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Electron Microscope Study of Length and Partial Denaturation of *Rhizobium* Bacteriophage DNA

FRANK MAYER, WOLFGANG LOTZ, AND DIMITRIJ LANG

Institut für Mikrobiologie, Universität Erlangen, Germany, and Institute for Molecular Biology, University of Texas at Dallas, Dallas, Texas 75230

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By electron microscopy and melting of the DNA of some bacteriophages from the soil bacterium *Rhizobium lupini*, it was found that molecular weights range between 27 and 50 Mdaltons and GC contents between 53 and 62%. All DNAs studied are linear; one has exposed single-stranded terminals. Partial heat denaturation of two phage DNA permitted mapping of AT-rich sites within unique nucleotide sequences. The maps are asymmetric with respect to the midpoints of the DNA.

Aside from morphology, physiology and genetics of bacteriophages, attention has been paid also to physical characterization of the phage nucleic acid. Electron microscopy provided an important tool for determining molecular weights of DNA by measuring contour lengths (6, 7) and detection of denaturation maps, that is the distribution of AT-rich nucleotide sequences along individual duplex DNA molecules (5). This paper reports molecular weights and denaturation maps, obtained by electron microscopy, and GC contents of the DNA from some representative bacteriophages of the soil bacterium *Rhizobium lupini*.

MATERIALS AND METHODS

Phages. The six *R. lupini* phage strains used in this study were isolated from garden compost on the basis of their host range and plaque morphology. Structure and general growth properties of bacteriophage 16-2-4 have been described (10). Phage S was isolated by U. Taubeneck of the Institut für Mikrobiologie und Experimentelle Therapie at Jena. It can be propagated in most of the *R. lupini* strains described by Heumann (4). Bacteriophages 16-6-6, 16-6-12, and 16-6-14 form plaques on bacterial strain 16-6 which was newly isolated from root nodules of agriculturally used sweet lupines, as was bacterial strain 16-14 which propagates phage 16-14-1. The morphology of phages S and 16-6-14 has been investigated (11). The phage strains were purified by four single-plaque passages and were harvested by the plate method (2). Crude phage lysates were subjected to three rounds of differential centrifugation. Low speed centrifugations were carried out at 10,000 rpm for 10 min in a Sorvall centrifuge, high speed centrifugations in a WKF-ultracentrifuge at 96,000 × *g* for 6 h in an angle rotor. The phage stocks so obtained had usually a titer of 10^{12} to 10^{13} plaque-forming units per ml. After sterile filtration through Sartorius membrane filters (pore diameter 0.2 μm), the stocks were treated with 1 μg of pancreatic RNase and DNase per ml, respectively (9), followed by a round of high speed centrifugation. Phage S preparations were further purified by banding in a CsCl density gradient.

DNA. For length measurements, the DNA was extracted by heating phages in 0.2 M ammonium acetate, 0.001 M Na-EDTA, to 53 to 60 C for 7 to 30 min. For GC determinations, DNA was extracted by phenol (13) and dialyzed against 0.01 M Na-phosphate buffer containing 0.001 M Na-EDTA, pH 7.8, or against 1/10 standard saline citrate buffer, pH 7 (1/10 0.15 M NaCl plus 0.015 M sodium citrate [SSC]. For partial denaturation, DNA was extracted in the denaturing solution by heating (see below).

DNA melting curves. GC contents were determined according to Mandel and Marmur (12) from absorbance-temperature profiles obtained with a Zeiss PMQ II spectrophotometer at 260 nm. The temperature of the DNA (in 1/10 SSC) was raised at less than 1 C per 10 min. Temperatures were measured in a blank cell by a YS1 Model 42 SC telethermometer. Assuming that only usual bases are present, GC contents were obtained from the melting temperature, Tm, using the relationship GC = 2.44 (Tm − 53.9)% for 1/10 SSC (12). Repeats of GC determinations differed by about 1%.

The fine structure of the melting curve of bacteriophage S-DNA was determined, in neutral phosphate buffer containing 0.018 M [Na^+] and 0.001 M Na-EDTA, as described earlier (3).

