Discrete length classes of DNA depend on mode of dehydration

(BAC electron microscopy/freeze drying/ethidium bromide/bacteriophages $\lambda$ and T7/ethanol, methanol dehydration)

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ABSTRACT The length of double-stranded coliphage $\lambda$ DNA, as determined by electron microscopy using the benzyl- dimethylalkyl ammonium chloride technique, depends on the mode of dehydration. The freeze-dried DNA form is the longest (16.5 $\mu$m), whereas dehydration in methanol (15.9 $\mu$m) or in ethanol (three forms: 15.2 $\mu$m, 13.9 $\mu$m, and 12.4 $\mu$m) results in progressively shorter molecules. These measured lengths of the freeze-dried, methanol-dehydrated, and shortest ethanol-dehydrated forms correspond to the axial rise per nucleotide pair in the B, C, and A forms of DNA, respectively. The remaining forms of ethanol-dehydrated DNA seem to represent novel intermediary conformations of DNA. In agreement with the predicted increment, DNA exposed to ethidium bromide and freeze-dried is elongated by 30% (22.9 $\mu$m). All size classes show the same relative distribution pattern of bound Escherichia coli RNA polymerase molecules (nucleoside triphosphate:RNA nucleotide transferase, EC 2.7.7.6), used as intramolecular markers, indicating that the dehydration-caused transitions are uniform.

The conformation of double helical DNA (1) has been studied by various methods, including x-rays, circular dichroism (CD), and electron microscopy. X-ray diffraction by oriented DNA fibers reveals three double-helical forms, depending on the relative humidity, the ionic strength, and the nature of the cation. The B form appears at relative humidity 92% for the sodium salts (2); the crystallinity of the fibers is best with lithium salts at 66% relative humidity (3). The A form (4) exists in the presence of sodium, potassium, or rubidium salt when the relative humidity is lower than 75% or after dehydration in 80% ethanol (5). With lithium salts, the A form of DNA could not be obtained but, when the relative humidity is reduced to about 66%, the C form appears (6). Table 1 shows various parameters of the A, B, and C forms of DNA.

By using wide-angle x-ray scattering, the structure of DNA in aqueous solution was found to be close to the classical B form (7, 8). CD also permits determining various forms of DNA in solution but requires a calibration to assign the CD spectra to the A, B, and C forms. Therefore, Tunis-Schneider and Maestre (13) obtained the CD spectra of the DNA in films under conditions of humidity and ionic strength comparable to those used in x-ray studies. These assignments of standard spectra allowed study of the transition from one form to another in different solvents—e.g., the CD spectra of a number of DNAs were studied as a function of alcohol concentration in aqueous solutions at 0.1-1 mM salt concentrations. With increasing concentrations of methanol, the CD changes reflect a B-to-C transition, whereas in 80% ethanol the CD spectrum resembles what one might expect for form A DNA (9, 12) (Table 1). However, it remains unclear whether aggregates of DNA molecules or mixtures of different DNA forms could mimic or mask the true spectrum and thus introduce artifacts (8, 9).

Although X-ray diffraction, X-ray scattering, UV absorbance, and CD studies supply helpful statistical information on the population of DNA molecules, they provide little information on the individual molecules. In contrast, electron microscopy permits direct examination of individual DNA molecules [e.g., effect of salt concentration on DNA length (14, 15)]. Lang (16) and Lang et al. (17) examined the effect of ethanol on the tertiary structure of individual DNA molecules and observed three distinct types of DNA superstructures, whereas Vollemeider et al. (18) investigated a salt-induced superstructure. In the present studies, we used the benzylidimethylalkyl-C12 and -C14 ammonium chloride (BAC) technique (19) to determine the effect of dehydration on the length of phage $\lambda$ DNA at very low salt concentration with either freeze drying or alcohol (methanol or ethanol) dehydration. DNA did not form any superstructures under the conditions used (18).

