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5-Bromodeoxyuridine Labeling of Monomeric and Catenated Circular Mitochondrial DNA in HeLa Cells

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Bromouracil labeling of the mitochondrial DNA in exponentially growing HeLa cells produces two hybrid mitochondrial DNA species, with density shifts of 41·9 and 54·0 mg/ml relative to unlabeled mitochondrial DNA, as well as heavy mitochondrial DNA, with a shift of 95·3 mg/ml. The two hybrid species result from the difference in thymine composition of the complementary strands of mitochondrial DNA. In addition, mitochondrial DNA with a density intermediate between the hybrid and unlabeled species was found. This *quarter heavy* mitochondrial DNA represents 25% (w/w) of the total DNA after eight hours of labeling, and forms two peaks with shifts of 20·6 and 27·0 mg/ml relative to unlabeled mitochondrial DNA. 70% (w/w) of the quarter heavy mitochondrial DNA is in catenated forms, while 30% (w/w) is monomeric. Degradation of the catenanes by shearing of purified quarter heavy mitochondrial DNA results in the appearance of hybrid and unlabeled mitochondrial DNA bands, demonstrating that the quarter heavy catenanes contain both hybrid and unlabeled submolecules. The implications of the structure of the quarter heavy catenanes on the mechanism of formation of catenanes are discussed.

1. Introduction

Density labeling has been used to observe the replication of mitochondrial DNA in Neurospora (Reich & Luck, 1966), using ^{15}N as the density label, and in rat liver (Gross & Rabinowitz, 1969), with bromodeoxyuridine. In both cases, the results are consistent with a semiconservative mode of replication, but the buoyant density shifts are too small (15 mg/ml or less) to permit the separation of the different labeled species. The purposes of this study initially was to examine the buoyant densities of duplexes and single strands of mitDNA† from HeLa cells grown in the presence of BrdUrd. One heavy and two hybrid duplex species were formed as expected for the semiconservative replication of a duplex DNA having strands of different thymine composition. In addition, an unexpected species with a buoyant density between hybrid and light mitDNA was formed. These *quarter heavy* molecules were found to be predominantly catenanes, and were shown to contain both hybrid and light monomer submolecules topologically bonded to each other. The implications of this new structure on the possible modes of formation of catenanes are discussed.

A study of the mitDNA in mouse L cells (Kasamatsu *et al.*, 1971) has shown that intermediates containing a replicating displacement loop (D-loop) occur at a high

† Abbreviations used: mitDNA, mitochondrial DNA; EthBr, ethidium bromide; nDNA, nuclear DNA.

frequency. Similar forms are present in HeLa cells. The buoyant densities of light molecules containing a small D-loop would not be significantly affected (approximately 2 mg/ml) if the short 7 S displacing strand were density labeled. The other replicating intermediates are present at too low a frequency to interfere with the examination of the mature density labeled molecules.

2. Materials and Methods

(a) *Isolation and purification of total mitochondrial DNA*

Suspension cultures of HeLa cells were grown in the dark in Eagle's phosphate medium (Grand Island Biological Co., Berkeley, Calif.) containing 5% calf serum, 20 μg (BrdUrd/ml (7×10^{-5} M) and (in initial experiments) 0·1 to 0·01 μg FUrd/ml (0·03 to $0{\cdot}003\times10^{-5}$ M). The cells were harvested by centrifugation at 1000 *g* for 3 min and kept at 0 to 4°C thereafter. After two washes with 0·14 M-NaCl, 0·005 M-KCl, 0·0007 M-Na_2HPO_4, 0·025 M-Tris, pH 7·4 (TD buffer), the cells were suspended in a 10-fold vol. of 0·01 M-NaCl, 0·0015 M-$MgCl_2$, 0·01 M-Tris, pH 7·4 (RSB buffer), and allowed to swell for 10 min before disrupting them with 2 to 4 strokes in a Dounce homogenizer. Sucrose was immediately added to a concentration of 0·25 M and nuclei were removed by pelleting at 1000 *g* for 3 to 5 min. The crude mitochondria were pelleted at 27,000 *g* for 10 to 15 min, taken up in MS buffer (0·21 M-mannitol, 0·07 M-sucrose, 0·001 M-EDTA, 0·01 M-Tris, pH 7·4), and sedimented through 1·0 M-sucrose to an interface with 1·5 M-sucrose in a Spinco SW27 rotor at 27,000 revs/min at 4°C for 45 min. After a wash with MS buffer, the mitochondria were taken up in 0·25 M-sucrose, 0·025 M-KCl, 0·0025 M-$MgCl_2$, 0·05 M-Tris (pH 6·7), and treated with 50 to 100 μg DNase I/ml and 100 μg RNaseA/ml for 30 min at 37°C. Digestion was stopped by chilling and adding EDTA to 0·04 M. The mitochondria were washed three times with MS buffer and lysed by adding sodium dodecyl sulfate to 1% for 5 to 10 min at 25°C. Sodium dodecyl sulfate was precipitated by adding CsCl to 1 M and chilling; the precipitate was removed by centrifugation at 27,000 *g* for 5 to 10 min. Ethidium bromide was added to 300 μg/ml, the density was adjusted to 1·65 g/ml with CsCl, and the solution was centrifuged at 35,000 to 40,000 revs/min for 24 h in an SW50.1 rotor. The entire DNA-containing region of each tube was recovered by drop-collection in ultraviolet light. EthBr was removed with a Dowex-50 column (0·5 cm × 2 to 5 cm); CsCl was removed by dialysis against 0·01 M-Tris (pH 7·5), 0·001 M-EDTA. In later experiments, small nuclear DNA fragments remaining after the DNase digestion were removed by velocity sedimentation. Samples of 0·3 to 0·6 ml were layered onto 4·0 ml of CsCl (density 1·40 g/ml), 100 μg EthBr/ml, 0·01 M-Tris (pH 7·5), 0·001 M-EDTA, and centrifuged in an SW50.1 rotor for 3 h at 38,000 revs/min at 20°C. The tubes were fractionated by drop-collection; the lower part of the gradient was consolidated and treated as described above to remove the EthBr and CsCl.

(b) *Preparation of nuclear DNA*

A small amount of the nuclei pelleted after the homogenization step above was lysed by adding sodium dodecyl sulfate to 1%. After 5 to 10 min at 25°C, sodium dodecyl sulfate was removed by chilling, adding CsCl to 1 M, and centrifuging at 27,000 *g* for 5 to 10 min. EthBr was added to 300 μg/ml, the density of the solution was adjusted to 1·65 g/ml with CsCl, and the solution was centrifuged as above. The DNA region was collected, EthBr was removed, and the sample was dialyzed as above. DNA obtained by phenol extraction gave the same buoyant density patterns.

(c) *Analytical centrifugation*

Buoyant density experiments were performed at 25°C for 24 h at either 44,000 or 44,770 revs/min in a Beckman model E ultracentrifuge equipped with a photoelectric scanner. Alkaline buoyant densities were measured in 0·05 M-K_3PO_4, 0·05 M-KOH (pH 12·5). The buoyant density gradient (Vinograd & Hearst, 1962) was used in the calculation of the results in both the neutral and alkaline experiments. The relative amounts of

DNA in different peaks were determined by ordinate summation, either enlarging the trace from the Offner recorder in the scanner or measuring directly from a scan recorded on a Moseley 7001 AM recorder. Correction was made for the sector shape of the centrifuge cell.

(d) *Analysis of DNA in CsCl gradients by fluorescence*

Density-labeled mitDNA samples were centrifuged in CsCl with a density of 1·68 g/ml at 35,000 revs/min for 48 h in an SW50.1 rotor. The gradients were collected into tubes containing 0·5 ml of 10 μg EthBr/ml, 0·1 M-NaCl, 0·01 M-Tris (pH 7·5), 0·001 M-EDTA. The fluorescence of each fraction was measured in a Hitachi Perkin–Elmer model MPF2A fluorescence spectrophotometer using a microcell. The excitation and emission wavelengths were 380 and 586 nm, respectively.