Electron microscopy of DNA. Specimen grids were prepared (8) at 0.2 ionic strength. To a clean test tube were added in this sequence: 0.015 ml of 20 μg of DNA per ml in 0.2 M ammonium acetate, 0.001 M Na-EDTA; 2.67 ml of 0.2 M ammonium acetate, 0.001 M Na-EDTA; 0.07 ml of 0.01% cytochrome c in 0.2 M ammonium acetate containing 0.001 M Na-EDTA, and 0.16 ml of 3.7% formaldehyde in water. From this solution, droplets of 0.04-ml volume were deposited on

a Teflon surface. After 20 min, specimen grids (Siemens-type) were briefly touched to the droplets (one grid per droplet) and then touched for 10 s to ethanol, dryed on filter paper, and shadowed with platinum. Lengths and molecular weights were determined as described earlier (7).

Partial denaturation of DNA (5) was carried out similar to Bujard's procedure (1) by heating a solution containing intact phage, 0.045 M sodium phosphate buffer, pH 7.0, 23.5% dimethylsulfoxide and 4.5% formaldehyde, for 3 to 8 min at 53 to 56 C, cooling in ice, and subsequent preparation of grids as described in the preceding paragraph. The lengths of single-stranded DNA regions are ionic-strength dependent and were corrected, by multiplying with 1.56, to that length they would have when in duplex form. This factor was taken from Fig. 2 of Bujard's paper (1) for 0.2 ionic strength. The total length of each DNA molecule was then normalized to 1 and positions along it were given in fractional lengths.

RESULTS

Melting curves and GC determinations. Table 1 shows the melting temperatures in 1/10 SSC, the increase of hyperchromicity with temperature, dh/dT at $T\text{-}T_m$, and the GC contents of several bacteriophage DNAs of the *R. lupini* system. It is assumed that these DNAs do not contain significant amounts of unusual bases. The GC values were reproducible to about 1%. Thus, the differences in GC contents between phages are significant. Figure 1 shows a normalized high-resolution melting curve and its derivative for bacteriophage S DNA. The transition has a slope at midpoint of $(dh/dT)_{T-Tm} = 31$ %/C and is among the steepest known; for T7 DNA a value of 28 %/C had been found (3). It shows a reproducible fine structure indicating nonrandom fluctuations of the local GC content, as is the case in DNA from other sources (3). Figure 1 also indicates an early melting range 6 to 3 C below the melting temperature.

Molecular weights. Modifications of the protein monolayer technique of Kleinschmidt and Zahn (6) have been calibrated under standard conditions at 0.2 ionic strength by DNA of known molecular weight resulting in a molar linear density of (207 ± 4) daltons per 10^{-8} cm contour length of duplex sodium DNA (7). This value has been used for Table 2, presenting contour lengths with sample standard deviations and molecular weights with errors computed as the geometric sum of sample standard deviations of lengths and the error of the molar linear density (±1.9%). Each phage DNA has a unimodal length distribution. This suggests that the phage isolation as described resulted in pure phages. All DNAs were linear except that from phage 16-6-12 which exhibited heat-labile circularity indicating exposed single-stranded terminals of complementary nucleotide sequence.

TABLE 1. *Melting temperatures and slopes of the melting transitions in 1/10 SSC and GC contents of DNA from R. lupini bacteriophages*

DNA from bacteriophage	Melting temperature (C)	Slope $(dh/dT)_{T-Tm}$ (%/C)	GC content (%)
S	78.2	31	59.3
16-6-12	79.2	30	61.9
16-6-14	76.7	22	55.6
16-6-6	75.8	22	53.4

Partial DNA denaturations. Figure 2 shows the result of two partial DNA denaturations of bacteriophage S. Horizontal lines represent normalized intact molecules, the rectangles indicate sizes and lengths of strand separation. The denaturation maps were constructed using the procedure of Gómez and Lang (3).

The molecules in the upper panel show on the average 3% total length of strand separation per molecule (molecules with no visible strand separation were omitted), on the lower panel 30% (all molecules were partially denatured), respectively.

