Formation of Rings from Segments of HeLa-Cell Nuclear Deoxyribonucleic Acid

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Duplex segments of HeLa-cell nuclear DNA were generated by cleavage with DNA restriction endonuclease from *Haemophilus influenzae*. About 20–25% of the DNA segments produced, when partly degraded with exonuclease III and annealed, were found to form rings visible in the electron microscope. A further 5% of the DNA segments formed structures that were branched in configuration. Similar structures were generated from HeLa-cell DNA, without prior treatment with restriction endonuclease, when the complementary polynucleotide chains were exposed by exonuclease III action at single-chain nicks. After exposure of an average single-chain length of 1400 nucleotides per terminus at nicks in HeLa-cell DNA by exonuclease III, followed by annealing, the physical length of ring closures was estimated and found to be 0.02–0.1 μm , or 50–300 base pairs. An almost identical distribution of lengths was recorded for the regions of complementary base sequence responsible for branch formation. It is proposed that most of the rings and branches are formed from classes of reiterated base sequence with an average length of 180 base pairs arranged intermittently in HeLa-cell DNA. From the rate of formation of branched structures when HeLa-cell DNA segments were heat-denatured and annealed, it is estimated that the reiterated sequences are in families containing approximately 2400–24000 copies.

One method that has been used extensively for investigating DNA base sequence complexity is renaturation kinetics. The rate of renaturation of DNA single chains depends, together with other parameters, on the numbers of different nucleotide sequences present in the DNA preparation (Marmur & Doty, 1961). For DNA from viruses, bacteria and mitochondria, renaturation follows second-order kinetics, and the rate of reassociation can be related to genome size determined analytically (Wetmur & Davidson, 1968; Wells & Birnstiel, 1969). It is deduced that most of the base sequences present in prokaryotic DNA are single-copy, although it is known that a small fraction of the genome in micro-organisms, for example, contains multiple copies of rRNA and tRNA cistrons (Yankofsky & Spiegelman, 1962; Goodman & Rich, 1962). In contrast, for many eukaryotes, a significant fraction of the DNA single chains reassociate heterogeneously and far more rapidly than predicted on the basis of their known genome complexity. This is interpreted as evidence that eukaryotic DNA contains multiple copies of certain closely related base sequences (Britten & Kohne, 1967). The remaining single chains renature at a much lower rate, consistent with genome analytical complexity, and with second-order kinetics. It has been suggested, by analogy with prokaryotes,

that DNA in this fraction contains unique base sequences (Laird, 1971). By comparing kinetic and analytical complexity of fast-renaturing fractions of eukaryotic chromosomal DNA, it has been deduced that some base sequences are represented 10^2 times per haploid genome complement, and in certain cases over 10^6 repetitions of particular sequences are present (Britten & Kohne, 1968).

An alternative method used to detect repeated base sequences in eukaryotic chromosomes is the technique of DNA-ring formation, used previously as a test for terminal redundancy and circular permutation of base sequence in bacteriophage DNA (Thomas *et al.*, 1968; Thomas & MacHattie, 1964). It has been shown that duplex eukaryotic DNA fragments, when partly degraded with exonuclease III and annealed, form large numbers of ring structures, which can be detected by electron microscopy (Thomas *et al.*, 1970). This observation suggests that the DNA single-chain termini of eukaryotic DNA fragments, exposed by exonuclease treatment, contain regions of complementary base sequence that are able to unite to form a duplex and result in ring formation. In order to explain the persistence of DNA rings after denaturation and annealing, their configuration and stability in denaturing conditions of high formamide concentration, it has been proposed that a large proportion

of the eukaryotic genome is organized into families of tandemly repeated base sequences (Bick *et al.*, 1973; Pyeritz & Thomas, 1973). In addition, Lee & Thomas (1973) have shown that *Drosophila* polytene DNA, extracted from salivary glands, forms rings with an efficiency almost equal to whole-fly DNA. This finding has been used to argue against the assertion that the highly repetitious base sequences, which can be detected by DNA reassociation kinetics, might be responsible for ring formation, because the 15% fast-annealing highly reiterated sequences normally present in *Drosophila* whole-fly DNA are decreased to 2–5% on polytenization (Gall *et al.*, 1971). This presents a problem, however, because the remaining 95–98% of *Drosophila* polytene DNA is thought to contain single-copy base sequence, determined from its rate of renaturation (Laird, 1971), and on this basis polytene DNA is expected to contain far fewer repeated sequences available for ring formation. To explain this anomaly, it has been suggested that the use of calculations based on DNA reassociation kinetics data is an unreliable method to assess the precise extent of base sequence repetition in DNA (Thomas, 1970; Lee & Thomas, 1973) and that the so-called unique DNA fraction in eukaryotes contains many regions of tandemly repeated base sequence whose presence cannot be detected by DNA reassociation experiments.

In the present study, the regions of repeated base sequence involved in the formation of rings from HeLa-cell nuclear DNA segments have been identified. The results suggest that, of the HeLa-cell DNA rings generated, most are closed by distinct regions of complementary sequence 50–300 base pairs in length. Branched DNA structures are formed simultaneously with rings during annealing reactions. The regions of suspected intermolecular complementary base sequence responsible for branch formation have also been identified, and are shown to be 50–300 base pairs long, with a length distribution almost identical with that found for ring closures. The results can best be explained by a model chromosome containing many repeated base sequences approximately 180 base pairs in length intermittently dispersed along segments of the chromatid. The rate at which the sequences reassociate to form branched DNA structures indicate they belong to the highly reiterated fraction of HeLa-cell DNA. These findings are discussed in relation to recent studies of DNA sequence complexity in *Drosophila*.

Materials and Methods

Chemicals, enzymes and radiochemicals

Unless otherwise stated, chemicals were of A.R. quality. Enzymes were obtained from Sigma Biochemical Co., Kingston-upon-Thames, Surrey, U.K. Radiochemicals were obtained from New England

Nuclear Corp., Boston, Mass., U.S.A., or The Radiochemical Centre, Amersham, Bucks., U.K.

³H-labelled HeLa-cell DNA

The DNA used was labelled with [³H]thymidine and isolated from HeLa-cell nuclei by a sodium dodecyl sulphate–Pronase method, and was a gift from Dr. B. Dancis and Dr. J. Soll. Sedimentation analysis with the use of neutral and alkaline sucrose gradients showed that for the preparation used for the present work the duplex chain length was in excess of 12 μ m, and the single chain length was an average of 1.5 μ m. The duplex length was confirmed by electron microscopy.

Haemophilus influenzae DNA restriction endonuclease

The enzyme was prepared as described by Smith & Wilcox (1970). Small amounts of contaminating exonuclease activity were totally eliminated by modification of the initial fractionation step (D. C. Gould & P. Henson, unpublished work). Samples of the enzyme used for this study were a gift from Dr. D. C. Gould and Dr. P. Henson.

