THE OCCURRENCE OF CIRCULAR STRUCTURES IN HIGHLY REITERATED DNA OF THE RAT

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1. Introduction

It seems that electron-microscopic investigation of reassociated molecules of DNA can give new information about the nature and arrangement of nucleotide sequences in the DNA [1].

This communication describes the molecular configuration of reassociated molecules possessing highly reiterated nucleotide sequences which recur in rat genome 2×10^6 times [2].

2. Materials and methods

DNA was isolated from the livers of three month old Wistar rats by the method of Savitsky and Stand [3]. DNA preparations were further purified by digestion with pancreatic RNase (5X crystallized, Sigma Chemical Co., St. Louis Mo., USA) for 30 min at an enzyme concentration of 50 μg/ml. The RNase solution was previously heated at 90° for 10 min. Subsequently DNA was digested with pronase B (500 μg/ml) at room temperature (pronase was the product of Calbiochem, Los Angeles, Calif., USA). Then the mixture was deproteinized by chloroform–isoamyl alcohol (24:1, v/v). DNA was sheared mechanically as previously described [4] down to about 6–10 $S_{20,w}$. Approximate sedimentation coefficients were calculated from band sedimentation in 5–20% sucrose gradient as described [5].

Sheared DNA was fractionated according to Britten and Kohne [6] (for details see also [4]), and arbitrary fractions renaturing at three kinetic rates have been obtained: fast, intermediate and slow [2, 7]. The fast fraction has been obtained at $C_0t - 10^{-3}$ mole sec l^{-1}. C_0t is defined as the product of initial DNA concentration expressed in moles of nucleotides and time of annealing [6].

The fast fraction also called the fraction of DNA with highly reiterated nucleotide sequences amounts to 5–10% of total DNA, its T_m in 0.02 M NaCl being 71°, the hyperchromic effect = 28.5%, and the buoyant density in CsCl, ρ = 1.695 g/cm^3. Under the same conditions, native, unfractionated rat liver DNA exhibits T_m of 72.5%, with a hyperchromic effect of 39.3%, and ρ = 1.695 g/cm^3.

For electron microscopy DNA was prepared by a modification of the cytochrome-film technique of Kleinschmidt and Zahn [8]. The fast-renaturing fraction of DNA was dissolved in 1.0 M ammonium acetate, at A_{260} = 0.1 and was mixed in an equal volume with a 0.02% solution of cytochrome *c* (Sigma Chemical Co.) in 1.0 M ammonium acetate. This mixture was layered over the surface of 1.0 M ammonium acetate previously cleaned and covered with talcum. The DNA-protein film was picked up on grids, fixed with ethanol, and grids shadowed in vacuum evaporator JEE-3B with platinum. The angle of shadowing was 6°. The grids were then examined and photographic pictures made in JEM-5Y (Japan

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Electron Optics Laboratory, Tokyo, Japan) under direct magnification of 10,000. The randomly chosen positives (final enlargement 100,000) served for examination of both shape and size of DNA molecules.

3. Results

Typical forms of DNA fragments found in fast-renaturing fraction are shown on figs. 1 and 2. Almost all molecules found in electron micrographs of fast renaturing fraction reassociated at $C_0t = 10^{-3}$, showed highly ordered conformation similar to that found in native DNA. The electron micrographs of DNA molecules with highly reiterated nucleotide sequences showed several different molecular types: linear molecules, molecules in the form of aggregates or entanglements, and lastly molecules in circular structures.

It is difficult to give the exact amount of each of the forms because molecules in the form of aggregates (fig. 1) were also observed. However, out of 530 molecules examined 54% were linear, 14% were in ring-