(e) *Hydrodynamic shearing of DNA*

Shearing of mitDNA was performed in a capillary shear apparatus (Yew & Davidson, 1968). The DNA samples were at a concentration of 0·2 to 0·8 μg/ml in 2 μg EthBr/ml, 0·01 M-Tris (pH 7·5), 0·001 M-EDTA. Each sample passed through the capillary 400 to 500 times under 150 lb/in^2 of nitrogen at 25°C.

(f) *Electron microscopy and fluorescence photography*

DNA was mounted for electron microscopy by a modified aqueous or formamide Kleinschmidt procedure followed by rotary shadowing with platinum/palladium. Grids were scored by the procedure described by Clayton *et al.* (1968), using a Philips EM300 microscope. Length distributions of the sheared samples were obtained by photography, enlarging and tracing the molecules with a Nikon 6F projection microcomparator, and measuring the contour length with a map measurer.

The camera system described by Watson *et al.* (1971) was used to photograph EthBr/CsCl gradients.

(g) *Reagents, enzymes and substrate DNA*

5-Bromodeoxyuridine was obtained from Schwarz/Mann, Inc., Orangeburg, N. Y. Fluorouridine was a gift from Hoffman-La Roche, Inc., Nutley, N. J.; ethidium bromide was a gift from Boots Pure Drug Co. Ltd, Nottingham, England. Optical grade cesium chloride was obtained from the Harshaw Chemical Company, Solon, Ohio. DNase I and RNase A were obtained from Sigma Chemical Co., St. Louis, Mo. DNase II was supplied by Worthington Biochemical Corp., Freehold, N. J. Closed PM2 viral DNA was a gift from Drs B. M. J. Révet and M. Schmir in our laboratory. All other chemicals were reagent grade.

3. Results

(a) *Assay for nuclear DNA contamination of total mitochondrial DNA preparations*

In the absence of density label, closed mitDNA is readily freed of nDNA as a lower band in an EthBr/CsCl gradient. This procedure is not applicable to BrUra-labeled mitDNA preparations because the density shift due to BrUra substitution is approximately the same as the EthBr-induced shift in closed, unlabeled mitDNA (Plate I(b) and (c)). We therefore adopted a procedure in which contaminating nDNA was digested to small fragments by DNase treatment of the mitochondria and removed in a later velocity sedimentation (Plate II) of the mitDNA sample. The amount of contaminating nDNA remaining after this treatment was assayed by examining the buoyant profiles of the total mitDNA from unlabeled cells (Plate III(a) and (b)). A small amount of high molecular weight nDNA appears at neutral pH as a slight skewing of the peak to the light side. The nDNA contamination estimated by this criterion is less than 5%(w/w). The nDNA bands between the two mitDNA

strands in alkaline CsCl and again accounts for 5% of the optical density. The flat baseline in both cases indicates that the sample is free of small nDNA fragments. When the sedimentation velocity step is omitted, fragments of nDNA give rise to broad, curved baselines (Plate III(c)).

(b) *Neutral buoyant densities of bromodeoxyuridine-labeled DNA species*

Buoyant density scans of BrUra-labeled mitDNA samples are presented in Plate IV. In each case, the band at the left is unlabeled mitDNA, which was used as an internal marker. We checked its buoyant density by adding *Micrococcus lysodeikticus* DNA to several preparations and obtained the value 1·6998±0·0005 g/ml (nine determinations), in good agreement with the value of 1·6996 g/ml found for human leukocyte mitDNA (Clayton *et al.*, 1970). The buoyant density of *M. lysodeikticus* DNA was taken to be 1·7257 g/ml (Bauer *et al.*, 1971). Similarly, unlabeled nDNA was used as an internal marker for density-labeled nDNA. Its buoyant density, also referred to *M. lysodeikticus* DNA present as a marker, is 1·6912±0·0009 g/ml (four determinations), slightly higher than the value of 1·6896 g/ml reported for human leukocyte nDNA (Clayton *et al.*, 1970). When the amount of unlabeled nDNA was insufficient (Plate V), hybrid nDNA was used as the reference.

Three major and two minor BrUra-labeled mitDNA species appear to the right of the unlabeled mitDNA in Plate IV. The band furthest to the right, with a separation of 95·3 mg/ml (Table 1), contains the heavy mitDNA and consists of molecules with both strands density labeled. A semiconservative mechanism for replication predicts two hybrid species for HeLa mitDNA because of the difference in thymine content of the two strands. The BrUra content and, therefore, the density of the hybrid molecule will depend on which strand has been labeled. The two major bands in the center have an average separation of 48·0±2·2 mg/ml, which is 0·50±0·02 of the heavy mitDNA separation. These bands, therefore, contain the two hybrid species.

The two minor species between the hybrid and unlabeled bands are called quarter heavy because their average separation, 23·8±1·3 mg/ml (Table 1), is 0·25±0·01 of the heavy mitDNA separation. They are also half way between hybrid and unlabeled

TABLE 1

Buoyant separations of bromouracil-labeled mitochondrial DNA species in neutral cesium chloride

Band	mitDNA (mg/ml)	mitDNA (FUrd)† (mg/ml)	Nuclear DNA (mg/ml)
Quarter heavy	20·6±0·8 (15)	not determined	not applicable
	27·0±1·0 (16)	not determined	not applicable
Hybrid light	41·9±1·5 (18)	44·2±1·8 (6)	52·7±1·2 (5)
Hybrid heavy	54·0±1·7 (18)	57·1±2·2 (6)	
Heavy	95·3±1·8 (8)	100·8±3·0 (5)	104·8±1·9 (3)

Separations for mitDNA are given relative to unlabeled mitDNA at 1·6998±0·0005 g/ml (nine determinations). Those for nuclear DNA are relative to unlabeled nuclear DNA at 1·6912±0·0009 g/ml (4 determinations). The numbers in parentheses after each value are the number of determinations which were averaged together.

† From cells labeled with BrdUrd in the presence of 0·01 μg of FUrd/ml.

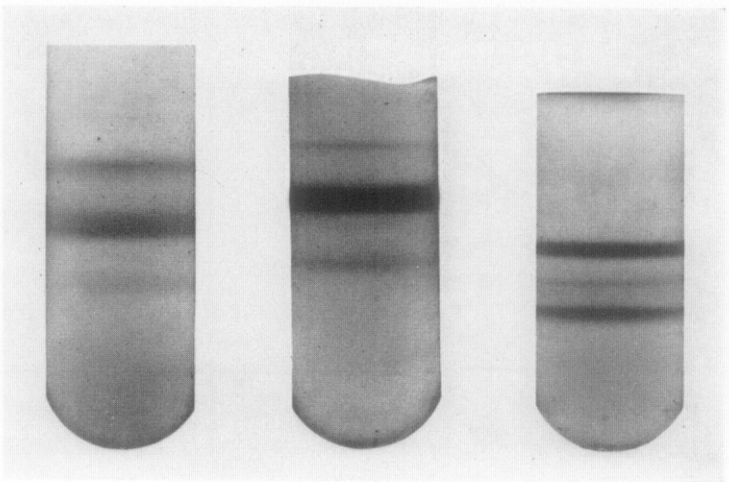

PLATE I. Fluorescent bands formed in EthBr/CsCl buoyant density gradients. (a) mitDNA from cells labeled with BrdUrd for 44 h; (b) nuclear DNA from cells labeled for 44 h; (c) mitDNA from untreated cells. Centrifugation conditions are described in Materials and Methods, section (a). The tubes were photographed in ultraviolet light as described in Watson *et al.* (1971).