Exonuclease III

The preparation method was as described by Lee & Thomas (1973). Samples were provided by Dr. C. S. Lee.

Preparation of ³²P-labelled bacteriophage ϕ X174-RFI DNA

Escherichia coli HF4704 was grown at 37°C to 10⁸ cells/ml in exponential growth phase in TPG2A medium (Lindquist & Sinsheimer, 1967) but containing only 0.023 g of KH₂PO₄/l. Cells were infected at 37°C with bacteriophage ϕ X174am3 (Hutchison & Edgell, 1971) at a multiplicity of 10:1, incubated for 2 min in standing culture, and for a further 14 min by shaking. Chloramphenicol was added to 30 μ g/ml and incubation continued for 5 min. Carrier-free [³²P]P_i was then added to give a final radiochemical concentration of 0.1–0.2 mCi/ml, and incubation was continued for a further 60 min. Cells (12 ml) were harvested by centrifugation at 5000g for 10 min at 4°C, resuspended in 2 ml of 0.3 M-EDTA–0.5 M-Tris–HCl buffer, pH 8.0, and treated with 0.1 vol. of lysozyme (0.2 mg/ml final concn.). After 15 min at 4°C, 10% (w/v) Sarkosyl NL97 (Geigy, Simonsway, Manchester, U.K.) was added (1:10, v/v) and the lysate was shaken briefly. Heat-treated pancreatic ribonuclease (2 mg/ml) was added (1:10, v/v) and the mixture was incubated at 60°C for 60 min. The lysate was then extracted twice with an equal volume of redistilled phenol previously saturated with 0.25 M-Tris–HCl buffer, pH 8.0.

Bacteriophage ϕ X174-RFI DNA was separated from labelled RNA and chromosomal DNA by sedimenting a portion of the lysate (1 ml) through a 36 ml

linear 5–20% (w/v) sucrose gradient containing 0.5M-NaCl, 0.03M-EDTA and 0.05M-Tris-HCl buffer, pH8.0, in the SW27 rotor in the Beckman L2-65B preparative ultracentrifuge (25000rev./min for 16h at 10°C). The intermediate band of radioactivity (approx. 20S) containing ^{32}P -labelled bacteriophage ϕX174 -RFI DNA was dialysed for 16h at 4°C against two changes of buffer (0.03M-EDTA–0.05M-Tris-HCl buffer, pH8.0).

^3H -labelled bacteriophage T_7 DNA

Bacteriophage T_7 DNA was labelled with [$\text{Me-}^3\text{H}$]-thymidine and extracted by using the method described by Thomas *et al.* (1970).

Procedure for the counting of radioactivity

Radioactive samples (0.1 ml) were mixed thoroughly with 10 ml of Triton-based scintillator fluor solution [composition: 1900 ml of toluene, 950 ml of Triton X-100, 200 ml of water, 15 g of 2,5-diphenyloxazole and 0.325 g of 1,4-bis-(4-methyl-5-phenoxazol-2-yl)-benzene]. Radioactivities of samples were counted at room temperature in a Beckman liquid-scintillation spectrometer.

Terminal digestion of DNA with restriction endonuclease

^3H -labelled HeLa-cell DNA or bacteriophage T_7 DNA (50 $\mu\text{g}/\text{ml}$) was digested to terminal segments after 60 min at 37°C in a reaction mixture containing (final concentrations) 6.7 mM-MgCl₂, 70 mM-NaCl, 7 mM- β -mercaptoethanol and 20 mM-Tris-HCl buffer, pH7.4, with 0.2 unit of *Haemophilus influenzae* DNA restriction endonuclease/ml (units are those defined by Smith & Wilcox, 1970). Terminal digestion was tested by digesting simultaneously 0.05–1.0 μg of ^{32}P -labelled bacteriophage ϕX174 -RFI DNA/ml in a parallel reaction, and observing the production of bacteriophage ϕX174 -DNA segments of a size characteristic of terminal reaction with the enzyme. Reactions were stopped by addition of 1 M-EDTA, pH8.0 (1:50, v/v), and heating at 60°C for 5 min.

Assay of DNA segment size

DNA segment size was determined by electrophoresis on low-concentration polyacrylamide gels containing agarose. Gels were polymerized in vertical channels of dimensions 2 mm $\times$ 10 mm $\times$ 140 mm in Perspex [poly(methyl methacrylate)] moulds consisting of two halves. A 3% (w/v) solution of acrylamide monomer was prepared from a stock solution of acrylamide-bisacrylamide (19:1, w/w) in distilled water (20%, w/v) by diluting the stock solution into an aqueous solution, held at 45°C and containing dissolved agarose (final concentration 0.5%, w/v) and 3-dimethylaminopropionitrile (final concentration 0.4%, v/v) in electrophoresis buffer (20 mM-sodium acetate – 10 mM-EDTA – 40 mM-Tris-HCl buffer,

pH7.2). Polymerization was affected by adding ammonium persulphate (final concentration 0.05%, w/v).

After being poured into the channels, the acrylamide-agarose solution was held at room temperature for 90 min to polymerize. Gels were pre-electrophoresed for 60 min in electrophoresis buffer at a current of 3 mA/gel. DNA samples (20 μl) in sucrose (5%, w/v) were applied to the top of each gel, and electrophoresis was carried out at room temperature with the same electrical current.

Exonuclease III degradation of DNA

Partial degradation of DNA using exonuclease III was carried out at 37°C in buffer containing 1 mM-MgCl₂ – 1 mM- β -mercaptoethanol – 67 mM-Tris-HCl buffer, pH8.0. The reaction was terminated by addition of 20 $\times$ SSC medium* to a final concentration of 2 $\times$ SSC medium, and cooling to 0°C.

The extent of DNA degradation was determined by measuring quantitatively the acid-soluble radioactivity released. A sample (10 μl) of reaction mixture containing radioactively labelled DNA was added to calf thymus DNA carrier (90 μl of 2.5 mg/ml in water). Then 100 μl of trichloroacetic acid (10%, w/v) was added and the mixture was shaken thoroughly. After 60 min at 0°C the precipitate was separated by centrifugation (1000 g for 10 min at 4°C). A portion of the supernatant (100 μl) was assayed for acid-soluble radioactivity by liquid-scintillation spectrometry.

Annealing conditions

DNA samples (0.2 ml) were annealed for various times by incubating at 65°C in a solution containing 4 $\times$ SSC medium, followed by quenching to 0°C in an ice bath.