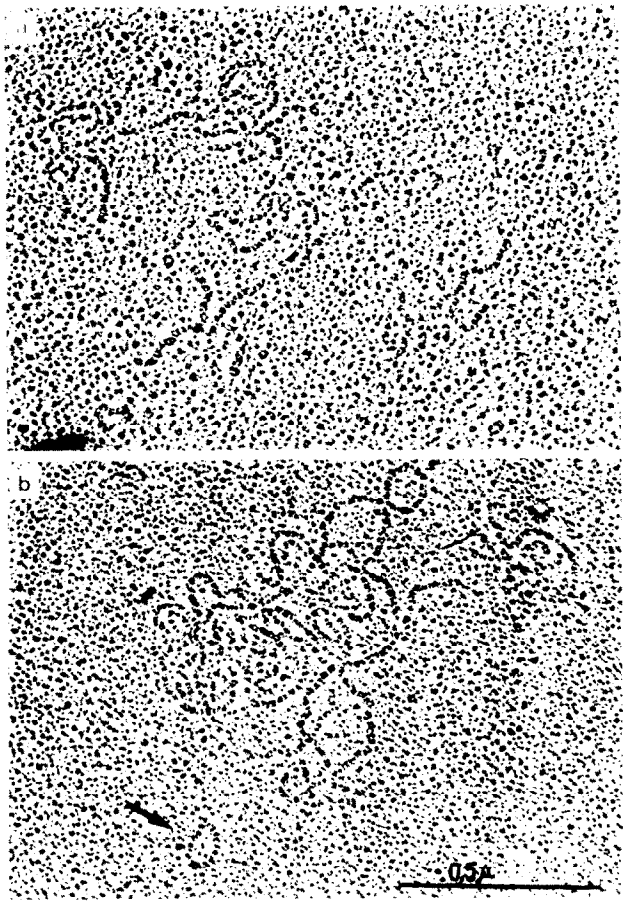

Fig. 1a, b. Electron micrographs of rapidly reassociated RNA of the rat. Sheared rat liver DNA at concentration A_{260} = 0.1–0.2 was thermally denatured (100°, 10 min) and subsequently reassociated in 0.125 M phosphate buffer at C_0t = 10^{-3}. Reassociated molecules were isolated by chromatography on hydroxyapatite column [4]. The pictures show randomly chosen areas. The arrow on fig. 1b indicates the molecule in ring-shaped configuration.

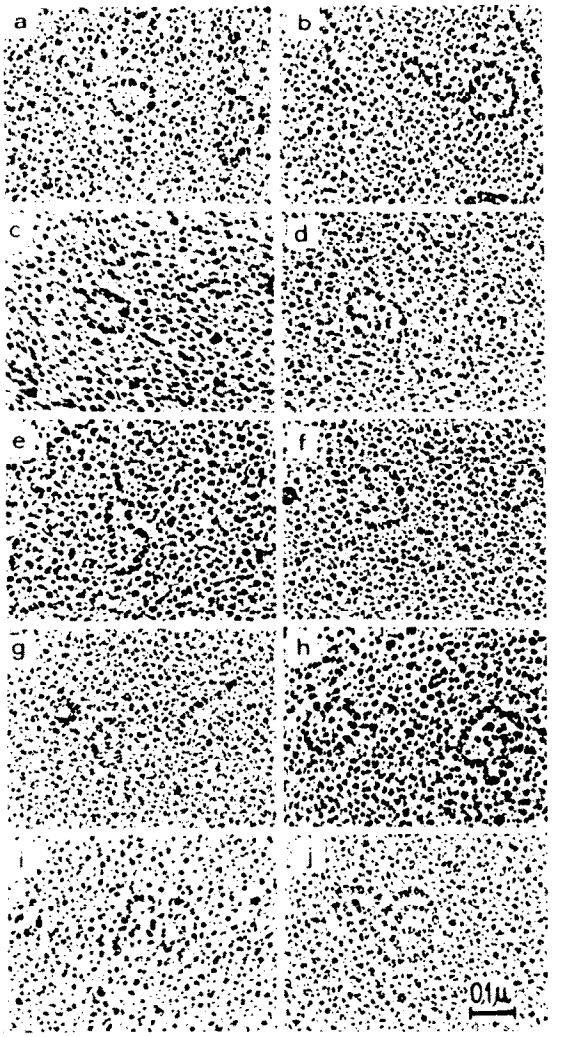

Fig. 2. Electron micrographs of several circular structures of rapidly reassociated DNA of the rat.

