded DNA connected by only a short segment of hydrogen bonding (4 to 7 nucleotide pairs). This "in register" polydeoxyribonucleotide pair then "zips up" (8) by forming all possible hydrogen bonds. The second reaction is of first-order kinetics.

In Fig. 3 the kinetic plot of bacterial DNA renaturation is shown. For at least 2.5 hr, renaturation is a bimolecular reaction. The corresponding kinetic plot for renaturation of the *ade-16* marker in phage DNA is shown in Fig. 4. Here the rate of renaturation was of first order. In repeat experiments with DNA at a concentration of 4.5 $\mu\text{g/ml}$, *ade-16* in phage DNA was shown to renature with first-order kinetics and with an equal rate constant (within a factor of 2). It is therefore possible that in renaturation of the *ade-16* marker DNA, the limiting reaction is the zippering-up reaction, whereas nucleation is fast and not measured by the transformation assay.

These results raise the possibility that the phage DNA molecules carrying the *ade-16* marker either carry a cross-link between complementary strands, or else are of a hairpin form. However, this would seem to be ruled out by the fact that the molecular weight of the majority of phage DNA molecules is halved upon denaturation (5). A direct search for cross-links would clarify the situation.

Ade-16-carrying PBSH DNA molecules are not cross-linked. A 35- μg amount of phage DNA in 0.2 N NaOH was sedimented through a 5 to 20% alkali (0.2 N NaOH) sucrose gradient. After dilution with distilled water, the fractions of denatured DNA were used without neutralization for transformation studies (Fig. 5). Addition of the small amount of alkali to the competent cell samples did not change their pH or their transformation efficiency. The denatured DNA fractions show two small peaks of activity (D),

FIG. 3. Second-order rate constant plot for renaturation of bacterial DNA. A 4.5 $\mu\text{g/ml}$ concentration of DNA was heat-denatured at 100 C for 12 min in SSC. The reaction rate is initially second-order, then changes to first order when it is about one-third complete. The initial rate constants are k_2 (adenine-16) = 27×10^{-4} ml per μg per min and k_2 (methionine) = 29.8×10^{-4} ml per μg per min. T_∞ , transformants after 9.5 hr of renaturation (see Fig. 2). T_t , transformants after indicated time of renaturation.

one at the position of the bulk of the sedimented DNA, coinciding with the optical density peak, and another at the position where denatured cross-linked molecules are expected, with a sedimentation value $1.4 \times$ that of the single strands of half the molecular weight (1).

After dialysis of these fractions against SSC and 8 hr of renaturation at 65 C, the peak of *ade-16*-transforming activity was observed to coincide with the bulk of the sedimenting material having separated strands (R). No peak with the increased sedimentation rate was found. The activity associated with the cross-linked DNA duplexes was 10% of that found for the renatured peak. Assuming 80% renaturation of the fractions at the DNA concentrations used and considering that the denaturation-resistant frac-

FIG. 4. First-order reaction rate plot (normalized) for renaturation of the *ade-16* marker in a heat-denatured PBSH DNA sample (Fig. 1). Rate constant: $K_1 = 3.7 \times 10^{-3}$ per min. Renaturation in SSC at 65 C. T_∞ , transformants after 10 hr of renaturation (see Fig. 1). T_t , transformants after indicated time of renaturation. T_0 , transformants of denatured samples.

tion re-forms its double helical structure immediately upon neutralization in the transformation mixture (1), we obtained a value of 8% for the fraction of cross-linked molecules in the phage DNA preparation. This value is similar to that reported for the fraction of cross-linked molecules present in bacterial DNA isolated from *Bacillus subtilis* phage PBS2 (2). However, when the number of cross-links per unit length of DNA is calculated, PBSH DNA is found to contain some 17-fold more cross-links than PBS2 DNA. The significance of this is not clear. Nevertheless, this experiment shows conclusively that the bulk of the phage *ade-16* activity resides in non-cross-linked molecules.