Electron microscopy

(a) *Aqueous technique.* DNA molecules were mounted for electron microscopy by using a modification of the Kleinschmidt basic protein film technique (Kleinschmidt, 1968). A 40 μl portion of a solution containing 1 μg of DNA/ml, 0.5 M-ammonium acetate, 1 mM-EDTA and 50 μg of cytochrome *c*/ml were spread on to a hypophase solution containing 0.25 M-ammonium acetate, pH7.5. The protein film was picked up on to copper electron-microscope grids, previously coated with a Parlodian support film, and stained for 30 s in a fresh solution of 50 μM -zinc uranyl acetate and 50 μM -HCl in 90% (v/v) ethanol. This was followed by washing for 10 s in 2-methylbutane, after which samples were air-dried.

DNA specimens were rotary-shadowed with Pt-Pd (4:1, w/w) and viewed in a Hitachi HS-7S electron microscope. Photographs were taken at an approximate magnification of 40000. The precise magnifica-

* Abbreviation: SSC medium, 0.15 M-NaCl–15 mM-trisodium citrate, pH7.0.

tion was determined in each experiment by using a standard diffraction grating.

(b) *Formamide technique.* The method used was as described above, except that the hypophase was a freshly prepared solution of 10% (v/v) formamide in 10mM-Tris-HCl buffer, pH8.5. The DNA spreading solution contained (final concentrations) 0.5–1.0 μ g of DNA/ml, 50 μ g of cytochrome *c*/ml, 10mM-EDTA and 0.1M-Tris-HCl buffer, pH8.5. Formamide was added to the required concentration. Grids were stained and shadowed as above.

DNA contour length measurement

DNA contour lengths were calculated from measurements made directly from magnified electron micrographs by using a Hewlett-Packard desk computer programmed for length measurement.

Results and Discussion

Formation of rings from HeLa-cell DNA after terminal digestion with *Haemophilus influenzae* restriction endonuclease

^3H -labelled HeLa-cell DNA and ^{32}P -labelled bacteriophage ϕX174 -RFI DNA were digested simultaneously to terminal segments with *Haemophilus influenzae* DNA restriction endonuclease.

A sample of the digest was applied to a 3% (w/v) polyacrylamide gel containing 0.5% agarose and electrophoresis was carried out for sufficient time to allow separation of the characteristic ^{32}P -labelled bacteriophage ϕX174 DNA segments. Fig. 1 shows the distribution of DNA segments observed after terminal digestion with restriction endonuclease. The segments produced from HeLa-cell DNA in such a reaction were markedly larger than those formed from bacteriophage ϕX174 -RFI DNA, and migrate as a single diffuse band in 3% polyacrylamide. The length distribution of the terminal HeLa-cell DNA segments was found by electron microscopy to be in the range 0.2–2.1 μm , or 600–6300 nucleotide pairs, with a number-average size and weight-average size of 1500 and 2340 base pairs respectively. Hence the larger HeLa-cell DNA segments, although heterogeneous in size, are not separated by electrophoresis under these conditions.

In a similar experiment, ^3H -labelled bacteriophage T_7 DNA duplexes were digested to termination with restriction endonuclease, in the presence of marker ^{32}P -labelled bacteriophage ϕX174 -RFI DNA. The size of the bacteriophage T_7 DNA segments, estimated by electrophoretic migration relative to bacteriophage ϕX174 DNA terminal segments, was found to be in the range 0.1–1.2 μm (200–3500 nucleotide pairs). A similar size distribution was obtained by direct measurement of the bacteriophage T_7 DNA segments by use of the electron microscope.

The terminal HeLa-cell DNA segments generated

as above, in a parallel reaction without bacteriophage ϕX174 -RFI DNA marker, were subjected to partial degradation with exonuclease III, a process termed resection. The exonuclease-resected DNA segments were then annealed and the DNA structures formed were observed in the electron microscope. For quantitative analysis, photographs of microscope grids were taken at random, and all structures in a given field of view were classified. A typical result is shown in Plate 1. The structures observed were classified as follows. In addition to linear molecules, many ring structures were found (R in Plate 1) and loops, or lariats were observed (L). Branched structures were also present (B). No attempt was made to interpret more complex structures, but these were classified separately (C). The results are summarized in Table 1, showing the relative frequency of structures obtained after exonuclease III resection. Few structures other than linear types were observed for HeLa-cell DNA segments before exonuclease III resection, whereas 20.1% of the total structures were ring-containing (rings+lariats) after removal of 235

Fig. 1. Characterization of terminal HeLa-cell DNA segments and bacteriophage ϕX174 -RFI DNA segments by polyacrylamide-gel electrophoresis

^3H -labelled HeLa-cell DNA (50 $\mu\text{g}/\text{ml}$) and ^{32}P -labelled bacteriophage ϕX174 -RFI DNA (0.5 $\mu\text{g}/\text{ml}$) were digested simultaneously with restriction endonuclease (0.2 unit/ml) as described in the text. Terminal DNA segments were applied to a 3% (w/v) polyacrylamide gel containing 0.5% (w/v) agarose, and electrophoresis was carried out for 3 h at room temperature with a current of 3 mA/gel. Then 2 mm gel slices were dissolved in 0.1 ml of '20 vol.' H_2O_2 solution by heating at 90°C for 90 min. Samples were immersed in Triton X-100-based scintillation fluid and counted for radioactivity by liquid-scintillation spectrometry. ----, ^3H radioactivity (HeLa-cell DNA segments); —, ^{32}P radioactivity (bacteriophage ϕX174 DNA segments). The numbers are the sizes of the terminal ^{32}P -labelled bacteriophage ϕX174 DNA segments which are the estimates in nucleotide pairs determined by Edgell *et al.* (1972).

EXPLANATION OF PLATE I

Electron micrograph of terminal HeLa-cell DNA segments after partial digestion with exonuclease III and annealing

The experimental details are as described in the text and Table 1. The electron micrograph shows DNA structures obtained on annealing after removal of 235 nucleotides per terminus from the DNA segments with exonuclease III. Structure classification is described in the text. R, Rings; L, lariats; B, branches; C, complex.

EXPLANATION OF PLATE 2

Structures observed by electron microscopy after use of the aqueous spreading solution containing formamide for HeLa-cell DNA after resection with exonuclease III at single-chain nicks

Structures were observed by electron microscopy using a spreading solution containing 60% (v/v) formamide, after removal of 1350 nucleotides per terminus with exonuclease III and annealing. Experimental conditions are as described in Table 2. R, Ring; L, lariat; H, H-branches; R,H, structure containing a ring and an H-branch; R', double ring.

