

Partial Denaturation Mapping of Phage T4 DNA at Low Temperature

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Phage T4, DNA Denaturation Map, DNA Circular Permutation

The partial-denaturation map of T4 DNA is obtained by using benzyldodecyldimethyl ammonium chloride in the presence of a high concentration of formamide. In this way suitable conditions for preparation of electron microscope specimens and partial denaturation within a temperature range low enough to minimize the endonucleolytic cleavage, are realized.

It is found that, under our experimental conditions, the denaturation increase depends mainly on the appearance of new denaturation sites rather than on the lengthening of DNA segments already denatured.

Because of the DNA circular permutation it is necessary to align the measured maps to obtain the denaturation pattern. This is done through a computer program and informations on the distribution of the regions with highest (A-T) content along the genome are obtained.

The results of contour length measurements of λ and T4 DNA's are also reported.

Introduction

The T4 genome consists of a double stranded, terminally repetitious, linear DNA molecule formed by $(169 \pm 3) \times 10^3$ nucleotide pairs corresponding to $(123 \pm 4) \times 10^6$ dalton. It is circularly permuted and contains 65.6 molar percent of (A-T) base pairs. Furthermore all C-bases are substituted by hydroxymethylcytosines, which are also glucosylated.

The electron microscope observation of partially denatured DNA molecules makes possible, as an alternative approach to enzymatic methods, to reveal the gross topologies of DNA sequences, to identify particular regions of interest in the genome and to investigate the circular permutation characteristics.

By using the BAC in the presence of high concentration of formamide we have attained a slower denaturation rate and a narrower distribution of the denaturation degrees than with other methods. Stationary conditions of denaturation are reached within 10 min, at a temperature value sufficiently low to reduce the endonucleolytic cleavage introduced by the heating of large molecules such as T4 DNA. We found that the increases in denaturation as a function of the temperature are mainly caused by the appearance of new denaturation sites rather than by the lengthening of the DNA segments already denatured.

In the second part of the present paper we report the denaturation map of T4 coliphage obtained by both BAC and cytochrome c methods to evidentiate possible differences. The circular permutation is taken into account by aligning the partially denatured molecules by using a computer. A noticeable asymmetry in the (A-T) site distribution along the molecule is resulted, so that it is possible to recognize six zones of major denaturation.

Materials and Methods

Materials

In the original protein-free spreading method [1] a mixture of Benzyldimethylalkyl ammonium chloride was used in which the *n*-alkyl group was about 40% C₁₄H₂₉-alkyl and 60% C₁₂H₂₅-alkyl. Since this mixture (BAC I) behaved somewhat erratically, we used pure Benzyldodecyldimethyl ammonium chloride in aqueous solution 50% w/v (BAC II). Horse heart cytochrome c (Sigma-Chemical Company) was used for classic spreading method. Formamide from Merck AG was used as long as the pH value remained between 8.0 and 8.4. All other reagents were analytical grade.

Phage propagation, purification and DNA preparation

T4 phage was propagated in *E. coli* B^E culture following the standard procedure [2]. The λ phage was prepared following the procedure reported in ref. [3].

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The DNA of both phages was extracted by redistilled phenol in the cold and its homogeneity was analyzed by sedimentation in alkali sucrose density gradient [4].

Commercial λ , SV40 and fd DNA's were also employed in some experiments (New England Biolabs).

Specimen preparation for electron microscopy

a) – *BAC spreading* – The final spreading solution contained 0.5 $\mu\text{g}/\text{ml}$ DNA, 0.01 M Tris HCl (pH = 7.5), 10^{-3} % BAC II, 66% or 50% or 33% formamide and was prepared following the procedure in ref. [1]. This solution was kept for 10 min in a thermostatic bath to allow for DNA partial denaturation at various temperatures. We did not observe significant differences among the denaturation percentages of specimens heated for 10, 30 and 60 min. The pH value remained steady at 8.0.

