The Anatomy of the SP50 Bacteriophage DNA Molecule

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Evidence is presented which indicates that SP50 bacteriophage contain non-permuted, linear, duplex DNA molecules with a molecular weight of \(100 \times 10^4\) daltons. Alkaline sucrose sedimentation studies show that about half of both of the SP50 single polynucleotide chains contain interruptions, apparently located at random, although the exact number of interruptions depends on the host strain.

**INTRODUCTION**

The *Racillus subtilis* bacteriophage SP50 was first isolated and characterized by Földes and Trautner (1964) and Földes and Molnár (1964). Studies by Földes and Trautner (1964) and Biswal *et al.* (1967) have shown that each SP50 bacteriophage contains a single, duplex DNA molecule. In 1966, Dr. Thomas Trautner informed us (personal communication) that the SP50 DNA molecule appeared to contain a significant number of single-strand interruptions. We suspected that this molecule might be similar to the T5 DNA molecule (Abelson and Thomas, 1966) and therefore an unusual case worthy of further study. In the following report, we confirm and extend the findings of Biswal *et al.* (1967).

**MATERIALS AND METHODS**

_Growth and purification of bacteriophage_. SP50 (obtained from T. Trautner) were grown by infecting at a multiplicity of 5:1 to 10:1 *B. subtilis* W-168 or W-23 (obtained from N. Sueoka) in the logarithmic phase of growth (concentration of host cells \(\leq 7 \times 10^6\)/ml). Strain W-168 was used in all experiments except where indicated otherwise.

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The medium used for growth of the host cells was either a tryptone broth containing 0.8% Difco Bacto Tryptone, 0.5% NaCl, 0.1% Difco Yeast Extract, 10\(^{-2}\) M MgCl\(_2\) and 10\(^{-4}\) M MnCl\(_2\) or a low phosphate medium containing 0.1 M Tris, pH 7.5, 0.1% Difco Casamino Acids, 0.1% Glucose, 0.05% NaCl, 1.6 \(\times\) 10\(^{-4}\) M Na\(_2\)SO\(_4\), 10\(^{-3}\) M MgSO\(_4\), 10\(^{-4}\) M CaCl\(_2\), 10\(^{-4}\) M MnCl\(_2\), and 10\(^{-2}\) M MgCl\(_2\). Radiotopes were added to the growing host cells several hours prior to infection at the following final concentrations: \(^3\)P0\(_4\) (Nuclear Consultants Corporation) \(\leq 7 \mu\)Ci/ml, thymidine-\(^3\)H (Schwarz Bio-Research, Inc.) = 15 to 20 \(\mu\)Ci/ml. After infection, aerated incubation at 37\(^\circ\)C was continued for 70–90 minutes.

Immediately after lysis, the SP50 lysate was centrifuged at 7000 rpm for 5 min in the number 30 rotor of the Spinco Model L ultracentrifuge. The supernatant was decanted into a second centrifuge tube and the phage suspension was centrifuged at 25,000 rpm for 0.5 hour in the above-mentioned rotor. The phage pellet was resuspended in 1 ml Z buffer (0.1 M Tris, pH 7.5, 0.1 M NaCl, 10\(^{-2}\) M MgCl\(_2\), and 10\(^{-4}\) M MnCl\(_2\)) by agitation on a Vortex Jr. mixer for less than 10 min. In some cases this differential centrifugation procedure was repeated a second time.

The resulting 1 ml phage suspension was loaded on top of a 5–25% (w/v) linear sucrose gradient in Z buffer. The zone sedi-
mentation purification was accomplished by centrifugation at 20,000 rpm for 20 min in a Spinco SW-25 swinging-bucket rotor. The phage band was collected and then dialyzed against 0.1 M NaCl and 0.01 M Tris, pH 7.5–8.0. The A260 per plaque-forming unit (PFU) was generally near 2.5 × 10^{-11} cm²/PFU.

T2 bacteriophage were grown and purified as described by Thomas and Abelson (1966). T7 bacteriophage were grown and purified according to the procedure in Thomas and Abelson (1966) as modified by Kelly (1968).