Table 1. *Classification of structures observed by electron microscopy for terminal HeLa-cell DNA and bacteriophage T₇ DNA segments after partial degradation with exonuclease III and annealing*

³H-labelled HeLa-cell DNA or bacteriophage T₇ DNA (50 µg/ml) was digested to terminal segments with restriction endonuclease as described in Fig. 1. DNA segments were partly degraded with exonuclease III and annealed for 2 h at 65°C at a DNA concentration of 5 µg/ml in 4×SSC medium. The DNA structures generated were observed by electron microscopy after use of the aqueous basic protein film technique, and were classified as described in the text. The average degree of exonuclease III resection in each case was determined from estimates of the radioactive acid-soluble nucleotides released. For HeLa-cell DNA segments, the number of nucleotides removed per terminus was calculated from the length for the weight-average DNA segment (0.78 µm or 2340 base pairs, assuming 1 µm = 3000 base pairs duplex length). This value was corrected to 1560 base pairs by multiplying by 2/3 to take into account resection taking place at the single-chain nicks present in the DNA preparation (one per duplex length of 0.75 µm). For bacteriophage T₇ DNA segments, the weight-average duplex length (0.6 µm or 1800 base pairs) was determined by gel electrophoresis and checked by centrifugation on neutral sucrose gradients. This DNA contained no nicks detectable by alkaline-sucrose-gradient centrifugation.

DNA	Nucleotides removed per terminus	No. of structures analysed	% of each structural type				
			Linear	Ring	Lariat	Branched	Complex
HeLa	0	397	94.2	1.3	1.5	2.8	0.3
HeLa	80	504	75.0	6.7	7.3	6.1	4.8
HeLa	235	456	73.4	10.9	9.2	4.8	1.6
T ₇	0	400	100.0	0	0	0	0
T ₇	240	400	93.5	1.5	0.5	4.5	0

Fig. 2. *Contour length distribution of structures formed from terminal HeLa-cell DNA segments after exonuclease III resection and annealing*

Experimental conditions were as described in Plate 1. Contour length distributions for 300 structures are shown for (a) linear structures, (b) rings, (c) lariats (total length) and (d) lariat rings.

nucleotides per terminus. Branched structures were less predominant under the same conditions (4.8% of total).

For restriction endonuclease-digested bacteriophage T₇ DNA segments, after a similar treatment with exonuclease III, which resulted in removal of 240 nucleotides per terminus (Table 1), ring-containing forms were only 2% of the total structures observed. Branched structures were more frequently found.

The contour length distribution of the structures formed from terminal HeLa-cell DNA segments after exonuclease III resection is shown in Fig. 2. The result for rings (Fig. 2b) is compared with linear molecules in the same micrographs (Fig. 2a). In the case of lariats, two measurements were possible. Results are expressed as the distribution of measurements for lariat total length (Fig. 2c) or, alternatively, the circumference of lariat rings (Fig. 2d).

Formamide 'melting' of rings formed from restriction endonuclease-digested HeLa-cell DNA segments

The observed frequency of formation of rings for HeLa-cell DNA segments as a function of the degree of exonuclease III resection is shown in Fig. 3. With the use of the aqueous spreading technique for specimen preparation, the frequency of rings+lariats increased sharply after removal of approximately 50 nucleotides per terminus. Ring frequency reached a maximum after removal of 200 nucleotides, and then began to fall gradually. The ring frequency obtained when electron-microscope grids were coated with DNA molecules from a solution containing 60% formamide was about one-half of that observed with aqueous solution at the same degree of exonuclease III resection. It is known that each 10% increase in formamide concentration produces a denaturing effect equivalent to raising the temperature by steps of 7.2°C (McConaughy *et al.*, 1969). DNA structures

Fig. 3. Frequency of ring-containing structures observed by electron microscopy for terminal HeLa-cell DNA segments as a function of the degree of exonuclease III resection

The ordinate represents the combined percentage of rings+lariats observed after partial exonuclease III resection and annealing of terminal HeLa-cell DNA segments. The number of nucleotides removed by exonuclease III per terminus was determined as described in Table 1. Annealing was carried out at a DNA concentration of 5 μ g/ml for 2 h at 65°C in 4 $\times$ SSC medium. ●, Ring frequency observed after use of the aqueous basic protein film technique; ○, ring frequency observed after use of a DNA spreading solution containing 60% (v/v) formamide, as described in the text.

observed in 60% formamide are therefore those that would be stable at 63.2°C, the equivalent temperature.

By preparing DNA specimens for electron microscopy from solutions containing an increasing formamide concentration, a formamide 'melting' curve was generated for rings formed from resected HeLa-cell DNA segments (Fig. 4). By this technique, the equivalent 'melting' temperatures for rings formed after removal of 180 and 430 nucleotides per terminus were found to be 45° and 56°C respectively.

Formation of rings from HeLa-cell DNA after partial degradation with exonuclease III at single-chain nicks

In addition to removing nucleotides from the 3'-ends of DNA duplexes, exonuclease III also acts at single-chain nicks (Richardson *et al.*, 1964). It is therefore possible to expose internal complementary single-chain regions in the isolated HeLa-cell DNA preparation without prior treatment with restriction endonuclease. More extensive resection of each exonuclease III-sensitive terminus is achieved, because the average distance between successive sites of resection is determined by the density of single-chain

nicks, assuming the DNA duplex to be infinitely long. With the HeLa-cell DNA preparation used presently, the duplex length was in excess of 12 μ m. Hence the average distance between sites of resection is approx. 1.5 μ m (4500 nucleotides), determined from the average single-chain length in alkaline sucrose gradients, compared with 1560 nucleotides for weight-average restriction endonuclease-digested HeLa-cell DNA segments (Table 1).

The DNA structures formed after exonuclease III resection and annealing were observed by electron microscopy, with 60% formamide in the specimen solution, to allow micrographs to be obtained of both single-chain and duplex regions of DNA molecules (Davis *et al.*, 1970). The structures generated were classified as described above and are shown in Table 2. After removal of an average of 1350 nucleotides per terminus with exonuclease III, 9.5% of the total structures observed contained rings. Of the 74 ring-containing structures analysed in detail (R in Plate 2), 58 each had two tails emerging from the closed ring. The remaining 16 each had a lariat configuration with only one tail. The branched structures observed were of two types, either H-branched, with

Fig. 4. Formamide 'melting' profile of ring-containing structures observed for terminal HeLa-cell DNA segments after partial degradation with exonuclease III and annealing

³H-labelled HeLa-cell DNA was digested to terminal segments by restriction endonuclease, partly degraded with exonuclease III and annealed at 5 μ g/ml for 2 h in 4 $\times$ SSC medium at 65°C. Samples were mounted for electron microscopy from a spreading solution containing formamide (0–80%, v/v). The ordinate is the frequency of rings+lariats observed at each formamide concentration relative to the frequency observed after use of the aqueous spreading technique, at the same degree of exonuclease III resection. The extent of exonuclease III resection was determined as described in Table 1. ●, 180 nucleotides removed per terminus with exonuclease III; ○, 430 nucleotides removed per terminus with exonuclease III.