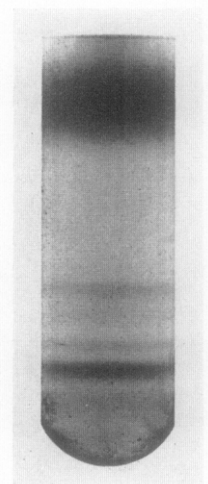

PLATE II. The purification of bromouracil-labeled mitDNA (lower bands) by velocity sedimentation to remove nuclear DNA fragments. The diffuse band of nuclear DNA near the meniscus at the top of the photograph was discarded. Centrifugation conditions are described in Materials and Methods, section (a). The tube was photographed as in Plate I.

[*facing p. 84*

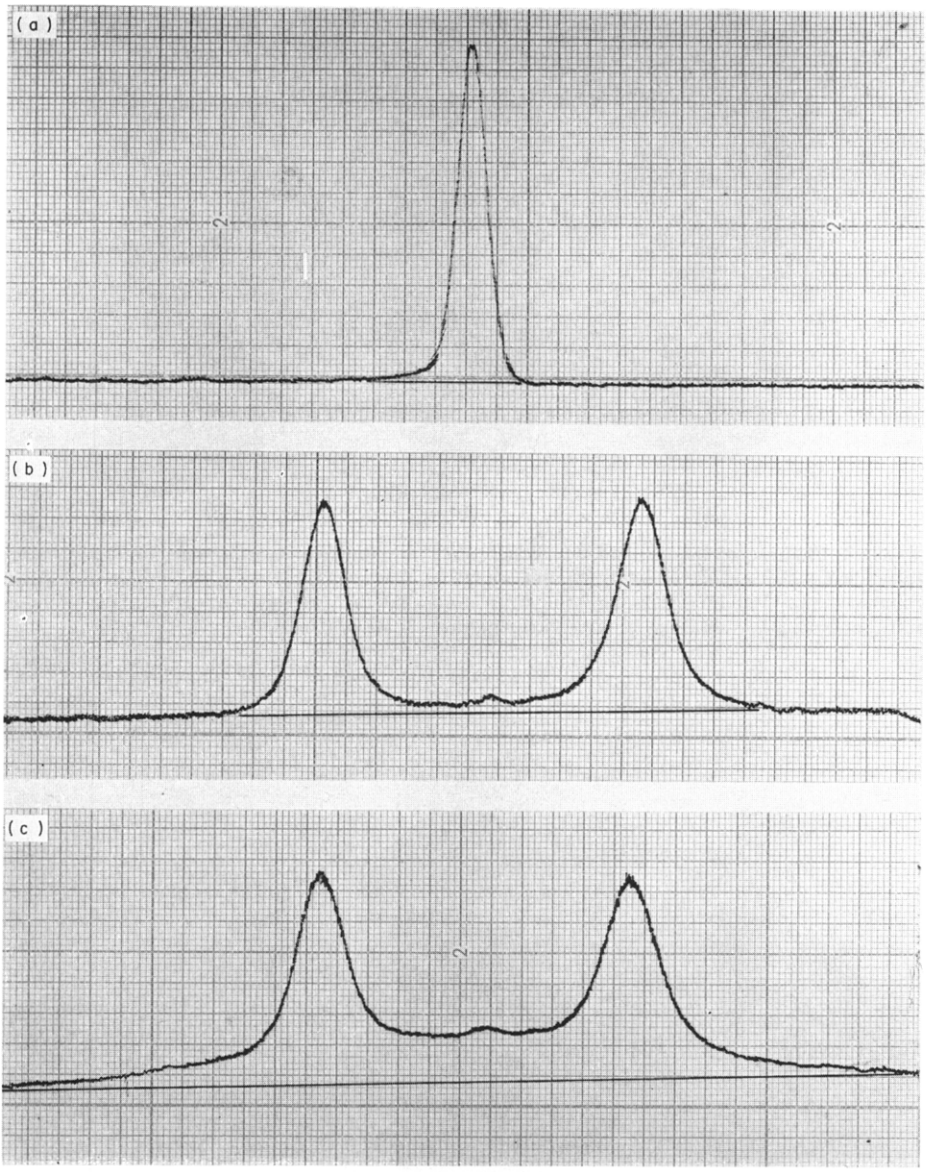

PLATE III. Photoelectric scans of (a) unlabeled mitDNA in buoyant neutral CsCl after velocity sedimentation to remove small nuclear DNA fragments; (b) the same sample; (a), in buoyant CsCl (pH 12·5), 0·05 M-K_3PO_4; (c) unlabeled mitDNA which had not been purified by velocity sedimentation, in alkaline buoyant CsCl, at 44,000 revs/min, 25°C. The field is directed to the right. The two bands in (b) and (c) contain the complementary strands of mitDNA. The entire scan was used in each case for drawing in the baseline.