**Extraction of DNA.** Native DNA was extracted using freshly redistilled phenol that was saturated with 0.5 M Tris, pH 8.0. Each bacteriophage suspension was mixed in equal 1.5-ml volumes with this redistilled phenol in a 50-ml conical screw-capped tube. The tube was rolled at 60 rpm for 30 min at room temperature. The phenol and aqueous phases were separated by centrifugation for 3 min at 3000 rpm in a Servall table-top centrifuge. The phenol layer was removed with a Pasteur pipette, and the procedure was repeated with fresh phenol. Residual phenol was removed by extensive dialysis against 0.01 M Tris, pH 8.0, 0.05 M NaCl.

The DNA concentration and spectrum were determined in a Zeiss spectrophotometer. The specific absorbancy at 260 μm (E260) was taken as 0.020 cm²/μg. In general the E260/E230 ratio was above 2.00 and the E260/E280 ratio was above 1.75.

Denaturation was accomplished by bringing the DNA solution to 0.1 M in either NaOH or Na3PO4. In some cases the SP50 DNA was simultaneously extracted and denatured by mixing the phage suspension with a freshly prepared solution of Na3PO4 to yield a final concentration of 0.1 M Na3PO4 (Abelson and Thomas, 1966).

3H P22 DNA, 32P T3 DNA, and 32P T5 DNA used in these experiments were gifts from M. Rhoades, D. Ritchie, and T. Pinkerton of this laboratory.

**SW-39 sucrose density gradient centrifugation.** The generation of and properties of SW-39 sucrose gradients have been described before (Blattner and Abelson, 1966). The following kinds of gradients were used in this study: (1) alkaline sucrose gradients—5–20% (w/v) linear sucrose gradients in 0.9 M NaCl and 0.1 M NaOH; (2) neutral sucrose high salt gradients—5–20% (w/v) linear sucrose gradients in 1.0 M NaCl and 0.01 M Tris, pH 8.5; (3) neutral sucrose gradients—5–20% (w/v) linear sucrose gradients in 0.1 M NaCl and 0.05 M PO4 buffer, pH 6.8.

**SW-25 sucrose density gradient centrifugation.** Linear, 5–25% (w/v) sucrose gradients in 1.0 M NaCl, 0.01 M Tris, pH 8.5, were formed in SW-25 cellulose nitrate centrifuge tubes by means of a mixing device similar to one described by Boek and Ling (1954). The DNA was extracted and denatured using Na3PO4 as described above. The denatured DNA solution was reneutralized with an appropriate volume of 1.0 M NaH2PO4 and 1.0 ml of this sample was loaded onto the top of the gradient. Centrifugation was as noted in the legends of Figs. 2 and 3. Samples were collected as usual and radioassayed by scintillation or Geiger counting.

**Electron microscopy.** Solutions containing about 2 μg/ml DNA in 2 × SSC (0.3 M NaCl, 0.03 M sodium citrate) were mixed with equal volumes of 0.04% cytochrome c (Sigma Chemical Corporation) in 7.2 M NaClO4, 0.1 M Tris, pH 7.2. The electron microscopy was accomplished as described by MacHattie and Thomas (1964) and Kleinschmidt et al. (1960).

**Hydroxyapatite chromatography of DNA.** The procedure used for distinguishing single-stranded from double-stranded DNA through the use of hydroxyapatite was done according to Bernardi (1965) and Miyazawa and Thomas (1965) as modified by Rhoades et al. (1968).

**RESULTS**

**Molecular Weight of Native SP50 DNA**

Phenol-extracted SP50 DNA labeled with either 32PO4 or thymidine-3H sedimented in neutral sucrose gradients as a single sharp zone which was unaccompanied by any noticeable amounts of leading or trailing material (Reznikoff, 1967). Therefore it is unlikely that these DNA preparations are contaminated by significant quantities of
TABLE 1
MOLECULAR WEIGHT OF NATIVE SP50 DNA
AS DETERMINED BY SUCROSE
SEDIMENTATION STUDIES*