Redistilled water with pH value ranging from 6.8 to 8.0 was used as hypophase. The spreading was performed at room temperature, in teflon cylindrical containers (diameters 70 mm and 120 mm) by a controlled flow of 10–30 μl of solution along an inclined wet microscope slide. Part of the film was picked up on carbon parlodion coated grids by touching the water surface about 5 mm away from the slide. The specimens were washed in water for a short time, stained for 35 s in a solution of 500 μM uranyl acetate, 500 μM HCl in 90% ethanol and washed again in 90% ethanol. Finally the grids were rotary shadowed with platinum under an angle of 10 degrees, at a sample-to-source distance of 8 cm.

b) – *Cytochrome c spreading* – The final spreading solution contained 0.5 $\mu\text{g}/\text{ml}$ DNA, 0.01 μM Tris-HCl (pH = 7.5), 85% formamide, 0.1 $\mu\text{g}/\text{ml}$ cytochrome c. The solution was maintained for 10 min at various temperatures to allow for partial DNA denaturation before adding the cytochrome c. Everything else was like the standard procedure.

Electron microscopy

The specimens were examined on AEI 801 and Philips EM 400 electron microscopes. The molecules were followed for their entire length and coarsely measured by a marker on the screen, in order to ascertain their measurability (start- and end-point well visible, no ambiguity by super imposed molecules or entangled parts). Molecules exceeding

13 μm for λ DNA and 45 μm for T4 DNA were photographed at 10000:1 nominal magnification. From time to time a carbon grating replica (Fullam, 2160 lines/mm) was recorded and in some spreading SV 40 DNA and fd DNA were included as standard length for double- and single-stranded DNA, respectively.

Quantitative image analysis

The electron microscope plates were enlarged to a final magnification of 210000:1 and the molecular tracks were measured by a Hewlett Packard 9864 A digitizer connected to a 9820 A calculator. The accuracy determined by measuring the same molecule several times turned out to be 0.5%.

Alignment of the denaturation maps

In order to construct the partial denaturation histogram of T4 DNA, the molecules have to be lined up with respect to their partial denaturation pattern rather than from their ends, because of their circular permutation. This was done by using a computer, according to the following procedure. The 85 partially denatured T4 DNA molecules were grouped into 3 sets according to the different denaturation percentages. One molecule for each set was chosen as reference for the alignment of the others in the set. The length of each unbroken molecule was normalized to the unity and divided into 1000 segments which were coded to indicate whether it was a native ($l=0$) or a denatured ($l=1$) region.

The relative shift (s) of the map of the molecule (j) with respect to the reference one (k) was determined by searching the minimum of the function $\Delta_{jk}(s)$, which represents the sum over the entire length (L) of the map, extended around a circle, of the differences between the codes $l(i)$ assigned to the i -th segment.

$$\Delta_{jk}(s) = \sum_{l_i}^L |l_j(i) - l_k(i \pm s)|$$

Since the left to right orientation is unknown two calculations were made for $+s$ and $-s$. In such a way the oriented shift for each molecule with respect to the reference one was obtained. Each oriented and shifted molecule had a second alignment operation with respect to the denaturation histogram of the preceding molecules and then added itself to the denaturation histogram. Before

beginning the alignment procedure a length equal to 1.7% of the total length was deleted at random from one end of each molecule to eliminate the terminal repetition (the measurement of the terminal repetition by homoduplex T4 molecules is reported in ref. [5]).

In order to check the goodness of the criteria used in the computation, partially denatured λ DNA molecules were measured and their maps were artificially permuted in a known way. After alignment the starting maps were obtained.

Results

Contour length measurements

Since the sites which can undergo the denaturation process at a given temperature are not uniformly distributed along the molecule, only entire molecules were analyzed and, also for this reason, the contour length distribution was measured. In Fig. 1 the length distributions of λ DNA and T4 DNA are shown. In Fig. 2 the same distribution for circular T4 DNA obtained by homoduplex annealing (ref. [5]) is reported. The mean values of the distributions are $L_\lambda = (18.8 \pm 1.1) \mu\text{m}$, $L_{T4} = (57 \pm 4) \mu\text{m}$ for BAC spreading, $L_{T4} = (59 \pm 4) \mu\text{m}$ for cytochrome c spreading and $L_{T4} = (60.0 \pm 1.5) \mu\text{m}$ for BAC spreading of circular molecules. The widths of T4 distributions, which are much larger than the experimental uncertainty of each length measure, could be related to an intrinsic variability of the molecular lengths [6–9]. Such a variability could

depend on the dehydration states of DNA, as reported by Vollenweider *et al.* [10] and therefore it would be preparation dependent [11].