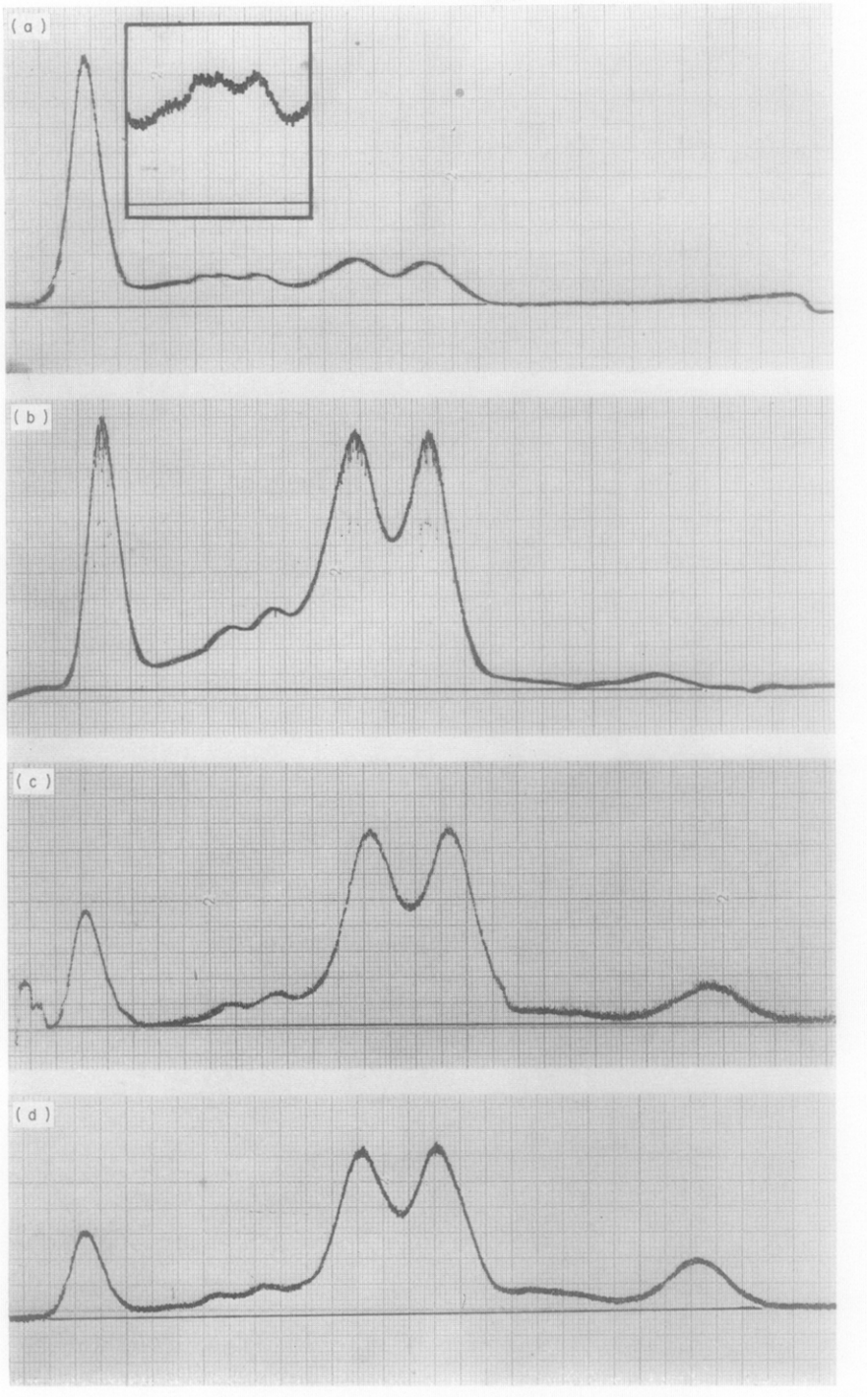

PLATE IV. Photoelectric scans of mitDNA from cells labeled with BrdUrd for (a) 8 h; (b) 16 h; (c) 24 h; (d) 44 h. The samples are in neutral buoyant CsCl at 25°C and 44,000 revs/min, except (b), at 44,770 revs/min. The field is directed to the right. In each case, the band at the left is the unlabeled mitDNA, the two peaks in the middle are the hybrid mitDNA and the band at the right (when present) is the heavy mitDNA. The quarter heavy bands are between the hybrid and the unlabeled mitDNA bands. The inset in (a) corresponds to the region immediately below it, scanned at a higher sensitivity.

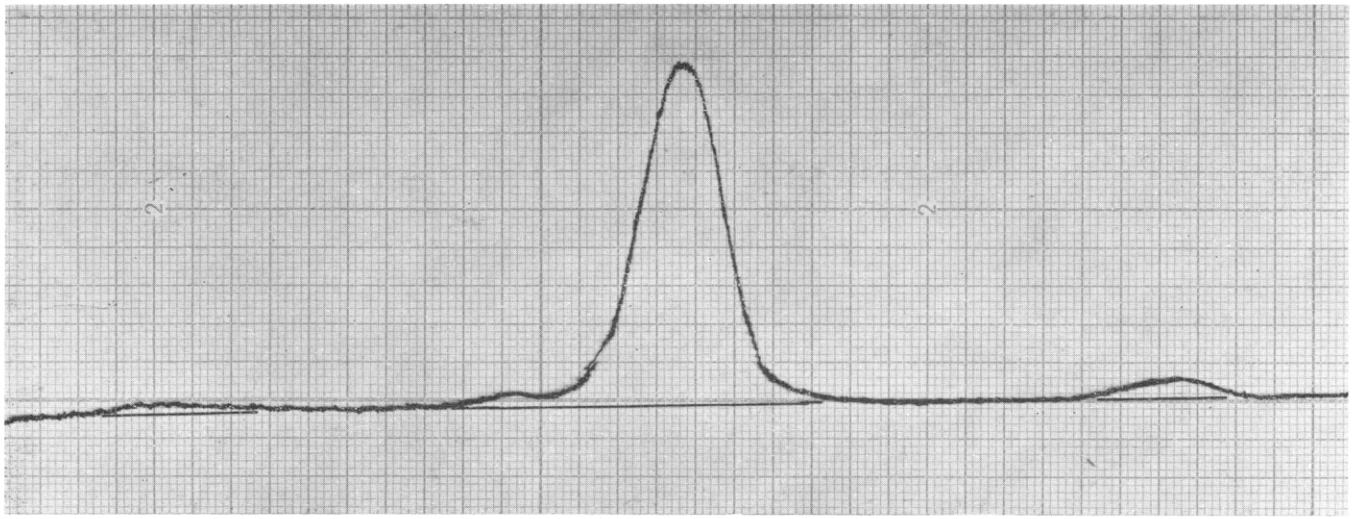

PLATE V. Photoelectric scan of nuclear DNA from cells labeled for 44 h with BrdUrd. The sample is in buoyant neutral CsCl at 44,000 revs/min, 25°C. The field is directed to the right. The large band at the center is hybrid nuclear DNA. A very small amount of unlabeled nuclear DNA appears at the left.

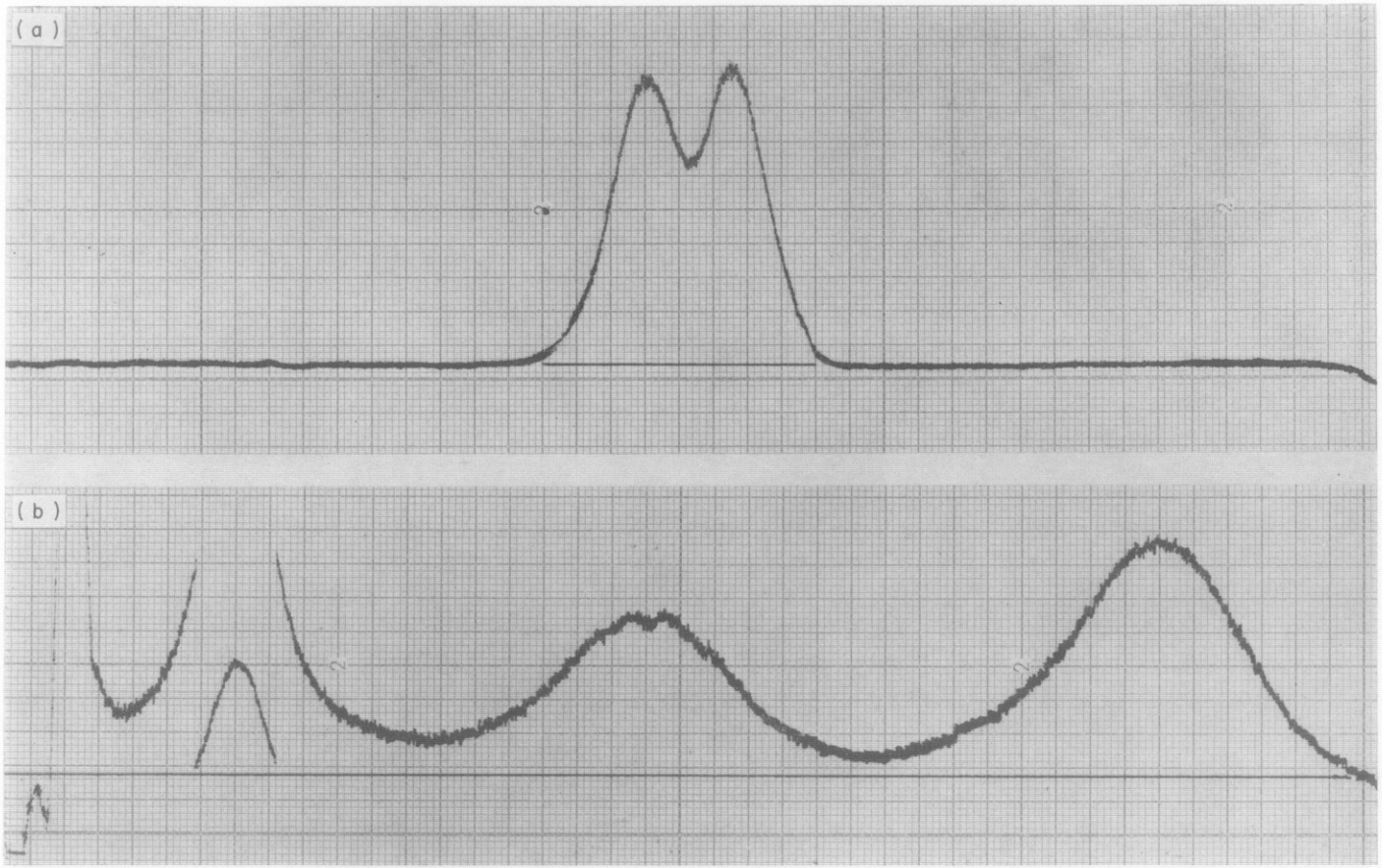

PLATE VI. Photoelectric scans of purified hybrid mitDNA in (a) buoyant neutral CsCl and (b) buoyant alkaline CsCl (pH 12·5), 0·05 M-K_3PO_4, at 44,000 revs/*min*, 25°C. The field is directed to the right. The two bands at the right in (b) are the BrUra-labeled strands. The heavy unlabeled strand is at the far left, while the light unlabeled strand is at the meniscus. The scale was changed during the scan so as to include the maximum of the band at the left.