MATERIALS AND METHODS

Materials. BAC was a gift from Bayer, Leverkusen, Germany. Glutaraldehyde sealed under nitrogen was obtained from Polysciences, Warrington, RI. Ethidium bromide was purchased from Calbiochem, San Diego, CA. All other reagents were analytical grade. Polystyrene latex spheres (0.481 $\mu$m ± 0.33%) and carbon grating replicas (0.463 $\mu$m ± 0.3% per line) were purchased from Fullam, Schenectady, NY. Restriction endonucleases EcoRI from Escherichia coli and HindIII from Haemophilus influenzae were a gift from W. Weisblum of the University of Wisconsin. E. coli RNA polymerase holoenzyme (nucleoside triphosphate:RNA nucleotide transferase, EC 2.7.7.6), a gift from R. R. Burgess, was a homogeneous preparation containing stoichiometric amounts of $\sigma$ subunit. Glassware was autoclaved prior to use. Sterile plasticware was purchased from Falcon Plastics, Oxnard, CA. Buffer solutions were passed through Millipore filters (250 nm pore size) and autoclaved.

Phage Propagation, Purification, and DNA Preparation. The techniques used for preparing $\lambda$B857ST DNA were essentially those outlined by Bövre and Szybalski (20) and Bövre et al. (21). The DNA (0.8 mg/ml) was stored in 0.01 M NaCl, 0.01 M Tris-HCl, pH 7.9, at 4°. Homogeneity of the phage was examined by equilibrium density gradient centrifugation in an analytical ultracentrifuge (Spinco model E). The homogeneity of the DNA was verified by cleavage with EcoRI and HindIII and subsequent electrophoretic fractionation of the fragments on agarose in the presence of ethidium bromide (22).

Preparation of the RNA Polymerase-DNA Complex. DNA (25 $\mu$g) and a 30-fold molar excess of RNA polymerase were incubated at 37° for 20 min in 100 $\mu$l of a solution containing

Abbreviations: CD, circular dichroism; (EtOH)$_2$N, 0.03 M triethanolamine-HCl, pH 7.8; BAC, benzylidimethylalkyl-C12 and -C14 ammonium chloride.

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0.03 M triethanolamine-HCl (pH 7.9) [EtOH]2N], 0.05 M KCl, and 0.01 M MgCl₂. The complex was then fixed (10 min, 37°) by addition of one-fifth volume of fixation buffer (0.5% glutaraldehyde in the above solution at pH 7.9), as described by Portmann et al. (23). To open the cohesive ends of λ DNA, 1 volume of dissociation buffer [7% formaldehyde and 0.1% glutaraldehyde in (EtOH)₂N] was added and the temperature was then raised to 57° for 20 min. The next step was the purification of the RNA polymerase-DNA complex on a Sepharose 4B column. The complex was eluted from the column with an elution buffer [0.1% glutaraldehyde in (EtOH)₂N]. The elution profile was recorded at 260 nm. The DNA-containing fraction was used for electron microscopy.

Specimen Preparation for Electron Microscopy. The procedure followed was as described by Vollenweider et al. (19) but with the stock solution of BAC diluted in elution buffer. The DNA or RNA polymerase-DNA complex was absorbed to carbon film-coated copper grids by touching the surface of a 50-μl droplet containing DNA (0.2 μg/ml), 2 × 10⁻⁴% BAC, and 0.1% glutaraldehyde in (EtOH)₂N. The specimens were then washed by floating on redistilled water for 10 min.

For experiments using ethidium bromide, DNA was simply diluted to 0.8 μg/ml in the elution buffer and heated for 15 min at 37° to dissociate the cohesive ends. After addition of ethidium bromide to 15 μg/ml, the DNA was adsorbed to film-coated grids, as above.

For the dehydration of DNA two methods were used: (i) the grids were submerged for about 10 sec in 90% methanol or 90% ethanol; (ii) the grids were deposited on a copper block immersed in liquid nitrogen, and the block was rapidly transferred into a vacuum chamber to sublime the ice at 1 × 10⁻⁶ torr (1.3 × 10⁻⁴ Pa).

The grids were rotary shadowed with uranium oxide (24) and then sprayed on the same side with latex spheres in ethanol.