Dependence of the denaturation rate on temperature and formamide concentration

We define the denaturation percentage (D) for each molecule as the sum of the lengths (l_b) of single stranded zones (denaturation bubbles) over the total molecular length (L).

$$D = \frac{\text{Sum } l_b}{L} = \frac{N_b \bar{l}_b}{L}$$

where N_b is the number of bubbles and \bar{l}_b is their mean length.

In Fig. 3 the denaturation percentages as a function of the temperature at different formamide concentrations for λ DNA and T4 DNA are shown. The data refer to different experiments and each point resulted from an average on at least five molecules. The T4 observations were performed only at high formamide concentration, namely at low temperature, in order to minimize the molecular cleavage. In Table I the values of some parameters which are relevant for the denaturation process are reported. T_0 and T_{50} represent the temperature values, as obtained from the denaturation curves, at which the denaturation starts and reaches the 50% value, respectively. In principle, T_{50} should correspond to the T_m value as obtained through adsorbance measurements. By extrapolating the three measured T_{50} values (column 4) for λ DNA at zero formamide

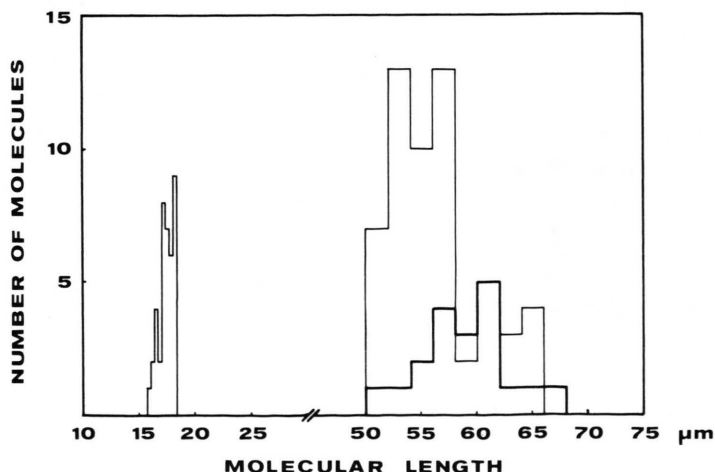


Fig. 1. Contour lengths for λ DNA and T4 DNA. Each bar corresponds to $0.25 \mu\text{m}$ class size for λ and $2 \mu\text{m}$ class size for T4. The mean values of distributions are $L_\lambda = (18.8 \pm 1.1) \mu\text{m}$, $L_{T4} = (57 \pm 4) \mu\text{m}$ for BAC spreading and $L_{T4} = (59 \pm 4) \mu\text{m}$ for cytochrome c spreading.

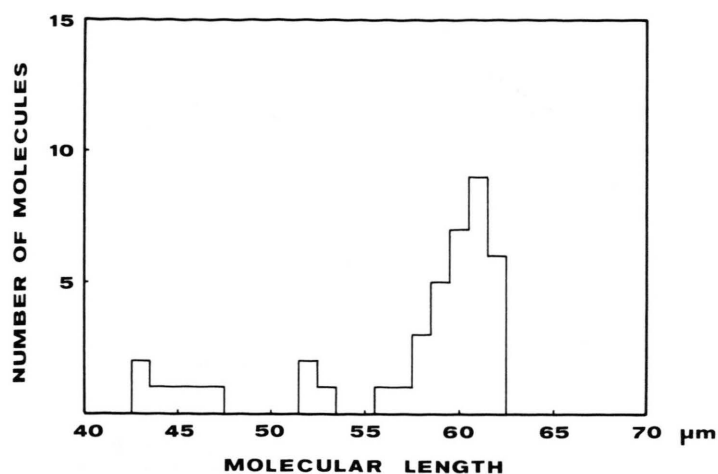


Fig. 2. Contour length for T4 DNA circular molecules. Each bar correspond to 1 μm class size. The mean value of the distribution is $L_{T4} = (60.0 \pm 1.5) \mu\text{m}$.