The denaturation maps obtained from Fig. 2 are presented in Fig. 3, for 3% (top) and 30% denaturation (bottom), respectively. At 3%, a main peak appears at 0.54 fractional length. About 50% of all measured molecules had a site between 0.50 and 0.55 indicating a prominently AT-rich site. At higher temperature (30% denturation, bottom of Fig. 3), this peak retains its position and approximately its size while other peaks are building up.

Figures 4 and 5 show 16 partially denatured DNA molecules of the bacteriophage 16-6-6 for 15% (top panels) and 60% denaturation (bottom panels), respectively. At 15% denaturation, most AT-rich sites are found in one half of the molecules (Fig. 4 and 5; top). Peak positions in Fig. 5 agree approximately for both, 15% and 60% denaturation.

DISCUSSION

Molecular weight and topology. Molecular weights of DNA of the six *R. lupini* bacteriophages listed in Table 2 range from 27 to 50 Mdaltons and are thus well within the range found for bacteriophages of other bacteria. The DNAs are linear. Phage 16-6-12 has a DNA with "sticky ends" as revealed by observation of circular molecules after DNA release by heat at 0.2 ionic strength. *E. coli* bacteriophage λ also has a DNA with sticky ends and about the same molecular weight (31.2 Mdaltons; D. Lang,