<table>
<thead>
<tr>
<th>Expt. No.</th>
<th>Standard DNA</th>
<th>M. W. of standard DNA (daltons)</th>
<th>( \frac{D_{20,6}}{D_{0.4}} )</th>
<th>M. W. SP50 DNA (daltons)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>T7</td>
<td>( 24.3 \times 10^6 )</td>
<td>1.652</td>
<td>101.6 ( \times 10^6 )</td>
</tr>
<tr>
<td>2</td>
<td>T7</td>
<td>( 24.3 \times 10^6 )</td>
<td>1.043</td>
<td>99.9 ( \times 10^6 )</td>
</tr>
<tr>
<td>3</td>
<td>T7</td>
<td>( 24.3 \times 10^6 )</td>
<td>1.714</td>
<td>112.8 ( \times 10^6 )</td>
</tr>
<tr>
<td>4</td>
<td>T3</td>
<td>( 24.3 \times 10^6 )</td>
<td>1.735</td>
<td>116.8 ( \times 10^6 )</td>
</tr>
<tr>
<td>5</td>
<td>T3</td>
<td>( 24.3 \times 10^6 )</td>
<td>1.703</td>
<td>110.8 ( \times 10^6 )</td>
</tr>
<tr>
<td>6</td>
<td>P22</td>
<td>( 26.5 \times 10^6 )</td>
<td>1.649</td>
<td>109.0 ( \times 10^6 )</td>
</tr>
<tr>
<td>7</td>
<td>T2</td>
<td>( 130 \times 10^6 )</td>
<td>0.937</td>
<td>108.0 ( \times 10^6 )</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>M.W. Ave.</td>
<td>= 108.6 ( \pm 6.0 ) ( \times 10^6 )</td>
</tr>
</tbody>
</table>

* Calculations assume the Burgi-Hershey (1963) relationship of \( D_1/D_2 = (M_1/M_2)^{0.33} \) where \( D \) is taken as the distance between the center of gravity of a given peak and the meniscus of the gradient in drop number and \( M \) is the molecular weight in daltons.

Molecular weight as reported in Thomas and MacHattie (1967).

Molecular weight as reported in Rhoades et al. (1968) and Thomas and MacHattie (1968).

low molecular weight substances such as teichoic acids.

The molecular weight of native, phenol-extracted SP50 DNA was measured by cosedimentation studies with T7, T3, P22, and T2 DNA's through neutral sucrose gradients. The formula of Burgi and Hershey (1963), \( D_1/D_2 = (M_1/M_2)^{0.33} \), was used to relate the molecular weights (\( M_1 \) and \( M_2 \)) of the two DNA species to the distances traveled (\( D_1 \) and \( D_2 \)). The results of several such experiments are presented in Table 1. These data indicate that the molecular weight of the SP50 DNA molecule is 108.6 \( \pm 6.0 \times 10^6 \) daltons.

Native SP50 DNA molecules were also visualized by means of electron microscopy. A histogram of all molecules photographed and traced is presented in Fig. 4C. All molecules were linear. Their average length was 49.1 \( \pm 2.2 \mu \), which, assuming a linear density of 192 daltons/angstrom, is equivalent to a molecular weight of 94.3 \( \times 10^6 \) daltons.

These results are in agreement with those of Biswal et al. (1967), who arrived at molecular weights of 95.4 \( \times 10^6 \) daltons (electron microscopic measurement), 102 \( \times 10^6 \) daltons (sedimentation measurement), and 97 \( \times 10^6 \) daltons (viscosity measurement) for the SP50 DNA molecule.

Sedimentation of Denatured SP50 DNA

A typical alkaline sucrose profile of alkalinenatured SP50 DNA is shown in Fig. 1: a sharp peak is followed by decreasing amounts of trailing material containing no recognizable peaks. In experiments not shown, denatured SP50 DNA was mixed with denatured T5 or T7 DNA labeled with an alternate radioisotope, and, in another experiment, denatured SP50 and T2 DNA's were sedimented in parallel alkaline sucrose gradients. The peak positions of these single polynucleotide chains are shown by arrows in Fig. 1. Using these as markers, and