Search for unique nucleotide sequence as an explanation for the anomalous renaturation behavior of the *ade-16* marker in PBSH DNA. An alternative explanation for the rapid first-order renaturation of the phage *ade-16*-transforming DNA was investigated. The model must accommodate two difficulties in the renaturation behavior. Renaturation of the *ade-16* marker follows first-order kinetics, so the second part of the renaturation reaction [the zippering up (8)] would be the rate-limiting step. In the absence of a cross-link, nucleation must therefore be so rapid

that it was not detected by our assay method. Nevertheless, the zippering-up reaction is known to be very rapid, whereas the *ade-16* marker renatured slowly. One explanation for the observed renaturation data is schematically shown in Fig. 6. If a homopolymer or alternating polymer exists in the same molecule with the *ade-16* marker, it would cause rapid nucleation, but biological activity would not be recovered until the two nucleated strands have been brought completely into register by means of a slippage mechanism (7). In our transformation assay, only fully native molecules have substantial activity, whereas partially single-stranded molecules are inactive (3, 13).

We sought evidence by various means for the existence of clusters of high adenine-thymine (A-T) or guanine-cytosine (G-C) homo- or copolymeric regions in DNA molecules containing the *ade-16* marker. Figure 7 shows two high-resolution CsCl density gradients of purified spore DNA and purified phage DNA run in a Spinco no. 50 fixed-angle rotor. Transformation of the gradient fractions revealed that DNA fragments transforming two of the markers tested, *ade-6* and *met*, had an average buoyant density different from that of the bulk by 0.004 g/cm³ (4 "density units"). The peak of *ade-6* activity occurred at a higher density, that of

FIG. 5. Alkali sucrose gradient of purified PBSH DNA. A 35- μ g amount of PBSH DNA was layered on top of a 5 to 20% alkaline (0.2 N NaOH) sucrose gradient. After 4.5 hr of sedimentation at 4 C and 37,000 rev/min, fractions were collected through the bottom of the siliconized tube. Fractions were diluted with distilled water and used in the denatured state to transform LMA-16 competent cells (D). After neutralization and renaturation of the fractions for 8 hr at 65 C, they were again used to transform the same recipient cells (R).

FIG. 6. Renaturation model to account for the renaturation data of *ade-16* phage DNA. Nucleation is rapid due to the presence of a repeating sequence somewhere along the DNA molecule. Slippage must occur during zippering up for biological activity to be restored. The slippage mechanism causes the first-order zippering-up reaction to slow down.

met activity at a lower density, than the bulk of the DNA. There was no difference between the density of the markers in PBSH DNA and in bacterial DNA extracted from spores. This experiment showed that if clustering of nucleotides exists in the PBSH DNA containing the *ade-16* marker, it may be assumed to be of low molecular weight. The dense marker (*ade-6*) probably owed its increased density to ribosomal genes which are located near *ade-6* (4) and have a base composition different from that of bulk DNA (9). (GC content of ribosomal RNA of *B. subtilis* is 55% and of bulk DNA, 43%.) We do not know the reason for the low density of the *met* marker.

Differential marker inactivation would detect the existence of high AT- or GC-containing clusters in phage DNA containing the *ade-16* marker. Samples of phage DNA and bacterial control DNA were incubated for 20 min at temperatures near the melting temperature and then rapidly quenched in crushed ice. The presence of a substantial and unique AT or GC cluster in *ade-16*-transforming phage DNA would cause denaturation and loss of the *ade-16* marker activity at a temperature respectively lower or higher than for the nonunique control DNA. No difference could be detected in marker inactivation between the two samples, confirming the fact that such clusters, if present, must be of low molecular weight (Fig. 8). The DNA mole-

cule was unstable at the inactivation temperature. When the biological activity of the partially inactivated molecule was measured, large variations were found from one experiment to the other, even when the temperature was controlled to within 0.1 C. This instability is reflected in the large variation seen in Fig. 8 at the inactivation temperature. The *met* marker had lower buoyant density and a lower temperature of inactivation than the *ade-16* marker (Fig. 7).