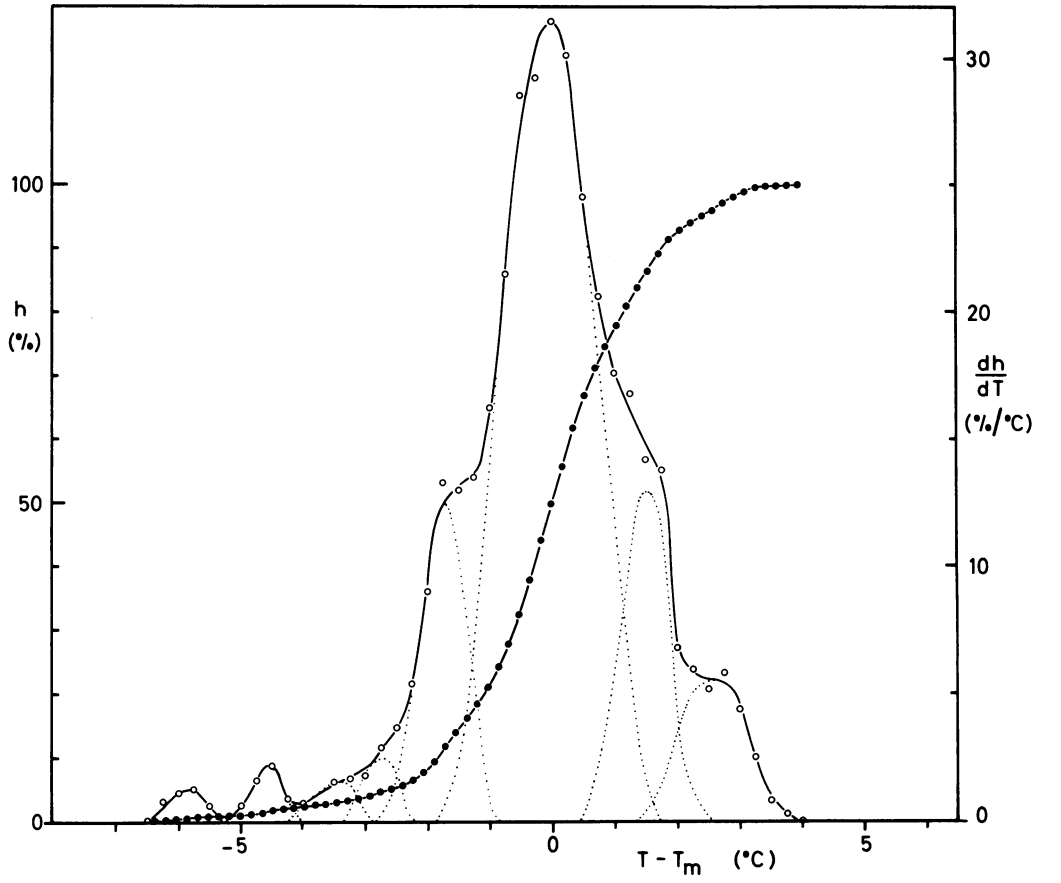


FIG. 1. *Normalized melting curve of bacteriophage S DNA.* ●: *fraction, h, of total hyperchromicity at 260 nm as a function of the difference of the temperature (T) and the melting temperature (Tm).* O: *the derivative, dh/dT, obtained by graphic differentiation of the melting curve, h. The dotted lines are suggested components of the derivative.*

TABLE 2 *Contour lengths and molecular weights of DNA from R. lupini bacteriophages*

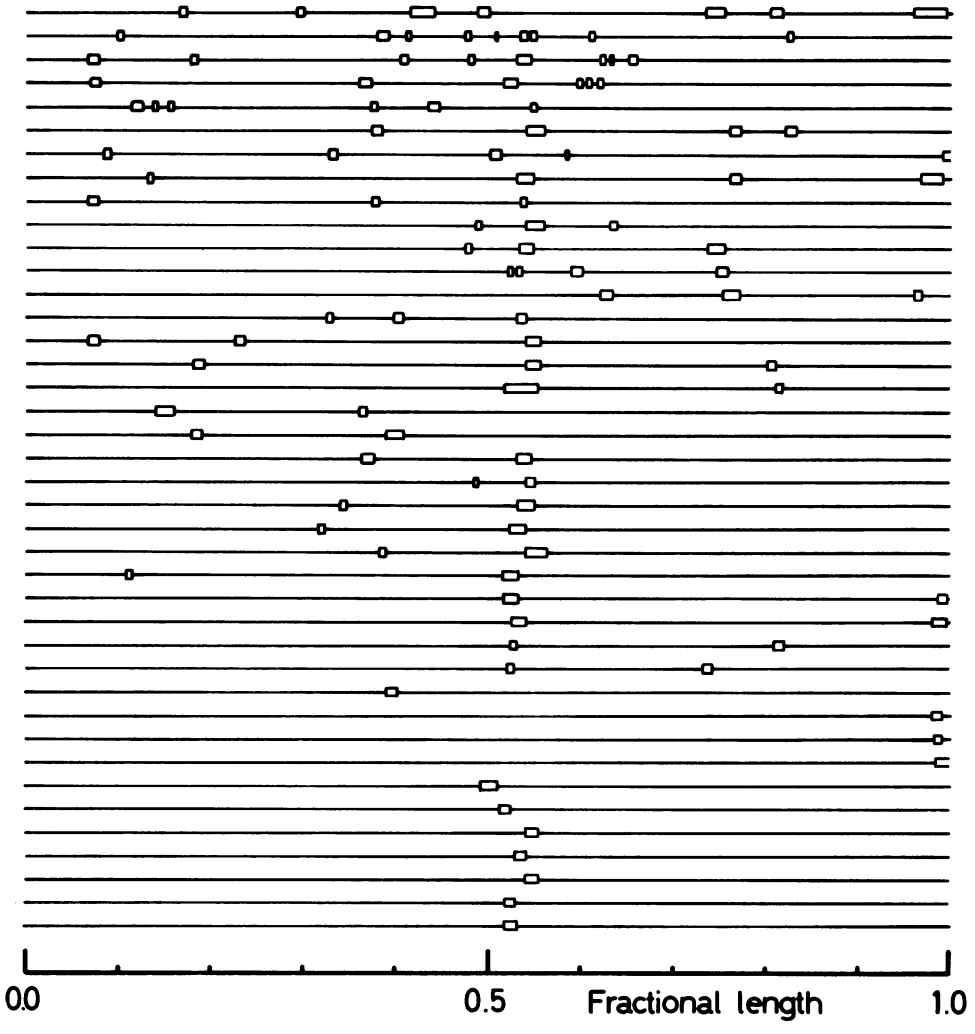
DNA from bacteriophage	Contour length (μm)	Molecular weight (10^6 daltons)
S	13.2 ± 0.2	27.3 ± 0.7
16-14-1	14.1 ± 0.3	29.2 ± 0.8
16-6-12	14.6 ± 0.3	30.2 ± 0.9
16-2-4	15.4 ± 0.6	31.9 ± 1.4
16-6-14	22.7 ± 1.0	47.0 ± 2.3
16-6-6	24.4 ± 0.8	50.5 ± 1.9