**Electron Microscopy.** Electron micrographs (Kodak electron image plates) were taken with a Hitachi Hu-11B at 75 kV, at X7000 (for samples containing ethidium bromide) or X10,000 magnification. Orientation of the grids was always the same and the focusing was on the DNA, uranyl oxide granules, or replica grating, but never on the latex spheres.

**Quantitative Analysis of the Micrographs.** Electron micrographs were projected with X13 enlargement and the contour lengths of the DNA molecules, the RNA polymerase binding sites, and the circumferences of the latex spheres were measured with a vertically mounted electronic graphic calculator with a counterbalanced arm (Mark 3, 264-3605-136; Numenics Corp., North Wales, PA). The measuring accuracy of this device was about ±0.5%, determined by measuring the same object several times.

**RESULTS**

When the contour lengths of a large number of λ DNA molecules, prepared by the BAC technique and ethanol dehydration, were measured, unexpectedly they fell into three distinct size classes (Fig. 1D). We undertook to study this phenomenon in detail and to determine the source of this apparent inhomogeneity.

**Homogeneity of the DNA.** The homogeneity of the λ phage was ascertained by two methods. Equilibrium density gradient centrifugation yielded only one narrow band, indicating less

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**Table 1. Physical parameters for the A, B, and C forms of DNA and helical conformations in various solvents**

<table>
<thead>
<tr>
<th>DNA Conformation</th>
<th>Axial rise per nucleotide pair (x-ray diffraction), Å</th>
<th>DNA conformations determined in specified solvents by:</th>
<th>X-ray diffraction</th>
<th>X-ray scattering</th>
<th>CD spectra</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>3.46 (Na) (2)</td>
<td>Water</td>
<td>Water</td>
<td>Water (low salt)(9)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3.37 (Li) (3)</td>
<td>(7, 8)</td>
<td>(5)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>3.31 (Li) (6, 10)</td>
<td>—</td>
<td>90% methanol</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>2.56 (Na) (4, 11)</td>
<td>80% ethanol</td>
<td>80% ethanol (9, 12)</td>
<td></td>
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</tr>
</tbody>
</table>

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**Fig. 1.** Effect of different modes of dehydration on contour lengths of intact λ DNA molecules. Each bar corresponds to a 0.5-μm class size. (A) Based on 139 freeze-dried DNA molecules (10 independent sets of measurements). (B) Based on 91 freeze-dried DNA molecules saturated with intercalated ethidium bromide (three independent sets of measurements). (C) Based on 139 methanol-dehydrated DNA molecules (14 independent sets of measurements). (D) Based on 183 ethanol-dehydrated DNA molecules (nine independent sets of measurements). Dashed lines indicate distribution of DNA molecules at junctions between peaks at 0.15-μm class size. Hatched areas indicate DNA molecules that were not included in calculation of mean contour length.
Table 2. Contour lengths and axial rises per nucleotide pair of various forms of dehydrated phage λ DNA as determined by electron microscopy

<table>
<thead>
<tr>
<th>DNA preparation</th>
<th>Absolute DNA length, μm</th>
<th>Average axial rise/nucleotide pair, Å</th>
<th>Relative length</th>
</tr>
</thead>
<tbody>
<tr>
<td>Freeze drying</td>
<td>16.48 ± 0.21</td>
<td>3.43 ± 0.08</td>
<td>100</td>
</tr>
<tr>
<td>Ethidium bromide intercalation and freeze drying</td>
<td>22.86 ± 0.29</td>
<td>4.75 ± 0.11</td>
<td>139</td>
</tr>
<tr>
<td>Dehydration in methanol (90%)</td>
<td>15.94 ± 0.20</td>
<td>3.31 ± 0.08</td>
<td>97</td>
</tr>
<tr>
<td>Dehydration in ethanol (90%)</td>
<td>I 15.24 ± 0.21</td>
<td>3.17 ± 0.08</td>
<td>92</td>
</tr>
<tr>
<td></td>
<td>II 13.93 ± 0.19</td>
<td>2.90 ± 0.07</td>
<td>85</td>
</tr>
<tr>
<td></td>
<td>III 12.42 ± 0.19</td>
<td>2.58 ± 0.07</td>
<td>75</td>
</tr>
</tbody>
</table>

* Confidence interval calculated at 0.99 confidence level and including magnification error (error of carbon grating replica and latex measurements) and the DNA contour length error (derived from Fig. 1). Confidence intervals arising from the standard deviations of measurements of the carbon grating replica squares, the latex circumferences, and the DNA contour lengths were geometrically added.