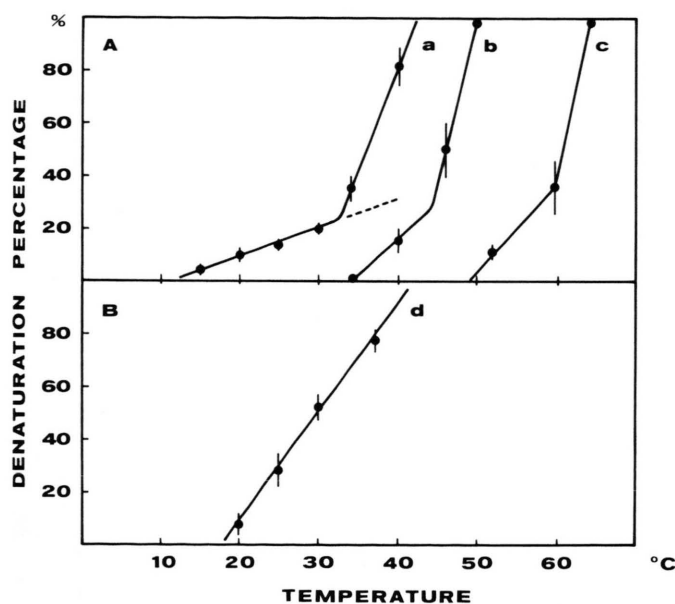


Fig. 3. Denaturation percentages versus temperature for λ DNA (A) and T4 DNA (B) for different formamide concentrations. a) and d): 66% formamide; b): 50% formamide; c): 33% formamide. The solid lines are only guide-lines through the points. The temperature uncertainty is of about 0.5 $^{\circ}\text{C}$. Each point resulted by averaging on at least five molecules and the error bar represents the S. D.

Table I. Values of some parameters relevant for the denaturation process.

DNA	Formamide [%]	T_0 [$^{\circ}\text{C}$]	T_{50} [$^{\circ}\text{C}$]	Δ [$^{\circ}\text{C}/\%$]	α [%D/ $^{\circ}\text{C}$]	$\frac{(\text{A}-\text{T}) \text{ content}}{(\text{G}-\text{C}) \text{ content}}$
λ	33	50	59 ± 2	0.90 ± 0.09	1.2	1.00
	50	34	46 ± 2	0.86 ± 0.06		
	66	13	36 ± 1	0.80 ± 0.03		
T4	66	18	30 ± 2	0.82 ± 0.05	4.2	1.94

T_0 is the temperature at which denaturation starts; T_{50} is the temperature corresponding to 50% denaturation; Δ is the T_{50} decrement with respect to the formamide concentration; α is the rate of denaturation increase with temperature.

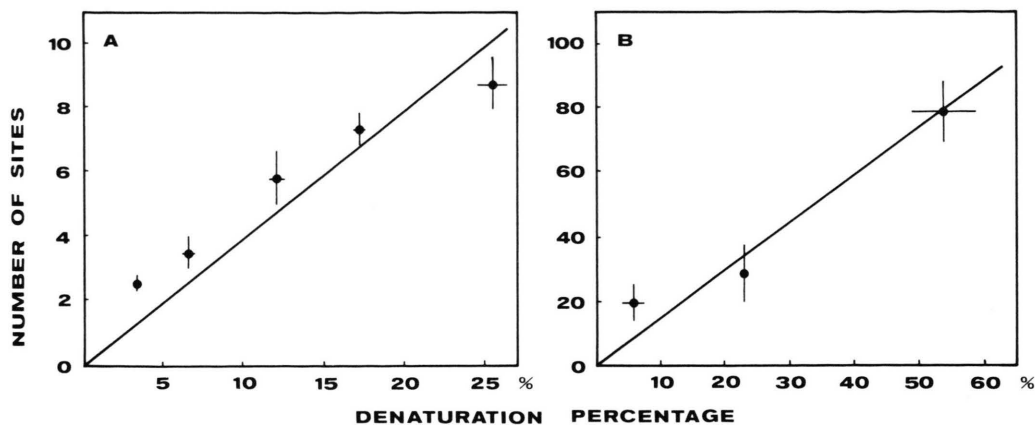


Fig. 4. Average number (\bar{N}_b) of the denaturation sites as a function of the average denaturation percentage for λ DNA (A) and T4 DNA (B) – The data refer to the 66% formamide experiments below 30 °C for λ and below 37 °C for T4 – The straight lines are calculated by the least squares method.