![Fig. 1. Alkaline sedimentation profile of denatured SP50 DNA. 32P-labeled SP50 phage was mixed with an equal volume of 0.2 M Na3PO4 and sedimented through an alkaline sucrose gradient in the SW-39 rotor by centrifugation for 2.25 hours at 35,000 rpm at 20°. Fractions were collected from the bottom of the tube. The arrows indicate the peak positions of indicated standard denatured DNA molecules when labeled with an alternate radioisotope and mixed with the SP50 DNA prior to centrifugation or, in the case of T2, when centrifuged in a parallel alkaline sucrose gradient. The dashed line denotes the hypothetical symmetrical peak of uninterrupted single chains. The counts at the bottom of the tube are thought to represent molecules which have collided with the walls of the tube during centrifugation (Abelson and Thomas, 1966).](image-url)
TABLE 2

Molecular Weight of Polynucleotide Chains Contained in the Leading Peak (LP) as Determined by Alkaline Sucrose Sedimentation Studies

<table>
<thead>
<tr>
<th>Expt. No.</th>
<th>Standard polynucleotide chain</th>
<th>M. W. of standard polynucleotide chain (daltons)</th>
<th>( D_{12}/D_{86} )</th>
<th>M. W. of leading peak polynucleotide chains (daltons)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>T5 (14 ( \mu ) chains)</td>
<td>14.9 ( \times 10^6 )</td>
<td>1.580</td>
<td>49.9 ( \times 10^6 )</td>
</tr>
<tr>
<td>2</td>
<td>T5 (34 ( \mu ) chains)</td>
<td>38 ( \times 10^6 )</td>
<td>1.135</td>
<td>52.8 ( \times 10^6 )</td>
</tr>
<tr>
<td>3</td>
<td>T7</td>
<td>12.1 ( \times 10^6 )</td>
<td>1.665</td>
<td>46.3 ( \times 10^6 )</td>
</tr>
<tr>
<td>4</td>
<td>T7</td>
<td>12.1 ( \times 10^6 )</td>
<td>1.689</td>
<td>48.1 ( \times 10^6 )</td>
</tr>
<tr>
<td>5</td>
<td>T2</td>
<td>65 ( \times 10^6 )</td>
<td>0.917</td>
<td>46.0 ( \times 10^6 )</td>
</tr>
</tbody>
</table>

* M. W. Ave. = 48.6 \( \pm 3.2 \times 10^6 \)

* Calculations assume the Abelson-Thomas (1966) relationship of \( D_2/D_1 = (M_3/M_1)^{0.38} \) where \( D \) is taken as the distance between the center of gravity of a given peak and the meniscus. The center of gravity of the leading peak and the T5 peaks were approximated based on the most prominent 3 fractions in each case.

* Molecular weights of standard polynucleotide chains derived from the molecular weights of native DNA's as found in Thomas and MacHattie (1967).

employing the relationship of Abelson and Thomas (1966), \( D_1/D_2 = (M_3/M_1)^{0.38} \), one may estimate the molecular weight of the single chains in the leading peak. The molecular weight calculated from the data shown in Table 2 averages to 48.6 \( \pm 3.2 \times 10^6 \) daltons, half the value of the native SP50 DNA molecule. Thus it appears that a substantial fraction of the SP50 single chains are uninterrupted.

One may estimate the fraction of unbroken chains by assuming that they sediment in a symmetrical peak as do other intact single chains derived from other phage. This is done diagrammatically in Fig. 1. Approximately 39.3% of the total label in the profile is in this peak. After correcting for the fraction of DNA that would be expected to collide with the walls of the tube (Abelson and Thomas, 1966), this would be increased to 41.7%. Duplicate runs and calculations of this sort give 48.2% and 51.0% when SP50 is grown on W-23, and 41.6% and 41.7% when W-168 serves as a host.