† Confidence interval calculated at 0.99 confidence level and including errors of magnification, DNA contour length, and φX174 RFII-to-λcl25 ratio (32).

than 2% variation in λ DNA length. Electrophoretic analysis of the λ DNA fragments produced by cleavage with the restriction endonucleases EcoRI and HindIII showed in both cases the expected patterns of only six sharp bands (data not shown), in agreement with published data (25, 26).

Standardization of Measurements and Absolute DNA Length. In order to correlate different experiments, latex spheres were sprayed onto the grids and used as an internal standard. Thus, independent sets of measurements (electron micrographs taken at different times at varying levels of lens excitation or from different grids) could be correlated by relating the DNA length measurements to the mean value of the latex circumference measurements, as done for Fig. 1.

Magnification was calibrated by spraying latex spheres on a carbon grating replica. The absolute DNA lengths (Table 2) are specified in relation to the carbon grating replica as an absolute standard, and the computations assume a normal distribution.

Ethanol- and Methanol-Dehydrated DNA. Measured lengths of λ DNA molecules photographed from different ethanol dehydrated grids always fell into one or more of three classes, depending upon the particular grid. No obvious reason could be found why a given grid showed preferentially one or two classes of contour lengths. We even found a few grids carrying representatives of all three classes (see analysis of one such grid in Fig. 2), which rules out a correlation error. The mean contour lengths of these three classes were 12.42, 13.93, and 15.24 μm (Fig. 1 and Table 2). When ethanol was replaced by methanol, only one class, of a uniform mean contour length of 15.94 μm, was observed (Fig. 1 and Table 2).

Freeze-Dried DNA. DNA molecules dehydrated by freeze drying displayed a uniform mean contour length of 16.48 μm, which was the longest class (Fig. 1 and Table 2). However, as expected, they could be further lengthened by up to 40% when the DNA was prepared from a droplet containing an excess of ethidium bromide. The mean contour length of such freeze-dried DNA was 22.96 μm (Fig. 1 and Table 2).

Assay for Uniformity of Length Changes. We elected to use the specifically bound RNA polymerase as an internal marker to determine whether particular modes of dehydration cause uniform or only local shrinkage in DNA, because the BAC method allows mapping of protein binding sites on the genomes (18, 27). No significant differences in the distribution of bound RNA polymerase could be discerned for the various size classes of DNA, as shown in Fig. 3, which clearly indicates that the entire DNA molecules are uniformly affected within the limits of resolution.

DISCUSSION

Freeze-dried DNA exhibited uniform contour lengths. Alcohol dehydration however, resulted in shorter size classes (Fig. 1 and Table 2), although the general shape of the DNA molecules was not perceptibly affected. This shortening does not appear to be caused by local shrinkage, as evidenced by the distribution of the bound RNA polymerase (Fig. 3). These results strongly suggest that, when absorbed to the grid, the wet DNA filaments are still mobile and therefore, upon dehydration by alcohols, may undergo structural transitions that cause a shortening along the helical axis.

Is there any relationship between the observed DNA size classes and the A, B, and C conformations (Table 1)? Bram (7) and Maniatis et al. (8) found that the wide-angle

![Fig. 2. Single-grid measurements of the contour length of λ (37 molecules) and T7 (26 molecules) DNA molecules dehydrated in 90% ethanol, shown in relation to the average length of the longest molecules. Each bar corresponds to about 2% of the length of the respective λ and T7 molecules (about 0.3 μm for λ).](image)
high concentrations of methanol, CD spectra are compatible with the C form (Table 1). On methanol-dehydrated grids we found only one uniform size class of DNA with a mean contour length only slightly shorter than that of the freeze-dried class (Fig. 1 and Table 2). Ethanol dehydration should result in an A form because Bram and Baudy (5) found for DNA fibers immersed in 80% ethanol the same x-ray fiber diagrams as for the A form, and CD shows A-form spectra at high ethanol concentrations (Table 1). However, on ethanol-dehydrated grids we detected three size classes (Fig. 1 and Table 2). Does one of these three forms represent an A form, and what are the others?