Table 2. Classification of structures formed from HeLa-cell DNA after partial degradation at single-chain nicks with exonuclease III, followed by annealing

Structure classification is described in the text. The number of nucleotides removed per terminus from HeLa-cell DNA by exonuclease III was determined from measurements of the acid-soluble radioactivity released, assuming that each exonuclease III-sensitive site was a single-chain nick occurring every 1.5 μm (4500 base pairs). Estimates were not corrected for resection at the ends of the duplex DNA molecules. After resection, DNA was annealed at a concentration of 5 $\mu\text{g}/\text{ml}$ for 2 h at 65°C in 4 $\times$ SSC medium, and mounted for electron microscopy from spreading solution containing 60% (v/v) formamide. The results for bacteriophage T₇ DNA were obtained after terminal digestion with restriction endonuclease, and after removal of 240 nucleotides per terminus with exonuclease III, as described in Table 1. Annealing and electron microscopy were carried out as described above after use of an aqueous spreading solution containing 60% (v/v) formamide.

DNA	No. of structures analysed	Nucleotides removed per terminus	% of each structural type				
			Linear	Ring+Lariat	H-branch	Y-branch	Other
HeLa	463	0	99.8	0	0	0.2	0
HeLa	356	900	66.6	16.8	3.8	7.0	5.8
HeLa	518	1350	67.9	9.5	9.3	7.2	6.4
T ₇	500	240	100	0	0	0	0

four distinct ends (H in Plate 2), or Y-branched, with three ends. More complicated structures, sometimes containing both rings and branches (R, H and R' in Plate 2), were classified separately.

By this treatment, significantly more branched structures were generated than for restriction endonuclease-digested HeLa-cell DNA segments. Fewer ring-containing structures were formed, and ring frequency decreased further after more extensive exonuclease resection.

From the results presented it is demonstrated that, when the complementary chains of HeLa-cell DNA are exposed by partial degradation with exonuclease III, the single chains associate during the annealing reaction to form rings and branched DNA structures. Bacteriophage T₇ DNA, when treated similarly, forms far fewer rings, but significant numbers of branches (Table 1). However, all the structures formed from bacteriophage T₇ DNA segments dissociate into linear forms in 60% formamide (Table 2), demonstrating that the single-chain associations in this case are weak, possibly non-specific, interactions. This result suggests that the formation of stable rings and branches is a special property of HeLa-cell DNA.

Fig. 5 illustrates how rings and lariat structures can be generated from linear DNA segments after exonuclease III treatment, provided that they contain two or more regions of repeated base sequence. Because DNA single chains are collapsed when specimens are prepared with the use of aqueous spreading solution, lariat tails must be partly duplex in order to be visible as extended chains when viewed in the electron microscope after this method has been employed. For this reason, it is suspected that such structures arise by annealing a resected single-chain end to a complementary sequence exposed by exonuclease III at nicks in DNA molecules, as depicted in Fig. 5. The HeLa-cell DNA preparation used contains a nick

every 1.5 μm , and, after terminal digestion with restriction endonuclease, HeLa-cell DNA segments 0.7 μm long would contain, on average, one nick per molecule. Removal of 235 nucleotides from each of three possible 3'-termini with exonuclease III would result in such molecules having a duplex length of 0.47 μm and a total single-chain length of 0.23 μm . On this basis, DNA molecules with an observed contour length less than 0.47 μm (Fig. 2a) would be expected to exhibit a decreasing tendency to form lariat structures, because fewer of these shorter molecules would possess a nick necessary to generate an internal single-chain gap with exonuclease. Similarly, competition of single-chain gaps and ends for the annealing reaction could explain why larger DNA molecules preferentially form lariat structures (Fig. 2c) as opposed to perfect rings (Fig. 2b). Rings less than 0.1 μm in circumference are difficult to distinguish from background contamination on the electron-microscope grids, and this is one explanation for the few rings scored for very small DNA molecules ($\approx 0.01 \mu\text{m}$).

At this point it is useful to consider possible alternative arrangements of repeated base sequences that can account for the formation of DNA rings. Two extreme models are shown in Fig. 6.

The first model (Fig. 6, I) is a tandem arrangement of repeated sequences proposed by Lee & Thomas (1973) to account for ring formation in *Drosophila* polytene DNA. In this case, the chromatid consists of a continuous series of nucleotide sequences, g_i nucleotide pairs in length, unrelated in base sequence to other regions of the chromatid. Within each structural unit of length is a variable region consisting of n_i tandemly repeated copies of a particular base sequence of length s_i . For a collection of appropriate DNA segments of length L , it is possible to form rings only from those molecules where

Fig. 5. Formation of rings and lariats from DNA segments containing hypothetical repeated sequences

In the first part of the Figure each chain of the DNA duplex is represented as a single solid line. 5'-Termini are those marked with a bar. The duplex DNA segment depicted has an internal single-chain nick in one chain, and contains three regions of repeated base sequence shown as —ABC— in one chain, and in the complementary chain as —abc—. Exonuclease III removes nucleotides from the 3'-termini to expose the regions of complementary sequence. During the annealing reaction, two exposed repeated sequences —ABC— compete for a single complementary sequence —abc— to form rings. In the final part of the Figure, the ring structures generated are shown as they would be observed by electron microscopy with the use of the aqueous basic protein film technique, with duplex portions extended and single-chain regions collapsed into bushes, represented as —●—. End-to-end annealing results in ring formation, and end-to-middle annealing results in the formation of a lariat structure.

$L > s_i$. Hence the minimum size, R , of DNA ring that can be generated is determined by the length s_i . Similarly, the maximum size of ring is limited by the length of the tandemly repeated region ($=n_i s_i$), so that for rings $s_i < R \ll (n_i - 1)s_i$. This model is thought to account for the dependence of ring formation on

DNA fragment size for *Drosophila* polytene DNA (Lee & Thomas, 1973).

In the alternative model, repeated sequences of length s_i base pairs are arranged intermittently along the chromatid, separated by a variable length of more complex base sequence, T_i base pairs in length. In this case rings can be formed from appropriate segments provided that $L > (s_i + T_i)$. Since the repeated sequences i are not necessarily regionalized, as in model (I), there is no theoretical upper limit to DNA ring size. Hence for rings the only boundary condition is that $(s_i + T_i) < R$.

These two models make different predictions with regard to the character of the complementary base sequences responsible for ring closure. In model (I), progressive exonuclease III resection of DNA segments exposes correspondingly larger regions of complementary sequence available for ring formation, as shown in Fig. 6. The model predicts that a heterogeneous collection of increasingly long ring closures, b , should be formed ($b \ll s_i, 2s_i, 3s_i$ etc.). By contrast, in model (II), extensive resection with exonuclease exposes only limited regions of complementary base sequences ($b \ll s_i$). It was conceivable that determination of ring closure lengths might distinguish between these possible models.