Micrococcal nuclease is known to hydrolyze AT-rich regions in DNA at low temperatures (15). By treating PBSH DNA and control bacterial DNA with this enzyme at 10 C and assaying for remaining transforming activity, we expected to be able to detect unique clusters in *ade-16* phage DNA by differential inactivation of

FIG. 7. High resolution CsCl density gradient of phage DNA and control DNA. A 20- μg amount of purified DNA was, in each case, run in a Spinco no. 50 fixed-angle rotor for 60 hr at 40,000 rev/min at 24 C. Fractions were collected through the bottom of the tubes and diluted with 0.3 ml of SSC. A 0.02-ml amount of DNA was used to transform 1 ml of competent cells. Recipient cells were LMA-16, LMA-6 (*leu*, *met*, *ade-6*, MU8U5U6), LMH-A (*leu*, *met*, *his-A*, MU8U5U3), and LMTyr (*leu*, *met*, *tyr*, MU8U5U7). The number of transformants obtained for each recipient cell was not normalized with reference DNA.

FIG. 8. Marker inactivation by heat. PBSH DNA in SSC and spore DNA in SSC, both at $4.5 \mu\text{g/ml}$, were heated to the indicated temperatures in tightly capped polycarbonate tubes for 30 min and quenched in ice. The denatured samples were used to transform strain LMA-16 to assay for differential marker inactivation. The relative temperature regulation was within 0.1°C . Broken lines, phage DNA. Solid lines, control DNA.

these molecules. No difference was detected. All markers tested showed a survival of $1.5 \times 10^{-4} \text{ min}^{-1}$, when the enzyme concentration in the reaction mixture was $0.1 \mu\text{g/ml}$ (see above).

DISCUSSION

Various experiments were made in an attempt to explain the anomalous renaturation behavior of *ade-16* phage DNA. On the basis of the existing correlation between transformation activity of *B. subtilis* DNA and its "nativeness," the *ade-16* phage DNA molecules were observed to undergo anomalous renaturation. The experiments described ruled out cross-linking of these molecules. Other possible explanations for the observed results are that the phage *ade-16* molecules contain a unique nucleation region or that only the *ade-16* molecules are cut in a unique way, from the chromosome origin up to a length corresponding to 1.2×10^7 daltons, during DNA packaging into phage heads, whereas molecules carrying other markers are cut and packaged in a random manner.

If a cluster high in AT or GC is indeed present in these molecules, it must be rather small or we should have detected it in one of the above experiments. The most sensitive method used to detect such a cluster was micrococcal nuclease digestion. It is possible, however, that a nucleotide cluster is present near the *ade-16* marker but positioned at an end of that chromosome segment. Rapid hydrolysis of such a cluster would not greatly reduce the total molecular weight of that segment. Therefore, no significant

decrease of *ade-16* activity would be detected by the transformation assay. It is clear, therefore, that if a nucleotide cluster is indeed present, it must be of moderate size or else of an alternating sequence,

... ACACAC ...
... TGTGTG ...

which we would not have detected.

Another possibility remains, that only the *ade-16* molecules are truly unique and double-stranded, whereas all other phage DNA molecules renature to form duplexes with single-strand ends which have reduced biological activity. The repeated denaturation and renaturation of PBSH DNA facilitated genetic separation of *ade-16* from other markers. It is hoped that these unusual *ade-16*-transforming phage DNA molecules will yield information on the structure of the bacterial DNA initiation region.

ACKNOWLEDGMENTS

This investigation was supported by grants NGR-05-003-020 from the National Aeronautics and Space Administration and E-517 from the American Cancer Society to the University of California, Berkeley.

We are grateful to Estelle Cook, Barbara Jansen, and Joyce Cerwin for their technical assistance and to Thomas H. Jukes for his interest and support.

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