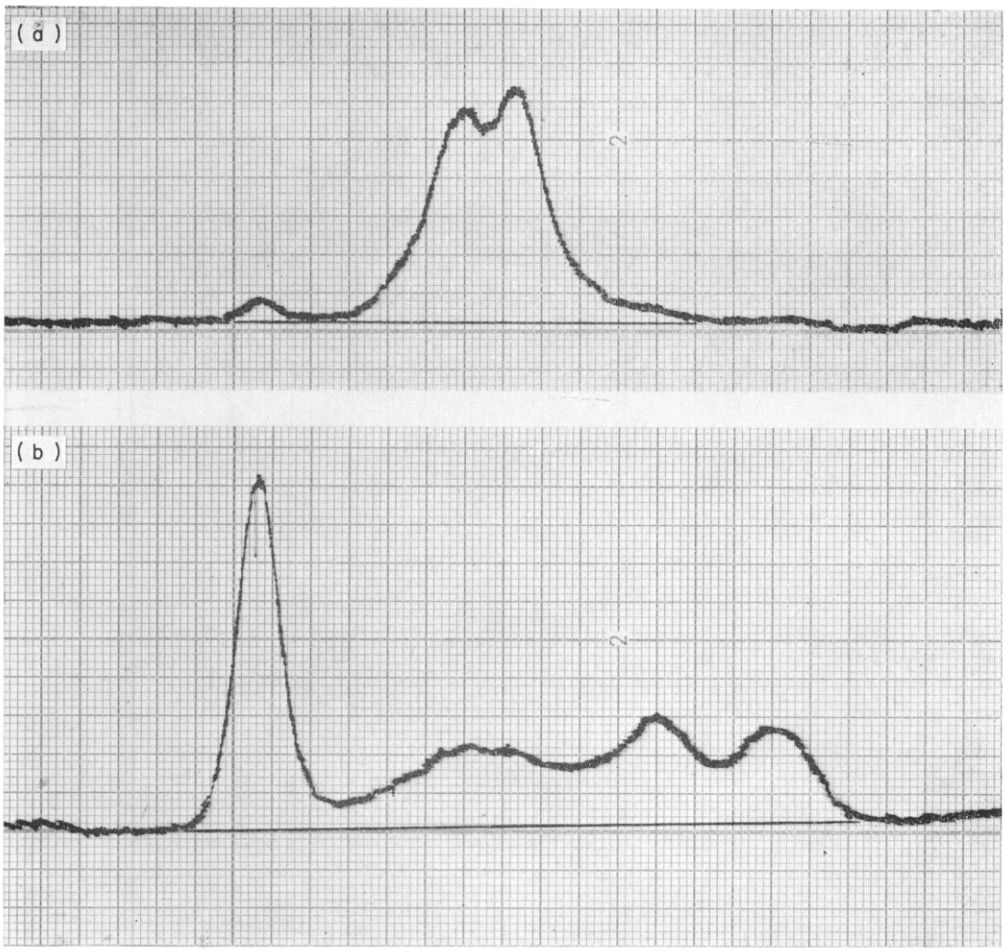

PLATE VII. Photoelectric scans of (a) the quarter heavy fraction isolated by combining the fractions shown in Fig. 1, and (b) the same fraction after shearing in buoyant neutral CsCl at 44,000 revs/min, 25°C. The field is directed to the right.

mitDNA. The separation between the two quarter heavy bands, 6·4 mg/ml, is approximately half the separation between the two hybrid bands, 12·1 mg/ml. The structure of these species will be considered in later sections.

The magnitudes of the buoyant separations can be used to estimate the extent of substitution of BrUra for thymine. The buoyant separation between poly[d(A-T)] and poly[d(A-BrUra)] is 200 mg/ml (Wake & Baldwin, 1962). Since the mitDNA contains 28% thymine (Wesley M. Brown, personal communication) and poly[d(A-T)] contains 50% thymine, the separation for heavy mitDNA would be 112 mg/ml if the substitution were complete. The observed separation of 95·3 mg/ml indicates that 85% of the thymine residues were replaced by BrUra. A similar calculation for the nDNA, with 30% thymine (Kin *et al.*, 1966) gives a value of 87% substitution, based on an observed separation of 104·8 mg/ml. Preparations of mitDNA from cells treated with FUrd gave a separation of 100·8 mg/ml indicating 90% substitution. The small difference observed on addition of FUrd led us to abandon its use.

The separations of the two hybrids from unlabeled mitDNA can be used to estimate the thymine bias between the complementary strands. Since the average of the separations between the hybrids and the unlabeled mitDNA is exactly half the separation between heavy and unlabeled mitDNA, the buoyant shift is proportional to BrUra content:

$$\Delta\theta = kB, \tag{1}$$

where $\Delta\theta$ is the buoyant density shift on BrUra incorporation, k is a constant, and B is the mole fraction of bases which are BrUra. The density shift for a hybrid in which the fraction of thymine residues on the labeled strand replaced by BrUra is a is:

$$\Delta\theta = akT, \tag{2}$$

where T is the thymine content of the strand before labeling. If we assume that the fractional extent of BrUra labeling, a, is the same for the labeled strands of the two hybrids, the ratio of the buoyant separations for the two hybrid species becomes

$$\Delta\theta_2/\Delta\theta_1 = T_2/T_1.$$

The thymine bias, T_2/T_1, obtained in this way is independent of the extent of labeling, a. The value of $1{\cdot}29 \pm 0{\cdot}05$ is in agreement with the value of 1·26 determined by base analysis (31·2 and 24·9% thymine in heavy and light strands (Wesley M. Brown, personal communication)). The agreement between these numbers indicates that the hybrids contain one labeled and one unlabeled strand and are, therefore, products of semiconservative replication.

(c) *Alkaline buoyant densities of density-labeled mitochondrial DNA*

Alkaline buoyant separations were calculated with the value of $1/\beta_b$ used for the calculations at neutral pH. Recent work by Schmid & Hearst (1971) indicates that this assumption is valid with a maximum error of 5%. The value for the separation between the two unlabeled strands is $42{\cdot}3 \pm 0{\cdot}6$ g/ml (Table 2; Plate III) which agrees fairly well with the value 41·0 mg/ml reported by Clayton *et al.* (1970) for human leukocyte mitDNA. When hybrid mitDNA was fractionated from a preparative CsCl gradient and banded in alkaline CsCl, four bands were formed. The separation between the lightest and heaviest bands was so large (0·149 mg/ml) that they could not all be banded in one analytical centrifuge cell at 44,000 revs/min. The three most

TABLE 2

Buoyant densities and separations between unlabeled and bromouracil-labeled mitochondrial DNA strands in alkaline cesium chloride

Strand	θ (g/ml)	Separation (mg/ml)
Light, unlabeled	1·737	
Heavy, unlabeled	1·779†	42·3±0·6 (4)
Light, BrUra-labeled	1·825	45·8±0·4 (3)
Heavy, BrUra-labeled	1·886	60·8±1·0 (3)

† Marker value from Clayton *et al.* (1970).

dense species are shown in Plate VI(b): from left to right, they are the unlabeled heavy strand, the BrUra light strand, and the BrUra heavy strand. The densities of the alkaline BrUra-labeled strands are 1·825 and 1·886 g/ml, referred to the unlabeled heavy strand at 1·779 g/ml (Clayton *et al.*, 1970). The ratio of the shift of the light strand on BrUra labeling to that of the heavy strand on BrUra labeling provides an estimate of the thymine bias of 1·20±0·02. This value agrees fairly well with the value of 1·26 determined from base composition analysis.