Use of DNA restriction endonuclease

It has been shown by Kelly & Smith (1970) that one of the DNA restriction endonucleases from *Haemophilus influenzae* cleaves the double helix at specific sites defined by the base sequence:

A small genome such as bacteriophage ϕ X174, which contains a limited number of such sequences, is cleaved into duplex DNA segments of characteristic size after terminal reaction with the enzyme (Edgell *et al.*, 1972). In the present experiments simultaneous production of characteristic segments of bacteriophage ϕ X174-RFI DNA was used as an assay for terminal reaction of restriction endonuclease with HeLa-cell DNA (Fig. 1).

Let us now suppose that DNA rings are generated with utilization of many different classes of repeated sequence, and that each sequence family contains a site for cleavage with restriction endonuclease. In each class, individual sequences are assumed to be identical, so that all copies of s_i would be cleaved at the same site. Since the DNA segments thus generated would all contain unique base sequence, the expected ring frequency for such segments would be zero, contrary to the results presented. The observed frequency of rings is approx. 20% (Table 1), compared with values of 17% obtained for rings formed from HeLa-cell DNA fragments generated by random

Fig. 6. Alternative models for organization of repeated sequences in DNA

Model (I): the tandem repeat model. The chromatid is considered to be organized into regions, g_i nucleotide pairs in length, unrelated in base sequence to other segments of the chromatid. A particular region g_i is subdivided into two parts, one containing n_i tandemly repeated copies of a particular base sequence of length s_i , and the other containing $g_i - n_i s_i$ base pairs of a more complex base sequence. An adjacent region of the chromatid of total length g_j base pairs is shown, containing n_j tandemly repeated copies of a different base sequence of length s_j . Model (II): the intermittent repeat model. Families of a given repeated sequence of length s_i are arranged intermittently along the chromatid, the distance between successive copies of repeated sequence i being T_i base pairs. In both models the arrows interrupting the DNA duplex are hypothetical sites of cleavage by restriction endonuclease. In each case rings can be formed from the appropriate DNA segments after exonuclease III resection and annealing.

shear breakage (N. Hardman, unpublished work). It is evident that many of the repeated sequences present in HeLa-cell DNA responsible for ring formation must, for some reason, be resistant to cleavage by restriction endonuclease. There are two likely explanations for this observation. First, the base sequence recognition site for cleavage with restriction endonuclease could be consistently absent from most of the repeated sequence families. Alternatively, successive copies of a given repeated sequence may not be identical, such that some members of a particular sequence family would not contain a site of cleavage by restriction endonuclease. Terminal segments could thus be generated containing two or more regions of closely related but not identical base sequence, and these DNA segments could form rings.

Ring stability

Fig. 3 shows that many of the repeated sequences involved in ring formation occur close to the ends of restriction endonuclease-digested segments of HeLa-cell DNA. This is indicated by the marked increase in ring frequency after only limited resection with exonuclease III. However, more than half of the rings thus formed are relatively unstable and dissociate to linear forms when exposed to 60% formamide.

The empirical equation presented by Thomas & Dancis (1973) relates the stability of a duplex polynucleotide to its length:

$$t_m = 81.5 + 16.6 \log M + 0.41 (\%G+C) - 820/b - 315p$$

where t_m is the 'melting' temperature of the duplex in $^{\circ}\text{C}$, M the molar concentration of univalent cation, $G+C$ the average guanine+cytosine content of the duplex, b its length in base pairs and p the fraction of mismatched bases. From this equation, and assuming that HeLa-cell DNA is 40% G+C, the 'melting' temperature for native infinitely long HeLa-cell DNA duplexes is 94.2°C under the conditions of DNA annealing presently reported ($M = 0.68\text{M}$). During DNA specimen preparation for electron microscopy, $M = 0.12\text{M}$ and t_m is thus lowered to 80.4°C .

By using the above equation, and by measuring ring frequency as a function of formamide concentration (Fig. 4), it is possible to determine the equivalent temperature at which one-half of the rings are dissociated (t_m for rings) and relate this to the length, b , of duplex DNA ring closures. The 'melting' temperature of rings formed after removal of 180 and 430 nucleotides from HeLa-cell DNA segments with exonuclease III is 45° and 56°C respectively (Fig. 4). Assuming a t_m of 80.4°C for HeLa-cell DNA under these conditions, the respective 'melting' temperatures are $t_m - 35.4^{\circ}\text{C}$ and $t_m - 24.4^{\circ}\text{C}$. Since annealing experiments were carried out at 65°C and $M = 0.68\text{M}$, equivalent to $t_m - 29.2^{\circ}\text{C}$, about one-half of the rings

observed by electron microscopy with the use of the aqueous spreading technique (Table 1) would be dissociated at the annealing temperature. This suggests that less specific base-pair interactions possibly occur when the temperature is lowered in the DNA specimen, after annealing and during sample preparation for electron microscopy. If it is assumed that the ring closures are perfectly matched sequences of average base composition for HeLa-cell DNA, then it can be calculated that, after removal of 180 nucleotides with exonuclease III, the average closure length for rings melting at 45°C is 23 nucleotide pairs. Similarly, rings formed after removing 430 bases per terminus, which 'melt' at 56°C, are closed by an average of 33 nucleotides.

However, the smaller DNA segments generated by restriction endonuclease, 600 base pairs in length, would be almost completely single-chain after removal of 300 nucleotides from each 3'-terminus with exonuclease III. For rings formed from such DNA segments, the length of DNA duplex determining ring stability might no longer be the closure length, b , formed by annealing repeated base sequences in the exposed single chains, but instead a smaller duplex length, b' , remaining in the molecule after exonuclease resection (Fig. 7c). Such a factor could account for the decrease in ring frequency observed after extensive resection of HeLa-cell DNA segments with exonuclease and their instability in formamide (Fig. 3). This could also provide an alternative explanation for the absence of rings shown in Fig. 2(b) for the smallest HeLa-cell DNA segments.

An attempt was made to overcome this ambiguity in ring-stability experiments by forming rings from HeLa-cell DNA before treatment with restriction endonuclease. Since nicks were present every 4500 nucleotides in the DNA preparation, it was possible theoretically to remove with exonuclease III an average of 2250 nucleotides per terminus before the complementary single chains would dissociate. In practice, after removal of an estimated 1350 nucleotides per terminus with exonuclease, as described in Table 2, some of the molecules dissociated in 60% formamide. This was suggested by the observation that, before resection with exonuclease, over 95% of the HeLa-cell DNA molecules examined by electron microscopy with the use of 60% formamide were larger than 12 μm , whereas after exonuclease treatment the contour length for linear molecules was in the range 0.5–4.0 μm . This indicates that the residual duplex regions of the DNA molecules were in some cases too small to remain stable in 60% formamide.