We can calculate the axial rise per nucleotide pair from the DNA length and the number of base pairs because Sanger et al. (31) recently determined that φX174 DNA contains 5386 bases (F. Sanger, personal communication). Since the ratio of the molecular lengths of double-stranded DNAs for φX174 RFII and λ DNA have been reported as 0.112 ± 0.002 (32), the number of base pairs in λ must be 48,100 ± 1.8%. Because we are dealing with a homogeneous population of DNA molecules, as verified by analytical ultracentrifugation and gel electrophoresis, we are able to calculate the average axial rise per nucleotide pair. Using the carbon grating replica as a length standard, we calculated the average axial rise per nucleotide pair for the different length classes at a 99% confidence level according to the law of propagation of a probable error (Table 2). Within the error of the mean, our freeze-dried form has the same axial rise per nucleotide pair, 3.4 Å, as the B form determined by x-ray fiber diffraction. This distance is increased by exactly 40% to 4.8 Å for the DNA molecules exposed to excess ethidium bromide and freeze dried. Analogously, the methanol form coincides with the C form, and the shortest ethanol form (form III) coincides with the A form (Tables 1 and 2). However, we also have to consider the significance of ethanol forms I and II. Because in the range 0%-65% ethanol CD reflects a B-to-C transition (9) and the C form is the least well-defined DNA form, we cannot exclude that ethanol form I might correspond to this DNA conformation. Ethanol form II does not seem to correspond to any known DNA conformation.

The presence of three discrete DNA forms induced by ethanol dehydration was further confirmed by experiments with T7 DNA (Fig. 2). Again, we obtained three contour length classes with the ratio 1:0.90:0.81, which is practically the same as for λ DNA (1:0.91:0.82).

All these results indicate that DNA can exist in several metastable forms and, when still flexible during the alcoholic dehydration process, it can "snap" from one form to another, depending on the dehydration condition. Only the freeze-drying method of dehydration preserved DNA in its B form, typical for fully hydrated DNA. The simplest hypothesis is that progressive dehydration results in stepwise shrinkage of DNA from the B form into the progressively shorter forms C, I, II, and A. Although the length changes are definitely stepwise, and not gradual, the process is uniform for each whole molecule, because the RNA polymerase marker experiments demonstrate that new size classes are not created by localized shrinkage of the DNA molecules. All size classes examined showed the same RNA polymerase distribution pattern (Fig. 3). This indicates that the DNA snaps uniformly from one into another form and implies some highly cooperative mechanism.

Note Added in Proof. Recently, the φX174 sequencing data (31) were used by other authors to determine the number of base pairs in λ DNA [48,000 (33), 48,300 (34), and 49,400 (35)]. However, the earlier 5975 nucleotide number (31) was used and some were two-step measurements (34, 35) made on the RFII form of circular DNA.

**FIG. 3.** RNA polymerase binding sites on the entire λ genome in terms of map units (relative position as percentage of the total contour length of the DNA molecule). The arm of the λ DNA molecule sparsely populated with RNA polymerases was always oriented toward the left in the diagram. Each bar corresponds to 2.5% λ class size. (A) Based on 90 freeze-dried DNA molecules with total of 930 bound RNA polymerases. (B) Based on 137 ethanol-dehydrated DNA molecules with total of 1345 bound RNA polymerases. (C) Based on 58 ethanol-dehydrated form I DNA molecules with total of 543 bound polymerases. (D) Based on 42 ethanol-dehydrated form III DNA molecules with total of 459 bound RNA polymerases.
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