(d) *Kinetics of labeling of mitochondrial and nuclear DNA*

HeLa cells were labeled for increasing lengths of time with BrdUrd, 20 μg/ml. The mitDNA was extracted and examined in neutral buoyant CsCl (Plate IV, Table 3). In this concentration of BrdUrd, the cells undergo only 1 to 1·5 doublings before growth ceases due to toxic effects of the analogue (Simon, 1961). It is clear that the mitDNA is labeled rapidly for the first 24 hours (1·2 generations for normal cells) but that the incorporation is greatly reduced during the next 20 hours. Similar results were obtained with nDNA after 44 hours (Plate V). Almost all of the nDNA is at hybrid density, with only 5% at the heavy position and less than 2% still unlabeled. In contrast, 10% of the mitDNA is still unlabeled after 44 hours, and another 10% is heavy. The unlabeled mitDNA could not have come from cells which did not grow in BrdUrd since such cells would have contained unlabeled nDNA. A mitDNA

TABLE 3

Buoyant density distributions of HeLa mitochondrial DNA as a function of labeling time in the presence of 5-bromodeoxyuridine

Time (h)	% of total mitDNA (w/w)				
	Unlabeled	Quarter heavy†	Hybrid	Hybrid-heavy†	Heavy
8	45	25	30	—	—
16	19	19	57	2	2
24	11	10	68	4	7
44	9	8	66	6	11

These density distributions were determined by summation of ordinates on enlargements of the photoelectric scans, as described in Materials and Methods, section (c).

† These categories include all material banding between the major species.

molecule therefore does not necessarily replicate once during each cell cycle; some molecules replicate twice and others not at all. Similar results have been found by Rownd (1969) for the replication of the R-factor in *Proteus mirabilis*.

The scans in Plate IV also show that the quarter heavy species appear as a major fraction of the mitDNA after eight hours (0·4 generation in normal cells) and then decrease in amount at later times. The kinetic behavior of these species corresponds to that of an intermediate in the labeling process. After 16 hours, a possibly equivalent material between the hybrid and heavy positions appears in small amounts.

(e) *Purification and properties of the quarter heavy mitochondrial DNA*

In view of the unusual buoyant density and labeling properties of the quarter heavy material, we decided to purify it and examine its properties in greater detail. Mitochondrial DNA from cells labeled for eight hours with BrdUrd was banded in a preparative CsCl gradient, and the fractions analyzed fluorometrically (Fig. 1). The fractions containing quarter heavy DNA were pooled as shown and banded in neutral buoyant CsCl in the analytical ultracentrifuge to assay for the presence of hybrid and unlabeled mitDNA (Plate VII(a)). In general, the amount of this contamination was 10% or less; if more, the amount was determined and taken into account in the evaluation of later results.

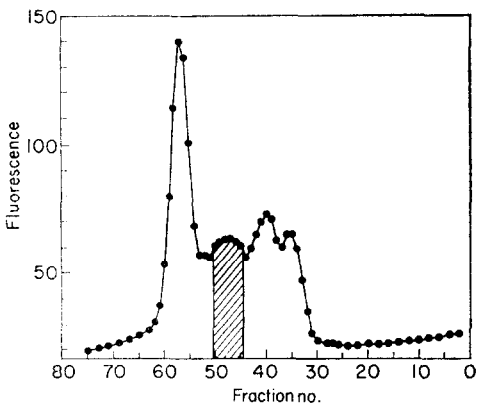

FIG. 1. Isolation of the quarter heavy mitDNA fraction.

Total mitDNA from cells labeled for 8 h with BrdUrd was banded in neutral CsCl for 48 h at 35,000 revs/min, 25°C, in an SW50.1 rotor. The tube was fractionated in 3-drop fractions into 0·5 ml of 10 μg EthBr/ml, 0·1 M-NaCl, 0·01 M-Tris (pH 7·5), 0·001 M-EDTA. The fluorescence of each fraction was determined using excitation at 380 nm and emission at 586 nm in a Hitachi Perkin–Elmer MPF2A fluorescence spectrophotometer. Fluorescence (arbitrary units) is plotted *versus* fraction number, with the field directed to the right. A baseline has been included for reference. The shaded area represents the fractions which were pooled to make the quarter heavy fraction. An analysis of this material in neutral CsCl is presented in Plate VII(a).

Electron microscopy showed that an unusually large portion of the DNA in this fraction is in the form of catenated molecules (Table 4). The several preparations of quarter heavy mitDNA contained about 70% (w/w) catenanes of all types, as compared with 37% (w/w) in the total mitDNA from cells labeled for eight hours and with 38% (w/w) in an unlabeled mitDNA preparation. Enrichment of catenanes in

TABLE 4

Electron microscope analysis of mitochondrial DNA from HeLa cells

Sample	Linear†	% of mitDNA in the sample (w/w) Monomer	Catenated dimer	Catenated trimer and larger forms	Molecules scored
Total mitDNA, unlabeled‡	n. s.§	62	22	16	1200
Total mitDNA,					
BrdUrd-labeled (8 h)	n. s.	63	22	15	1200
(a) Hybrid (8 h)	n. s.	78	15	7	2710
(b) Unlabeled (8 h)	n. s.	80	15	6	1320
(c) Quarter heavy, (8 h), purity‖					
90%	n. s.	35	35	31	1350
90%	n. s.	31	40	29	590
95% (Expt 1)¶	n. s.	27	46	27	1320
91% (Expt 2)	n. s.	26	45	26	760
80% (Expt 3)	n. s.	50	31	13	920
(d) Quarter heavy, sheared	54	44	2·1	—	450
(Expt 2)¶	50	47	2·1	0·5	850
(Expt 3)	38	60	2·4	0·3	1620

† All the linear molecules were assumed to be half the monomer length in determining the % (w/w). The value for experiment 3 was corrected for the presence of small linear fragments.

‡ These data were obtained from C. J. B. Tibbetts, this laboratory.

§ n. s., Not scored. Linear molecules of up to monomer length were seen, but did not constitute more than 10% (w/w) of the sample.

‖ Purity was determined by measuring the fraction of the quarter heavy mitDNA in buoyant CsCl in the analytical ultracentrifuge. Hybrid and unlabeled mitDNA were present as impurities.

¶ The experiment numbers correspond to the experiments listed in Table 5.

the quarter heavy region is at the expense of catenanes in the hybrid and unlabeled regions. The isolated hybrid and unlabeled peaks contained about 20% (w/w) catenanes (Table 4). A rough estimate of the fraction of the catenanes in the quarter heavy region can be obtained by weighting the catenane level in each band by the fraction of the total mitDNA in that band (Tables 3 and 4). About 50% (w/w) of the catenanes in the total sample are in the quarter heavy fraction; also, about 80% (w/w) of the density-labeled catenanes are in this region.

Analytical band sedimentation experiments were performed on the quarter heavy fraction in an attempt to confirm the electron microscope results. The measured sedimentation velocities agree well enough with the published values (Brown & Vinograd, 1971) to allow identification of the peaks. An accurate determination of the amount of catenanes in the sample was not possible because of the poor resolution between the doubly nicked catenated dimer and the closed monomer. The results indicated that between 50 and 80% (w/w) of the mitDNA in the quarter heavy fraction was in the form of catenated molecules.