Biswal et al. (1967) also noted that the portion of intact SP50 single strands varied depending upon the host used to grow the phage. However, their results differed from those presented in this paper in that they were unable to detect any intact single chains when they grew SP50 on W-168, and only 30% of the chains appeared to be uninterrupted when the phage was grown on W-23. Cosedimentation studies of denatured \(^3\)H-labeled SP50 DNA from phage grown in the W-168 strain used in this work and \(^32\)P-labeled DNA from SP50 grown in the W-168 strain used by Biswal et al. (1967) indicated that both DNA preparations produced identical alkaline sucrose sedimentation profiles, suggesting that the differences in our reported observations cannot be attributed to a minor difference in host strains.

Efforts to resolve hidden components in the trailing material by sedimentation through neutral sucrose gradients (containing 1.0 \( M \) NaCl) failed to reveal any structure although the molecular weight dependence on \( S \) was seen to be more pronounced as shown by Studier (1965).

Efforts to find extraction or growth conditions to eliminate this trailing material met with no success. In our work two different radioisotopes (thymidine-\(^3\)H and \(^32\)PO₄), two different growth media (a tryptone broth and a low phosphate medium), and two different extraction procedures (phenol extraction and simultaneous extraction and denaturation with Na₃PO₄) were used all with no affect on the sedimentation pattern. Moreover, T2 and T7 DNA’s were not found to contain interruptions after the same treatments. In the experiments by Biswal et al. (1967), the single-strand interruptions were
found, even though no radioisotopes were used, in DNA which had been extracted by either the phenol or the perchlorate procedures and after either alkaline or neutral denaturation.

Reannealing of Isolated SP50 Polynucleotide Chains

There are two general models which would explain the alkaline sedimentation profile of denatured SP50 DNA. The first possibility is that only one of the chains is (or can be) intact and that the other chain is always broken. The other model is that some of both chains are intact and some of both are broken. One method of distinguishing these alternatives is to determine whether there exist polynucleotide chains in the leading peak which are complementary to each other. The same question may be asked about the single strands composing the trailing material.

Figure 2 describes an experiment designed to study this problem. Denatured \(^{32}\)P-labeled SP50 DNA was centrifuged through a neutral sucrose gradient in the higher capacity SW-25 rotor. Fractions from the leading peak were pooled (I) as were those from the trailing material (II). These two samples were adjusted to have the same concentrations of DNA (cpm/ml) and sucrose. They were then mixed as indicated, either stored at 5° or reannealed by heating at 65° for 24 hours, subjected to ultrasound, and studied with regard to their hydroxylapatite elution properties.

As shown in Fig. 2, over 88% of the counts in the stored samples were eluted from the hydroxylapatite by the 0.2 \(M\) PO\(_4\) buffer wash indicating that these samples were primarily single-stranded in character. However, in all three of the reannealed samples, greater than 90% of the counts failed to elute from the hydroxylapatite until the concentration of the PO\(_4\) buffer was raised to 0.3 \(M\). By this criterion the I + I reannealed sample was judged to be 92% duplex, the II + II reannealed sample was judged to be 92% duplex and the I + II reannealed sample was judged to be 96% duplex. The fact that the material in the leading peak reannealed so efficiently with itself, as did

![Diagram](image)

**Fig. 2.** Extent of complementarity within and between the leading peak and the trailing material. \(^{32}\)P-labeled DNA was extracted and denatured in 0.1 \(M\) NaPO\(_4\) and reneutralized with NaH\(_2\)PO\(_4\). A volume of 0.9 ml of this preparation was loaded onto a preparative neutral sucrose high salt SW-25 gradient and was centrifuged 13.75 hours at 10,000 rpm. Fractions were collected into tubes from the bottom. Fractions 7 through 9 were pooled (I) as were fractions 21 through 27 (II). I and II were adjusted to the same radioactivity and sucrose concentrations. They were mixed as indicated, either reannealed or stored, subjected to ultrasound, and applied to 1-ml columns of hydroxylapatite. The hydroxylapatite–DNA complexes were washed with 0.2 \(M\) PO\(_4\) buffer, pH 6.8, until no further counts were eluted and then with 0.3 \(M\) PO\(_4\) buffer, pH 6.8, until no further counts were eluted.

the trailing material, demonstrates that each of the two regions of the SW-25 sedimentation profile contains material from both complementary chains.
Electron Microscopy of Molecules formed by Reannealing the Polynucleotide Chains Isolated from the Leading Peak

If both polynucleotide chains are present in the leading peak and if the leading peak is composed of intact polynucleotide chains, it should be possible to reconstitute intact full-length duplex molecules by reannealing the polynucleotide chains located in the leading peak. Furthermore, one could predict that if the SP50 DNA molecules were circularly permuted, the reconstituted native length duplex molecules should be circular in structure (Thomas and MacHattie, 1964). If, on the other hand, the SP50 DNA molecules were non-permuted, then the above mentioned reconstituted native length duplex molecules should be linear in structure (Ritchie et al., 1967).