unpublished data) and a long, flexible phage tail as found in 16-6-12 phage (F. Mayer and W. Lotz, unpublished data), but its DNA shows higher fluctuations in GC content, with $(dh/dT)_{T-Tm}$ = 8 %/C (3), as compared to 30%/C for 16-6-12 DNA (Table 1).

Melting curves and GC contents. The GC values of *R. lupini* phage DNA listed in Table 1 are not unusual, they range from 53 to 62%. The absorbance-temperature profile of S DNA (Fig. 1) shows that with increasing temperature an "early," limited melting occurs at about 6 to 3 C below the melting temperature, followed by a rapid increase of hyperchromicity, comparable with that of T7 DNA (3). It can be concluded from the partial denaturation (3%) map of S DNA (Fig. 3, top) that the early melting fraction suggested by Fig. 1 is mainly located at 0.54 fractional length of the S DNA.

Maps of partial denaturation. The fact that denaturation maps can be constructed with reference to one end of the linear S- and 16-6-6 DNA molecules proves that their nucleotide sequences are unique and not circularly permuted.

Partial denaturation by heat according to Inman (5) has in all published studies led to the detection of denaturation maps. However, the position of sites of strand separation fluctuated more than expected from measurement errors alone. The extent of denaturation per DNA molecule also varied widely within the same



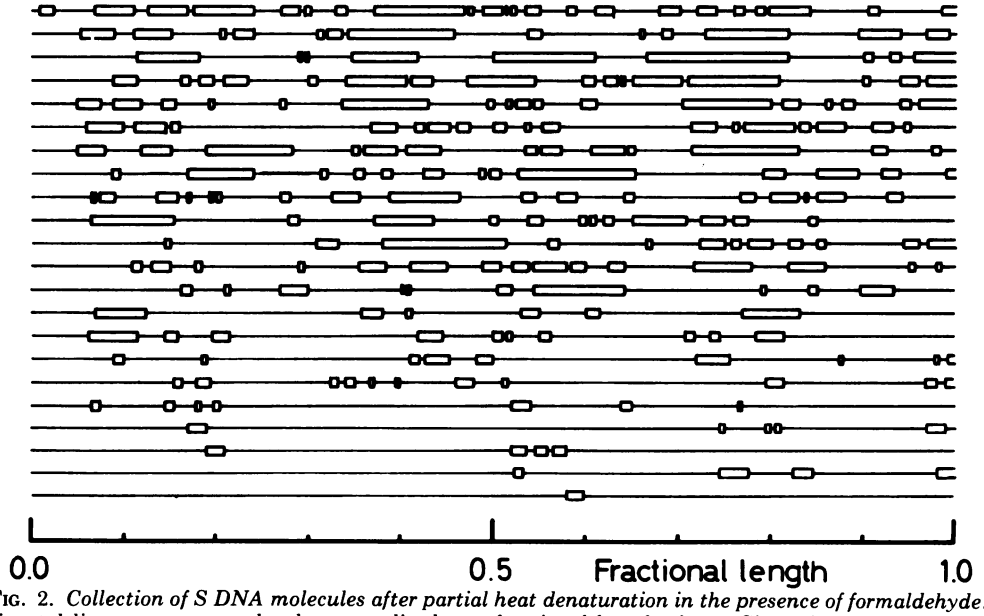


FIG. 2. *Collection of S DNA molecules after partial heat denaturation in the presence of formaldehyde. The horizontal lines represent molecules normalized to a fractional length of one. Sites of strand separation are indicated as boxes or terminal forks. The average length appearing denatured per molecule are 3% (top panel) and 30% (bottom panel).*