As an alternative approach to overcome these difficulties, direct observation of ring-closure lengths was attempted. If the complementary base sequences responsible for ring closure always occurred at the ends of the annealed single chains, as is the case for terminal base sequence repetitions in bacteriophage

Fig. 7. Chain diagrams for rings and branched DNA structures

In (a) the terminal ends of an exonuclease III-resected DNA segment are considered to be exactly complementary, forming a perfect ring with no single-chain ends. In (b) the regions of paired complementary sequence occur at some distance of each end of the resected DNA segment, such that two single-chain ends define the region of ring-closure, b . The molecule depicted in (c) is similar to that in (b) except that resection with exonuclease is more extensive, resulting in a residual duplex length, b' , shorter than the duplex length, b , that annealed to form the ring-closure. In (d) two resected DNA segments with a region of common sequence have annealed to form a branched structure. The distance, r , between the junctions of tail pairs defines the region of common sequence.

T₇ DNA (Ritchie *et al.*, 1967), the perfect rings with no single-chain ends are expected, as shown schematically in Fig. 7(a). Conversely, if ring closures are made by base sequences at some distance from each end of the exposed single chains, duplex closures would be bracketed by non-complementary regions, resulting in the formation of rings with two tails visible in the electron microscope under conditions where single chains were extended (Fig. 7b). For these molecules, it is possible to measure the distance between the two tails along the circumference of the ring and so determine the physical length of the region of complementary sequence. It was found that 58 out of 74 of the rings shown in Table 2 after removal of 1350 nucleotides by exonuclease III fall into this category (see Plate 2, R). The distribution of closure lengths for rings, estimated by this method,

Fig. 8. Size distribution of the suspected regions of repeated sequence responsible for the formation of rings and branches in HeLa-cell DNA

In (a) and (b) HeLa-cell DNA was partly degraded with exonuclease III and annealed as described in Table 2. Data were from electron micrographs similar to Plate 2, after removal of 1350 nucleotides per terminus with exonuclease III and annealing, followed by electron microscopy with the use of the spreading solution containing 60% (v/v) formamide. The lengths, b , for the inferred regions of ring closure are shown in (a), and the lengths, r , for regions of suspected common sequence in H-branches are represented in (b). In (c) restriction endonuclease-digested terminal segments of HeLa-cell DNA were heat-denatured, and annealed to $C_0t = 0.1 \text{ mol} \cdot \text{s} \cdot \text{l}^{-1}$ as described in Table 3. H-branched structures were observed by electron microscopy with the use of a spreading solution containing 60% (v/v) formamide, and the regions of suspected common sequence were measured as in (b).

is shown in Fig. 8(a). All of the ring closures formed a narrow size distribution of 0.02–0.1 μm (50–300 base pairs), considerably less than the average DNA single-chain length of 1350 nucleotides exposed by exonuclease. By reference to Fig. 6, this result is more readily consistent with an arrangement of repeated sequences such as that depicted in model (II), where limited regions of complementary sequence are exposed after extensive exonuclease resection.

Origin of branches

Branched structures are generated from HeLa-cell DNA segments after partial resection with exonuclease III (Table 1). These are probably not formed as a result of weak base-pair interactions, because many branches are also observed in 60% formamide (Table 2). This suggests that regions of specific complementary base sequence occur within single-chain portions of different segments of HeLa-cell DNA, which can anneal to form stable duplexes. For H-branches, the annealed regions of intermolecular complementary sequence would presumably occur at some distance from the ends of the DNA molecules, such that they are bounded by four tails, as shown schematically in Fig. 7(d). The distance between the junctions of tail pairs therefore defines the region of common sequence, r . The distribution of measurements for r , obtained for 45 H-branches formed after

removal of 1350 nucleotides per terminus by exonuclease, is shown in Fig. 8(b). A striking similarity between the length estimates for b and r is evident. This strongly suggests, but does not prove conclusively, that similar types of base sequence repeats may be responsible for the formation of rings and branches in HeLa-cell DNA. The formation of DNA branches and their configuration is also in accord with an intermittent model for the distribution of repeated sequences, shown in model (II). Branched structures can be generated between two individual DNA segments provided that each contains a region of common repeated sequence. The tandem arrangement of repeated sequences cannot account for the configuration of such large numbers of stable H-branched DNA structures. Moreover, because by definition such sequences are considered to be confined to a unique region of the chromatid, on the basis of this model members of a particular repeated sequence family would be found in few DNA segments after cleavage with restriction endonuclease. Contrary to this expectation, the rate of formation of branches (Table 3) shows that individual copies of a given repeated sequence are probably distributed, on average, among many thousands of DNA segments, as described below.

A further distinction can be made between the two models. It has already been stated that, in order to

account for the abundance of rings formed from HeLa-cell DNA segments generated by terminal digestion with restriction endonuclease, most repeated sequence families responsible for ring formation must be resistant to cleavage by the enzyme. If such sequence families were tandemly arranged in HeLa-cell DNA, as in model (I), then denaturation followed by annealing should, in most cases, re-form the original DNA duplexes with the single-chains 'slipped' (Thomas *et al.*, 1970), and thus such molecules should be able to form rings efficiently. In contrast, denatured single chains containing intermittently repeated base sequences would preferentially form heteroduplex structures with non-complementary regions of more complex sequence remaining as single-chain tails. This prediction was tested by annealing heat-denatured terminal HeLa-cell DNA segments and classifying the structures generated as a function of annealing time, by using electron microscopy after use of the aqueous solution containing formamide. The results are shown in Table 3. The branched structures formed were H and Y types, as before. For H-branches, the regions of suspected complementary sequence, r , were measured. The size distribution of measurements is shown in Fig. 8(c). The results correlated closely with ring closure lengths, b , and H-branch complementary sequences, r , obtained for exonuclease-resected HeLa-cell DNA (Figs. 8a and 8b). This result is consistent with the predictions of the intermittent repeat model but not the tandem repeat model. What was at first thought to be a contradictory result was the observation of 3–4% residual rings after denaturation and annealing (Table 3). It is suspected that this might be a special class of rings formed by intrachain annealing in some of the denatured HeLa-cell DNA single chains, because approximately equal numbers were formed at zero annealing time after a tenfold dilution of the

DNA specimen at 4°C ($C_0t = 0$; Table 3). These structures are possibly generated from intermittent repeated sequences periodically reversed between the two DNA chains similar to the situation in mouse satellite DNA (Flamm *et al.*, 1969).

The weight of evidence therefore indicates that many of the repeated sequences responsible for the formation of rings and branches in HeLa-cell DNA are probably arranged intermittently along the chromatid. If the assumptions made in interpretation of the data are valid, then the intermittent repeated sequences are probably about 50–300 base pairs in length. The hexanucleotide sequence necessary for cleavage with the restriction endonuclease would be expected to occur within a collection of polynucleotides of random base sequence once every $4^2 \times 4^2 \times 2^2 = 1024$ nucleotides. Hence, assuming a Poisson distribution of hits, the fraction of repeated sequence families of average length 180 base pairs which might be expected to escape cleavage by the enzyme would be given by the zero-order Poisson term, $e^{-180/1024} \approx 0.84$; i.e. most of the repeated sequences would be resistant to endonuclease digestion, in accord with the present findings.