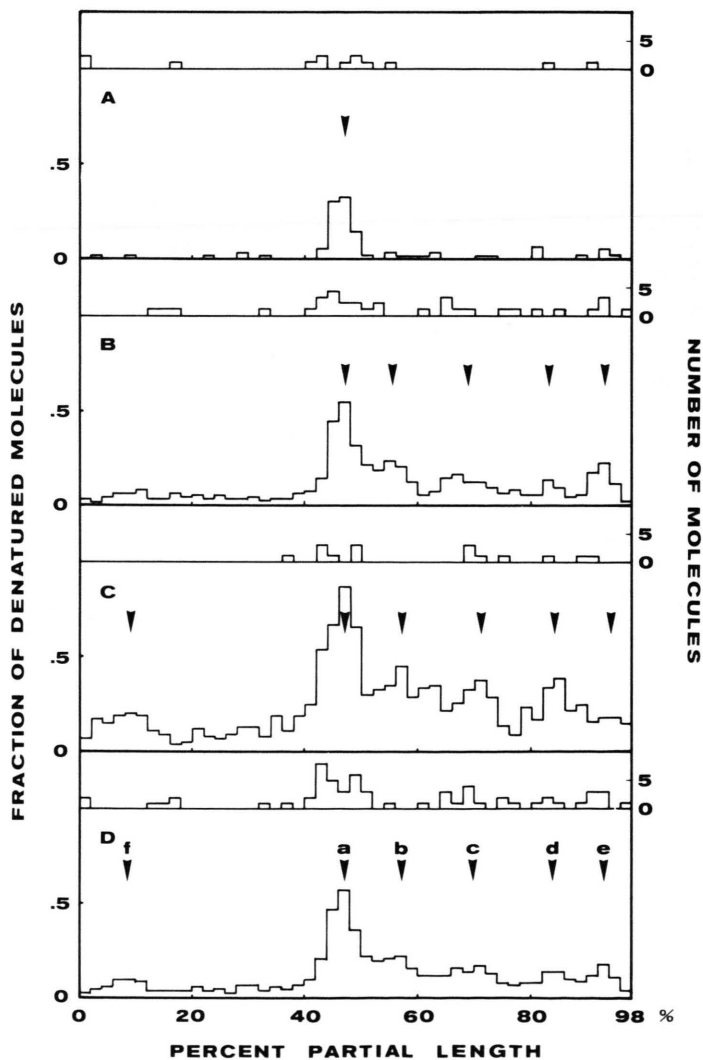


Fig. 5. T4 DNA denaturation histograms obtained by BAC spreading for various denaturation percentages (3% (A), 12% (B), 25% (C) and total (D)) after alignment (see text). The abscissa is expressed in terms of fraction of the genome length. The upper part of each panel shows the distribution of the molecular initial-points.

concentration one obtains $T'_m = (82 \pm 4)^\circ\text{C}$ which has to be compared with the quoted $T_m = 89^\circ\text{C}$ [12].

The rather close agreement between T'_m and T_m permitted us to calculate the T_{50} decrement with respect to the formamide concentration (Δ in column 5), starting from $T_m = 89^\circ\text{C}$ for λ DNA and $T_m = 84^\circ\text{C}$ for T4 DNA [13]. The quoted values [14] for Δ are placed between 0.6 to 0.73 $^\circ\text{C}$ per formamide percent. At high formamide concentration, both for λ and T4 (Fig. 3a and d), the points at low temperature appear to have a linear behaviour. The slope of the straight line (α in column 6) represents the rate of increase in denaturation with the temperature. The α -value for T4 DNA is larger than for λ DNA as might be expected from the different (A-T) content (column 8) of the two molecules.

In Fig. 4 the average numbers (\bar{N}_b) of denaturation bubbles for λ DNA and T4 DNA as a function of the average denaturation percentage (\bar{D}) are

shown. The data refer to the 66% formamide experiments below 30°C for λ and below 37°C for T4, which corresponds to the linear part of the denaturation function vs temperature, shown in Fig. 3.

Since $\bar{D} = \frac{l_b}{L} \bar{N}_b$, where \bar{N}_b is obtained averaging, over all molecules, the measured N_b 's at the same temperature, a linear relation between \bar{D} and \bar{N}_b involves no change in the average length of the bubbles with increasing denaturation. As one can see in Fig. 4, our data indicate a linear behaviour of \bar{D} vs \bar{N}_b , thus implying that the denaturation increase depends mainly on the appearance of new denaturation sites rather than on the lengthening of the already denatured ones.