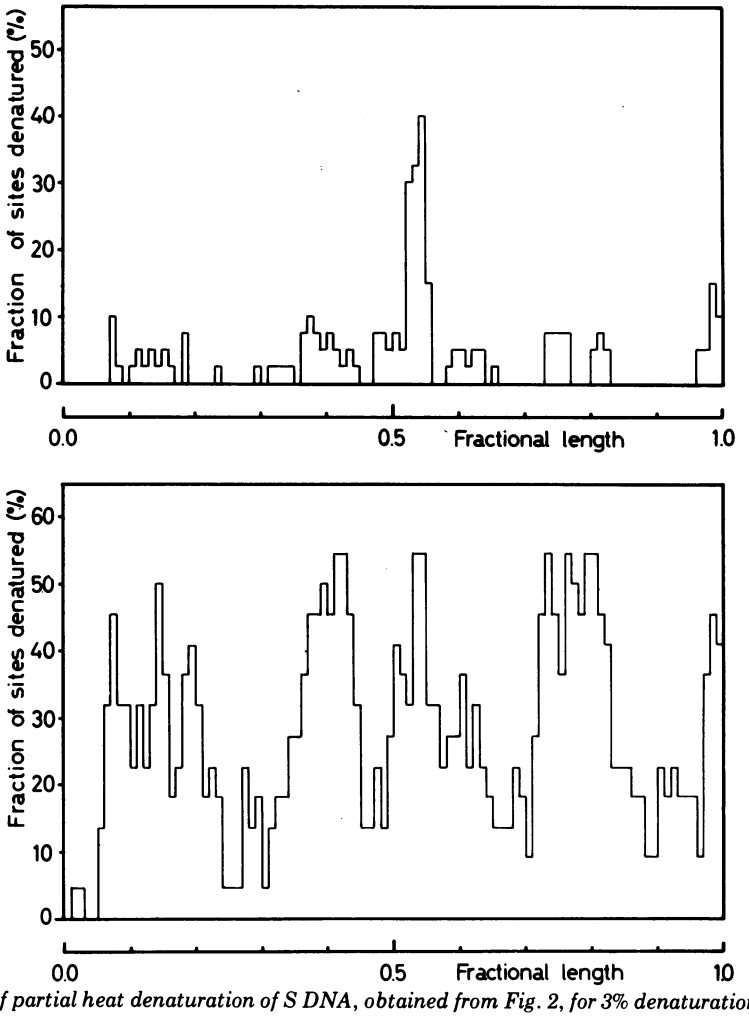


FIG. 3. *Map of partial heat denaturation of S DNA, obtained from Fig. 2, for 3% denaturation (top) and 30% (bottom).*