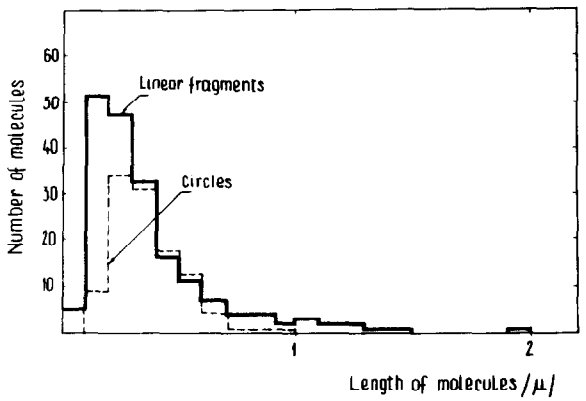

Fig. 3. Length distribution of linear and circular fragments of rapidly reassociated molecules.

shaped configurations, 8% were probable circles, and 24% were in the form of aggregates or entanglements. Various circular structures observed are shown on fig. 2. According to the nomenclature given by Thomas et al. [1] we may distinguish typical rings (fig. 2a), twisted circular forms (fig. 2b), "lariats" (fig. 2c, d, e), double rings (fig. 2f, g, h), and polyrings (fig. 2i, j). The length distribution of circular and linear fragments is illustrated by histogram (fig. 3). The most numerous linear molecules were of 0.1–0.3 μm in length, whereas the most frequent circles were of 0.2–0.4 μm in contour. Only a small number of both linear and circular molecules were of 0.7 μm in length. The mean length of linear fragments was 0.37 μm, and that of circles 0.36 μm (table 1). The relatively high value of standard deviation (S.D.) for both circular and linear forms could result from primary polydispersity of sheared DNA fragments, or what is more probable, from elongation of sheared fragments during reannealing by concatenation-like processes.

4. Discussion

The studies of Thomas et al. [1] have shown that artificial rings and circular structures of the eucaryotic DNA (salmon sperm, trout, Necturus, calf thymus) could be obtained either by "folding" or "slipping" processes. The prerequisite of ring formation is the occurrence of tandemly-repeating nucleotide sequences in the DNA molecule [1, 9]. The formation of circles by "folding" technique involves partial digestion of either 3′ or 5′ ends of DNA by specific exonucleases, whereas "slipping" technique is based on reannealing of two previously separated complementary chains which are mutually "slipped" by one or more repeated units. Under the same experimental conditions no circular forms of phage T_7, *Escherichia coli*, and *Bacillus subtilis* DNA are observed. Thus, besides kinetic measurements of renaturation rate [6], the ability of DNA to form the circles seems to be the most adequate way to reveal reiterated DNA sequences as pointed out by Thomas et al. [1]. On the other hand, one may expect that molecules which are involved in cyclization processes are physically in equilibrium with linear molecules and with higher linear aggregates (concatemers) derived from the latter. In their studies on the probability of ring closure of λ DNA, Wang and Davidson [10] showed that when the DNA concentration becomes greater than $A_{260} = 0.20$ and temperature is below 60°, significant quantities of cyclic *n*-mers should be present. However, as the concentration of DNA increases, linear *n*-mers of high *n* become the dominant species. In former studies it was observed that at high concentration of calf thymus fast reannealing DNA submitted to reassociation at $A_{260} = 2.0$, linear concatemers were dominant [4], with low proportion of circular structures [11]. In the recent experiments made by the "slipping" method [1] the concentration of reassociated DNA was low and ranged in $A_{260} = 0.10$ to 0.20.

The cyclization of molecules carrying highly reiterated nucleotide sequences is only possible when sheared fragments are smaller in length than the length of the region of tandemly-repetitious sequences. According to Thomas et al. [1] the fraction of segments containing tandemly repetitious sequences that are capable

Table 1
The size of circular and linear fragments of rapidly reassociated fraction of rat DNA.

Forms of DNA	Mean (μM)	S.D. (μM)	Range (μM)	Number of measured molecules
Circular fragments	0.36	0.13	0.17–1.01	113
Linear fragments	0.37	0.17	0.06–1.94	191

of cyclization is equal to $(F-L)/F$, where L is the length of sheared fragments, and F is the length of repeated copies. Following this reasoning we may calculate the length of the block. If we assume that the fraction capable of cyclization is equal to 0.14 (relative amount of evident circles) and L is equal to 0.37 μm (the mean contour length, see table 1), then F amounts to about 0.43 μm, which corresponds to about 1200 nucleotide pairs. This value may be underestimated due to the fact that some of the fragments which are capable to form circles are recognized as probable circles and entanglements (aggregates). On the other hand the real amount of circles could be lower as we cannot estimate how many sheared fragments are included in a single entanglement.

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