(f) *Structure of the quarter heavy mitochondrial DNA*

In view of both the buoyant properties and the high catenane content of the quarter heavy fraction, it appeared likely that this fraction contained catenanes composed of hybrid and unlabeled submolecules. The dimers of this type would be

expected to form two bands midway between the hybrid and unlabeled positions, since the hybrid submolecule could have either of the two densities. Catenated trimers and higher forms containing both hybrid and unlabeled forms would also band in this region.

The most direct way to test the proposed structure for the quarter heavy mitDNA is to degrade the catenanes with double-strand scissions so that the submolecules can separate. Banding in neutral buoyant CsCl would then show peaks at the hybrid and unlabeled positions. We first tried enzymic degradation using the cleaving nuclease, DNase II. Examination of the digestion products of closed PM2 DNA by sedimentation velocity showed that the enzyme introduces many single-stranded nicks for each cleavage. Careful calibration of the enzyme was necessary to minimize fragmentation of the DNA due to this nicking. Despite these precautions, a sedimentation analysis showed that the quarter heavy mitDNA was fragmented to small linear molecules. In neutral buoyant CsCl the bands were broader than usual; nevertheless, it was clear that a conversion of quarter heavy material to hybrid and light DNA had occurred (Table 5, experiment 1).

TABLE 5

Density distributions of degraded quarter heavy mitochondrial DNA fractions

Sample	% of the mitDNA fraction (w/w)			
	Unlabeled	Quarter heavy	Hybrid 1	Hybrid 2
Expt 1 (DNase II)	31	38	17	14
Expt 2 (shearing)	33	32	17	19
Expt 3 (shearing)	29	38	16	18
Average	31	36	17†	17‡

Distributions were determined by ordinate summation as in Table 3, and were corrected where necessary for the presence of contamination in unlabeled and hybrid mitDNA.

† Average separation from the unlabeled mitDNA peak was $40{\cdot}9 \pm 1{\cdot}5$ mg/ml (3 determinations).

‡ Average separation from the unlabeled mitDNA peak was $54{\cdot}1 \pm 0{\cdot}9$ mg/ml (3 determinations).

Mechanical shearing was then used to degrade the catenanes. The DNA solution was driven back and forth through a fine capillary by nitrogen gas at high pressure (Yew & Davidson, 1968). This method has several distinct advantages over enzymic digestion. It is simple and reproducible, since the shear breakage depends only on temperature, DNA concentration, number of passes through the capillary, and shear stress (proportional to nitrogen pressure). For a given shear stress, the size of the product approaches an asymptote as the number of passes increases. Product size can, therefore, be controlled by using a constant (large) number of passes and varying the shear stress. The shear stress necessary to break a catenated dimer should be roughly the same as that required to break a 10×10^6 dalton linear DNA in half, since the effective lengths in the shear gradient are probably similar. The linear mitDNA molecules resulting from the first rupture of the catenanes in the shear gradient should also be broken only once, to 5×10^6 daltons. Monomer mitDNA circles should not be broken under these conditions.

Three preparations of quarter heavy mitDNA were sheared under conditions appropriate to shearing $\lambda b_2 b_5 c$ DNA (mol. wt, 25×10^6) into quarters. EthBr at a concentration of 2·0 μg/ml was added to relax the closed molecules. Electron microscope examination of the sheared DNA showed that only 2% of the molecules were still catenanes; most of the molecules were either monomer circles or linear (Table 4). A histogram of the lengths of the linear molecules was constructed using the monomer circles as an internal standard (Fig. 2). The peak in the distribution is at 0·5 monomer length, as expected. The peak at 0·1 length (0·5 μm) is variable in amount in different preparations. These smaller linear fragments accounted for less than 2% (w/w) of the sheared quarter heavy mitDNA preparation. In all other preparations the amount was still less. The small fragments probably represent residual nDNA. The electron microscope analysis, therefore, demonstrates that the shearing process degraded the catenanes without extensive fragmentation of the mitDNA.

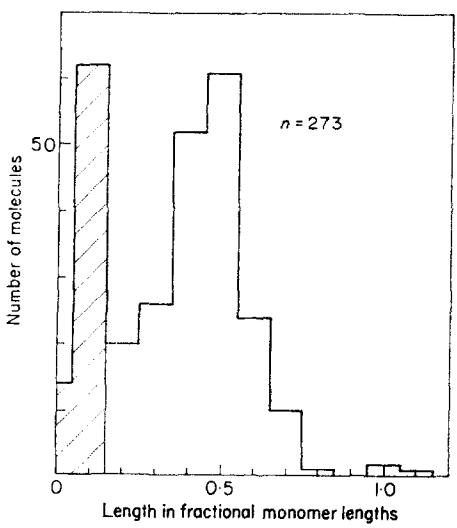

FIG. 2. Length distribution of the linear molecules in the quarter heavy fraction after shearing. The sheared quarter heavy fraction was mounted for electron microscopy by a modified Klenschmidt procedure. Linear molecules were photographed randomly, traced and measured. Monomer circles were also measured as an internal standard. The number of molecules seen is plotted against their length in fractions of the monomer circle length. The shaded area represents small fragments which account for less than 2% of the mass of DNA in the fraction.

The sheared samples, when banded in neutral buoyant CsCl, gave profiles like that in Plate VII(b). Clearly, shearing released hybrid and unlabeled DNA from the quarter heavy region. There was still some DNA at the quarter heavy position, despite the fact that there were no catenanes left. Measurements of the areas (Table 5) show that about 35% of the DNA was not shifted by shearing. This number is quite close to the percentage of monomers in the unsheared material, suggesting that part of the quarter heavy peak consists of monomers with one strand partly BrdUrd-labeled. The quarter heavy mitDNA, therefore, consists mainly of catenanes having hybrid and unlabeled submolecules, but contains incompletely labeled hybrid monomers as well.

4. Discussion

(a) *Extent of substitution*

Several factors indicate that the substitution of BrUra for thymine is almost complete under our conditions. The density shifts for both mitDNA and nDNA are very large. The mitDNA shifts are five times those previously seen in BrUra-labeled animal cell mitDNA (Karol & Simpson, 1968; Gross & Rabinowitz, 1969). The extent of substitution was estimated using the base compositions and the density shift of BrUra substitution in poly[d(A-T)] (Wake & Baldwin, 1962). The values of about 85% obtained in this way are only approximate for several reasons. The separation between poly[d(A-T)] and poly[d(A-BrUra)] in CsCl is so large that it is difficult to measure accurately. Moreover, synthetic polynucleotides may not be appropriate as standards inasmuch as the buoyant density of poly[d(A-T)] does not correspond to its base composition (Schildkraut *et al.*, 1962). Nevertheless, this calculation does indicate that the substitution is extensive. The addition of FUrd to the cultures in an attempt to block endogenous thymidine synthesis increased the density shifts only slightly to a value of 90% substitution as calculated by the above method. This observation, together with the fact that the densities do not increase at longer labeling times, suggests that the substitution may be virtually complete under our conditions.

(b) *Semiconservative replication and thymine bias*

The buoyant density data are in excellent agreement with the semiconservative model. The separation for heavy mitDNA is exactly twice the average of the separations for the two hybrids, which is consistent with the idea that the heavy species has two labeled strands and the hybrid only one. The two hybrids predicted by the difference in thymine content of the two strands are found. The hybrid and heavy peaks appear relatively homogeneous and do not shift with time, indicating that the mature molecules formed have discrete buoyant densities.