Of course, this conclusion can be drawn only for moderate denaturation values such as those reported in Fig. 4, when the probability of fusion of two individual bubbles can be assumed to be sufficiently low. The least squares fit to the data (the

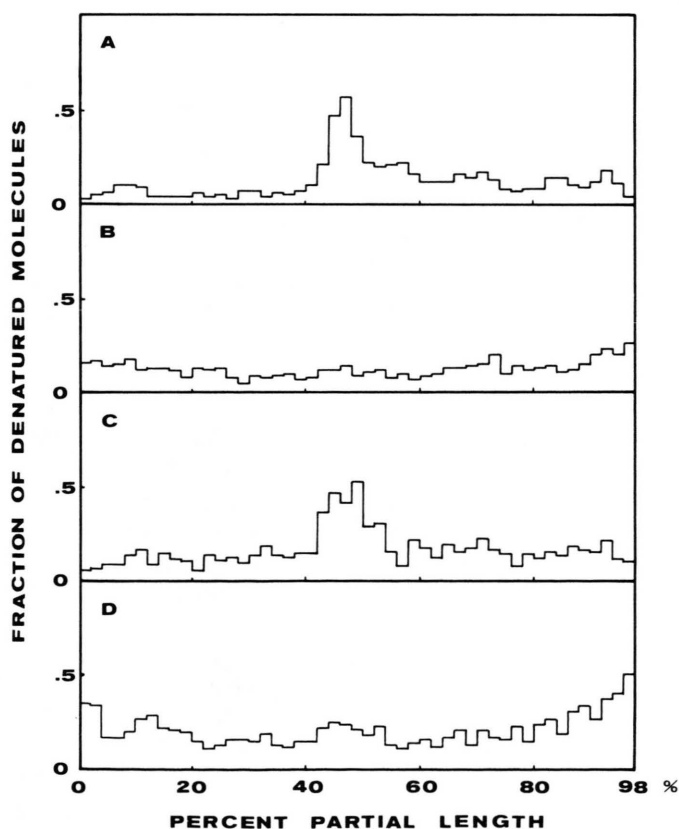
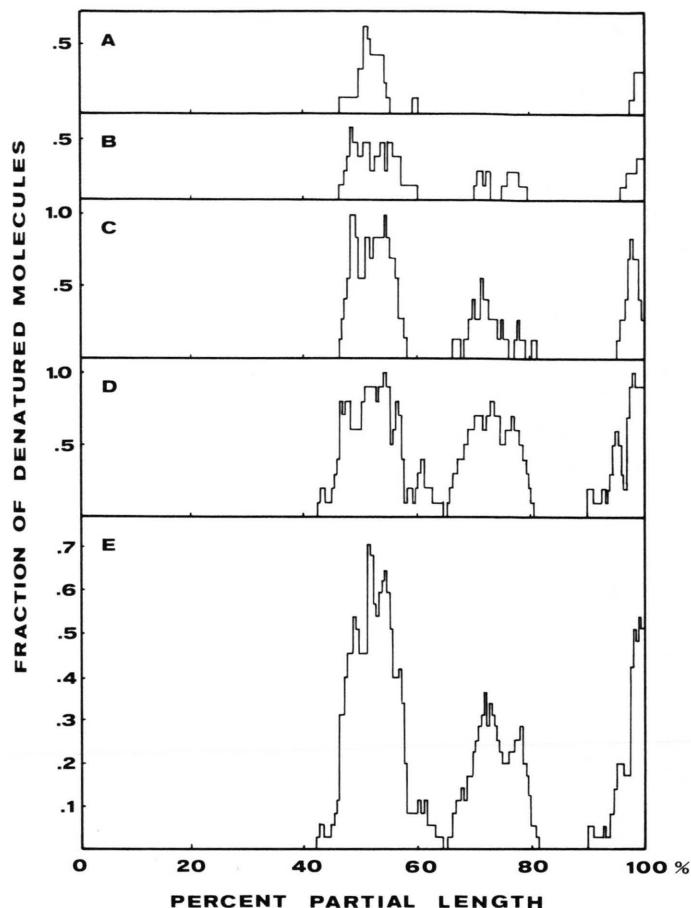


Fig. 6. Total histograms before and after the alignment obtained by BAC and cytochrome c spreadings. A: BAC, aligned; B: BAC, not aligned; C: cytochrome c, aligned; D: cytochrome c, not aligned.