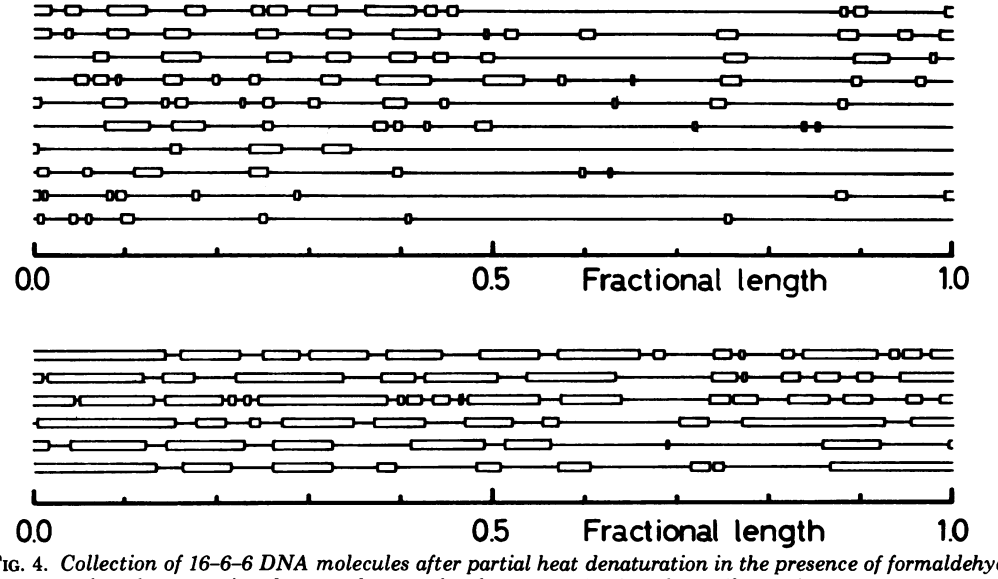


FIG. 4. *Collection of 16-6-6 DNA molecules after partial heat denaturation in the presence of formaldehyde. The average lengths appearing denatured per molecule are 15% (top) and 60% (bottom).*

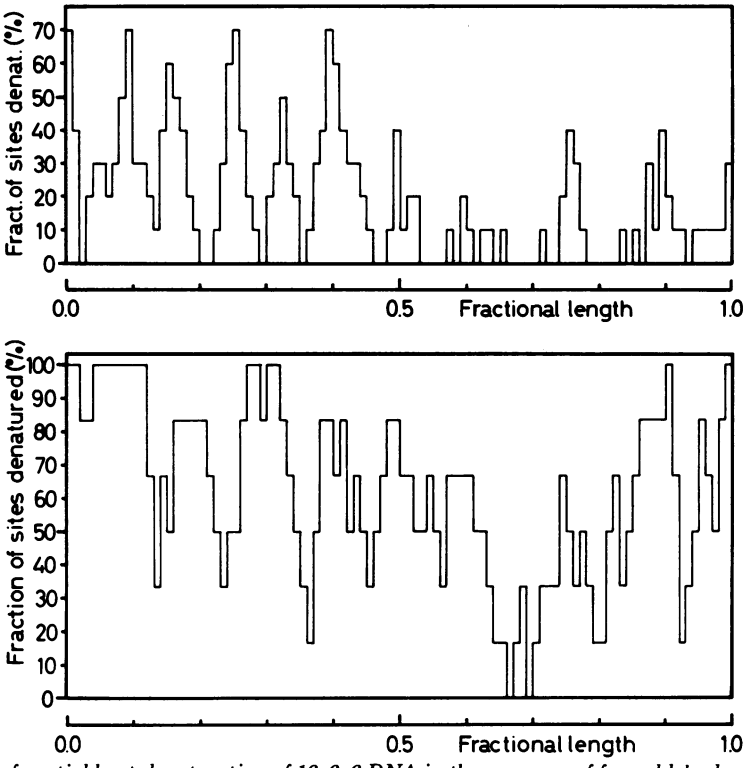


FIG. 5. *Map of partial heat denaturation of 16-6-6 DNA in the presence of formaldehyde, obtained from Fig. 4, for 15% denaturation (top) and 60% (bottom).*

sample of a homogeneous population. The DNA of S- and 16-6-6 bacteriophage is no exception. These variations in S DNA (Fig. 2 and 3) are smaller in the early melting range than in the steep transition range of the absorbance-temperature profile.

As expected, peaks in the denaturation maps at low extent of average denaturation per molecule (Fig. 3 and 5, top panels) are always found at similar positions in samples of more extensive denaturation (Fig. 3 and 5, bottom panels), but with different relative peak sizes. The peak between 0.50 and 0.55 fractional length of S DNA is much higher than the other peaks at 3% average denaturation (but not at 30%) and has therefore a much higher AT content than the rest of the molecule. Such a single main peak does not occur with 16-6-6 DNA, but this DNA has significantly more AT basepairs in one of its halves.

Both the existence of an early asymmetric denaturation site at 0.50 to 0.55 fractional length in S DNA and the general asymmetry of AT-rich nucleotide sequences in 16-6-6 DNA provide a physical discrimination between one end of a DNA molecule and the other end. This may be of help if transcription and replication of these DNAs are to be studied.

The distribution of visible strand separations shown in Fig. 2 and 3 indicate that the melting of S- and 16-6-6 DNA, under the conditions applied, is a multinucleated process.

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