If it is supposed that these intermittent repeated sequences are responsible for most of the rings formed from HeLa-cell DNA segments (Table 1), examination of ring contour lengths can reveal information about their distribution. The size distribution of perfect rings (Fig. 2b) and lariat rings (Fig. 2d) closely resembles that observed for linear molecules under the same conditions, so combined ring frequency (approx. 20%) is a measure of the fraction of HeLa-cell DNA containing the repeated sequences. This is likely to be an underestimate, since the efficiency of ring formation is probably less than 100%, and in addition the analysis excludes branches and other DNA segments containing only one copy of a

Table 3. Classification of structures observed by electron microscopy after use of aqueous spreading solution containing formamide after heat-denaturation and annealing of restriction endonuclease-digested terminal HeLa-cell DNA segments

³H-labelled HeLa-cell DNA ($C_0 = 1.4 \times 10^{-5}$ mol of nucleotide/l) was digested to termination with restriction endonuclease, as described in Table 1, and heat-denatured for 10 min at 100°C in $4 \times$ SSC medium. A sample (0.5 ml) of the solution was taken and plunged into ice-cold $4 \times$ SSC medium for the $C_0t = 0$ time-point. The denatured DNA solution remaining was cooled to 65°C and incubation was continued at this temperature. Samples (50 μ l) were taken periodically and diluted into 0.5 ml of $4 \times$ SSC medium at 4°C to minimize further annealing, after which each specimen was prepared immediately for electron microscopy with the use of the spreading solution containing 60% (v/v) formamide. The structures generated were classified as described in the text.

No. of structures analysed	% of each structural type				C_0t (mol·s·l ⁻¹)
	Linear	Ring+Lariat	Branched	Other	
500	93.5	3.8	2.4	0.2	0
500	89.2	5.0	5.0	0.8	0.01
500	91.0	2.4	6.6	0	0.10
500	87.1	3.6	8.6	0.8	0.50

given repeated sequence. Hence the minimum fraction of the HeLa-cell genome containing the repeated base sequences is probably about 20%. From a value of 7×10^9 base pairs for the human haploid genome (Saunders *et al.*, 1972) and 1500 base pairs for the number-average restriction endonuclease-digested HeLa-cell DNA duplex, 4.6×10^6 terminal segments are generated per haploid HeLa-cell genome by restriction endonuclease. On the assumption that two repeated sequences of average length 180 base pairs are required for ring formation, the total number of repeated sequences in 20% of the segments is 1.8×10^6 , which is equivalent to 3.2×10^8 base pairs or about 4–5% of the haploid HeLa-cell genome.

By following the kinetics of reassociation of denatured HeLa-cell DNA segments in forming branches (Table 3) it is also possible to deduce the average number of repeated sequences in each of the individual sequence families. The kinetic complexity, N , of a given polynucleotide sequence in a diverse population is related to the time for half of that particular class of complementary sequences to re-associate:

$$N = A \times C_0 t_{\frac{1}{2}}$$

where C_0 is the DNA concentration, $t_{\frac{1}{2}}$ the time for half-renaturation and A is a constant (Britten & Kohne, 1968; Wetmur & Davidson, 1968; Laird, 1971). Fragments of *E. coli* DNA ($N = 4.5 \times 10^6$ base pairs), with an average single-chain length of 780 bases, renature under conditions similar to those presently reported with a $C_0 t_{\frac{1}{2}}$ value of $3.5 \text{ mol} \cdot \text{s} \cdot \text{l}^{-1}$ (N. Hardman & A. Smith, unpublished work). Hence, by comparison, $C_0 t_{\frac{1}{2}}$ values of 0.01 – $0.1 \text{ mol} \cdot \text{s} \cdot \text{l}^{-1}$ in the present study for the annealing reaction of denatured HeLa-cell DNA segments to form branches (Table 3) correspond to sequences with approximate kinetic complexities of 1.3×10^4 – 1.3×10^5 base pairs. Since there is a total of approx. 3.2×10^8 base pairs of such repeated sequences, the frequency of repetition for each sequence family is in the range 2400–24000. These values are possibly only correct within an order of magnitude for several important reasons. For example, precise measurements of $C_0 t_{\frac{1}{2}}$ for branches have not been carried out, and it is presently not known whether such a kinetic analysis can in fact be applied to an annealing reaction involving only partly complementary single chains.

It is noteworthy that, similarly to the present study, in DNA from *Drosophila* pupae Wu *et al.* (1972) have discovered a class of repeated sequences of approximate length 150 base pairs distributed intermittently along the chromatid. In a study of centric heterochromatin in *Drosophila*, highly reiterated sequences of length 2.5×10^3 – 10×10^3 base pairs were found to be spaced by more complex sequences 5×10^3 base pairs long (Kram *et al.*, 1972). It seems

that the density of highly reiterated sequences may be one feature that distinguishes heterochromatic from euchromatic portions of the eukaryotic chromatid. In *Drosophila* DNA, the centric heterochromatin is not endo-replicated at polytenization, and this probably accounts for the absence of a large fraction of highly repetitious base sequences from polytene DNA (Gall *et al.*, 1971). However, if polytene DNA contains 3–5% dispersed highly reiterated base sequences among more complex, possibly unique, sequences, such as appear to be present in HeLa-cell DNA, their presence might explain the ability of *Drosophila* polytene DNA to form rings and at the same time account for the observation that most of the sequences are single-copy by reassociation kinetics.

Mouse satellite DNA has many properties in common with the highly reiterated fraction of *Drosophila* DNA described above. Isolated mouse satellite DNA is known to contain base sequences which are highly reiterated, and these hybridize with mouse main-band DNA to a marked extent. The hybrid molecules formed a band in a CsCl density gradient at the position expected of main-band DNA (Flamm *et al.*, 1969). This demonstrates that some satellite sequences are present in DNA single chains of predominantly main-band composition, suggesting that they are at least partly dispersed within mouse chromosomal DNA. Human DNA is also known to contain highly reiterated satellite components (Saunders *et al.*, 1972). It is therefore a possibility that the interspersed highly reiterated base sequences presently reported for HeLa-cell DNA might correspond to dispersed satellite sequences. Consistent with this idea, certain satellite sequences have been found to be resistant to cleavage with restriction endonucleases (Southern & Roizes, 1973). For guinea-pig α -satellite, the DNA probably consists of a simple hexanucleotide sequence with successive copies arranged in tandem (Southern, 1970). The present experiments do not rule out the possibility that the intermittent repeated sequences in HeLa-cell DNA might correspond to dispersed satellites, in which case it is conceivable that they contain internal tandem repeats of simple sequences. For example, average total repeat lengths of 180 base pairs could accommodate about 30 copies of a hexanucleotide sequence in tandem.

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