Fig. 7. λ DNA denaturation histograms obtained by BAC spreading for various denaturation percentages (3% (A), 8% (B), 16% (C), 22% (D), total (E)).



straight line in Fig. 4) gives $\bar{l}_b = 0.46 \mu\text{m}$ for λ DNA and $\bar{l}_b = 0.38 \mu\text{m}$ for T4 DNA, corresponding to about 1000 pairs of bases.

It must be noted that this result seems to be not in agreement with the findings of ref. [15] for SV 40 and Φ X174 DNA, where the denaturation length is assumed to be variable with the denaturation percentage.

Denaturation map

In Fig. 5 the denaturation histograms of T4 DNA molecules grouped in 3 sets of increasing denaturation (central values: 3%, 12% and 25%) are shown. For the handling and mapping of data we followed the procedure indicated by Inman [16–18]. The molecules were aligned as previously described. The abscissa of the histograms is expressed in terms of fraction of the genome length, *i.e.* the molecular length minus the terminal repetition. The distributions of the initial point position (*i.e.* the shift of

each molecule with respect to the reference one) are drawn in the upper part of the panels.

Six regions of considerable denaturation can be recognized along the genome (a–f). The zone a is probably the richest in (A–T) content, since it is present also at low percentage of denaturation. On the contrary there is a zone between the f and a regions, as long as about 20% of the whole genome, which remains practically undenatured.

The total histograms before and after the alignment obtained by BAC and cytochrome c spreadings are reported in Fig. 6. The cytochrome c histograms seems to be a little more broadened than the BAC histogram.

The histogram of not aligned molecules shows a little abundance at the edges, which could be due to spurious denaturation at the ends of the molecules. This is supported by the thickening of initial points in the major denaturation region. However this effect could be also interpreted by supposing that the

cutting mechanism of DNA is favoured, as the denaturation process, by the presence of high (A–T) content.

The denaturation hystograms of λ DNA, which were measured to check both the experimental and computational methods, are shown in Fig. 7.

Discussion

The use of BAC II and formamide at high concentration appears to be a very convenient method since it shows both a low reaction rate and a small DNA degradation. Moreover experimental conditions of denaturation equilibrium are obtained which are not met when formaldehyde [16–18] or glyoxal [19] are used.

Although we did not try to search for the best formamide content in the solution, from the gradual development of the λ denaturation map with increasing temperature, we can conclude that the BAC plus formamide method is more convenient than the alkali-denaturation procedure (complete denaturation from pH = 11.0 to pH = 11.4) and the heat-denaturation procedure with formaldehyde [17] (complete denaturation from $T = 54^\circ\text{C}$ to $T = 59^\circ\text{C}$).

The bubble mean length remains constant during increasing denaturation percentage (straight line in Fig. 4) and this could be interpreted in the following very simplified way. For a given temperature the denaturation starts at the sites where the (A–T) content is appropriate for that temperature. The denaturation, once started, proceeds up to a fixed (average) length of strand separation.

Such a length, indeed, appears to be independent of the temperature, but rather dependent on the

BAC plus formamide effects on the DNA molecular structure. On the other side, when the temperature increases, more and more sites, corresponding to zones with a lower (A–T) content, become accessible to the melting and therefore the denaturation process moves further on.

The mean values of the T4 and λ DNA molecular lengths as obtained by contour measurements, are in agreement with the values quoted in the literature [6, 9, 20, 22]. For the T4 DNA the comparison between the measured values after cytochrome c and BAC spreading, proves that there is no influence of the preparation method on the length measurement results.

The T4 DNA denaturation pattern becomes clearly visible after the alignment of the molecules and it shows six regions of high denaturation. However the information which can be extracted from the denaturation map, above all with the uncertainties due to the permutation, cannot be exhaustive by themselves. On the other hand the distribution of the initial points of the molecules, which could give some information on the permutation step [23], appears to be somewhat random in our cases. This is probably due both to the limited statistics, considering also the length of the molecule, and the alignment uncertainties.

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