From the SW-25 sedimentation profile of denatured $^{32}$P-labeled SP50 DNA shown in Fig. 3, fractions which represented the leading peak were pooled into one sample and dialyzed against 0.5 $\times$ SSC. After dialysis, the preparatively isolated sample was found to sediment in a high salt neutral sucrose gradient as a unimodal peak to a position in the gradient that would be predicted for intact single strands. The dialyzed sample was concentrated fourfold to a final concentration of 2.1 $\mu$g of DNA/ml in 2 $\times$ SSC by allowing a stream of compressed air to blow gently on the surface of the DNA solution. The concentrated sample was then reannealed at 65° for 40 min and examined by electron microscopy.
Fig. 5. Reannealed leading peak molecule with no associated single-stranded material. Preparatively isolated leading peak polynucleotide chains were reannealed in 2 × SSC for 40 min at 65°. A photomicrograph of this preparation shows a complete linear molecule with no apparent single-stranded material which has a traced length of 52.5 μ. A second molecule is partially shown in the upper right-hand corner. This molecule is also linear and has no apparent associated single-stranded material. It has a traced length of 50.9 μ.
Fig. 6. Reannealed leading peak molecules with associated single-stranded material. Preparatively isolated leading peak polynucleotide chains were reannealed in 2 X SSC for 40 min at 65°. This photomicrograph shows 2 molecules seen in the electron microscopic analysis of this preparation. One molecule appears to have single-stranded material at one end while the other has single-stranded material at both ends. The duplex length of the first molecule is 18.9 μ, and the second contains duplex material whose length is 31.1 μ.

Over 200 molecules were examined. All were found to be linear in structure. Thirty-one molecules were photographed and traced. Twenty-six of these molecules were chosen at random and five were chosen because they did not contain single-stranded material at their ends. These 31 molecules were found to consist of two groups as indicated in Fig. 4.

Sixteen molecules, such as that shown in Fig. 5, were found to have no single-stranded material associated with either end. With two exceptions, the sizes of these molecules clustered around the native length (average size = 49.5 ± 3.0 μ). The existence of these linear molecules is most easily explained by the reannealing of full length complementary single strands originating from a nonpermuted collection of molecules.

The remaining 15 molecules contained single-stranded material at one or both ends (see Fig. 6 for two examples). The lengths of the duplex regions of these molecules varied but were all shorter than the length of native control molecules. This kind of molecule is thought to have originated by the reannealing of an intact chain with a fragment or of two fragments together. Fragments probably arose due to breakage of some of the single strands during the handling of the leading peak sample. In fact, high salt neutral sucrose sedimentation analysis of the rean-
nealed leading peak preparation subsequent to a denaturation treatment confirmed that both intact and broken single chains were present.

The simplest explanation of the electron microscopic results just presented is the following: (1) The leading peak in the denatured sedimentation profile contains single polynucleotide chains that are intact. (2) Both of the two types of complementary polynucleotide chains are represented in this collection of intact chains. (3) The intact polynucleotide chains in the leading peak originate from a population of nonpermuted double stranded molecules.

CONCLUSION

The SP50 DNA molecule as isolated from mature bacteriophage particles is a duplex, linear molecule with a molecular weight of approximately 100 X 10^6 daltons. A collection of SP50 DNA molecules contain apparently random single-strand interruptions in approximately half of both chains although the exact frequency of single-strand interruptions appears to vary with the host used to grow the phage. Each SP50 DNA molecule is part of a non-permuted collection of molecules.

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