The thymine bias determined from the ratio of the separations of the two hybrids, 1·29, is very close to that determined from the base compositions of the separated strands, 1·26. If there were any BrUra on the "unlabeled" strand, the thymine bias determined from the buoyant shifts would be low. Hybrid catenanes containing submolecules of both hybrid densities would also tend to *reduce* the separation between the two hybrid peaks and give a low estimate of the thymine bias. These calculations are not affected by the extent of substitution, provided that it remains constant for the two strands.

The hybrid doublet gives rise to four bands in buoyant alkaline cesium chloride. Two of these bands appear to be completely unlabeled, since they have the same separation as that obtained from unlabeled mitDNA. These band widths also appear to be normal. The other two bands are the density-labeled strands. The thymine bias determined from the separations between the density-labeled strands and the corresponding unlabeled strands is 1·20, in fair agreement with the value determined by base analysis. The band widths of both density-labeled strands are larger than those of the unlabeled strands from the hybrid mitDNA. The increased band widths could result from faster alkaline hydrolysis of the density-labeled strands than the unlabeled ones. Alternatively, hydrolysis of the density-labeled strands may result in fragments of heterogeneous buoyant density due to differences in BrUra content.

(c) *Composition and structure of the quarter heavy mitochondrial DNA*

Electron microscope analyses show that the quarter heavy mitDNA fraction contains 30% (w/w) monomers and 70% (w/w) catenanes. The quarter heavy catenanes consist of hybrid submolecules catenated to unlabeled ones. The evidence for this structure comes from buoyant density and electron microscope analysis before and after shearing. The buoyant density analyses show that the quarter heavy mitDNA has an average buoyant density exactly half way between the hybrid and unlabeled species (Plates IV(a) and VII(a)). The quarter heavy region was enriched in catenanes as compared with the 40% (w/w) in the total sample. Examination of the other fractions confirms that catenanes were concentrated in the quarter heavy region.

The buoyant analyses revealed two peaks at the quarter heavy position with a separation exactly half that between the two hybrid peaks. Catenated dimers containing one hybrid and one unlabeled submolecule form two peaks because the hybrid submolecule can have either of two densities. The separation between these two peaks is expected to be half that between the hybrid species due to the contribution of the unlabeled submolecule. Most of the quarter heavy catenanes are dimers. Electron microscope analyses show that the quarter heavy catenanes are 63% dimers, 22% trimers, 7% tetramers, and 9% higher forms (w/w). The effect of the larger forms will be simply to broaden the bimodal distribution of the quarter heavy dimers. For example, the trimers containing either one or two hybrid submolecules will band in this region because they will be one-sixth and one-third heavy. The two buoyant densities for the hybrid submolecules will cause further splitting into bands too close to appear as separate peaks.

The strongest evidence for the structure of the quarter heavy catenanes comes from the shearing experiments. After degradation of the quarter heavy fraction into monomer circles and half-length linear molecules, 70% (w/w) of this mitDNA bands in the hybrid and unlabeled positions. Furthermore, approximately equal amounts of mitDNA appear at the hybrid and unlabeled positions. The hybrid material is evenly distributed between the two hybrid positions. Since 70% (w/w) of the quarter heavy fraction was catenated before shearing and only 2% (w/w) remains in this form after shearing, the hybrid and unlabeled mitDNA have apparently been released from catenanes composed of approximately equal numbers of unlabeled and hybrid monomer submolecules. The existence of discrete, sharp hybrid and unlabeled peaks after shearing excludes the possibility that the quarter heavy catenanes are composed of partially hybrid submolecules containing strands which are density labeled along part of their length. The quarter heavy catenanes are predominantly composed of hybrid and unlabeled submolecules.

Approximately 30% (w/w) of the quarter heavy fraction are monomers. They are probably not degraded by the shearing process, and so represent the 30% (w/w) of the quarter heavy fraction remaining at the quarter heavy position after shearing (Plate VII(b)). The broadness of this peak suggests that these monomers are a heterogeneous population containing one strand which has been partially density labeled. These probably represent molecules which had undergone various extents of replication when the BrUra incorporation began. Such molecules would be expected to have heterogeneous buoyant densities intermediate between the unlabeled and hybrid densities. They would form a peak at the quarter heavy position in the

sheared sample because the quarter heavy fraction contains a selected population of these molecules.

(d) *Implications of the structure of the quarter heavy catenanes*

HeLa cell mitDNA contains about 60% (w/w) monomeric circular duplexes and about 40% catenanes, of which 24% are dimers, 9% are trimers and 7% are tetramers and higher forms. Both the fraction of the mitDNA in this catenated form and the relative amounts of the various catenated oligomers have remained constant for over 500 cell generations. There must, therefore, be mechanisms which generate catenanes as the cells multiply and maintain the frequencies of the different catenated forms. New catenanes could be formed either from monomers or from pre-existing catenanes. Formation from monomers would entail either an aberrant form of replication in which the progeny molecules fail to separate or a linking process in which monomers become interlocked. A double recombination event is an example of such a linking step; two monomers recombine to form a circular dimer which can then form a catenated dimer *via* a second, internal, recombination event. Broker & Lehman (1971) have developed a double recombination model which avoids duplex cleavage. A double recombination event could also occur between two submolecules in a catenane, resulting in unlinking (Hudson *et al.*, 1968). An equilibrium condition might be established in which the frequency of the larger catenated forms is small because the proximity of the submolecules renders them more susceptible to unlinking.

Alternatively, new catenanes could be formed from pre-existing catenanes by replication of submolecules. This possibility must be considered seriously because catenanes containing submolecules with D-loop structures have been seen frequently during the course of this study. If linearization does not occur during replication, both of the progeny submolecules necessarily remain directly interlocked with the unreplicated submolecule(s). For example, replication of one submolecule in a catenated dimer would lead to a linear trimer. A subsequent unlinking process would be necessary, both to prevent the conversion of all the catenanes into large and complex forms and to produce new catenated dimers. The formation of new catenanes from pre-existing ones therefore requires replication of submolecules to form tetramers or higher oligomers followed by unlinking.

The quarter heavy catenanes could not have been formed by an aberrant type of monomer replication in which the progeny become linked into a catenated dimer, since this process would result in hybrid rather than quarter heavy dimers. This result is in disagreement with the replicative mechanism proposed for the formation of catenated dimers of SV40 DNA (Jaenisch & Levine, 1972) and is consistent with the recombinational mode of formation of ϕX RF-DNA (Benbow *et al.*, 1972).

If catenanes are formed from monomers by a linking process, the rate must be high in order to form 60% "new" (quarter heavy plus hybrid) dimers after eight hours. If the process is a random one, the frequencies of the different density-labeled dimer forms should approximately reflect the frequencies of the different density-labeled monomer units. After eight hours, 54% of the monomer units (including those in catenanes) are unlabeled, 39% are hybrid and 8% are intermediate in density. Linking of a hybrid and an unlabeled monomer would be more probable (0·49) than linking of two unlabeled (0·34) or two hybrid (0·18) monomers. We have calculated from the electron micrograph analysis and buoyant density distributions that about 40% of the dimers are quarter heavy, 38% are unlabeled and 21% are hybrid at

eight hours, in good agreement with the above calculations. Finally, if the linking process is a result of double recombination, the two events must occur very close together on the mitDNA molecule. Otherwise, a section of the hybrid monomer would be transferred to the unlabeled one (and *vice versa*) so that shearing would not have resulted in clean hybrid and unlabeled mitDNA. The transfer of as little as 4% of the monomer would have caused a measurable reduction in the buoyant separations, and no such change was observed.

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REFERENCES

Bauer, W., Prindaville, F. & Vinograd, J. (1971). *Biopolymers*, **10**, 2615.
Benbow, R. M., Eisenberg, M. & Sinsheimer, R. L. (1972). *Nature New Biol.* **237**, 141.
Broker, T. R. & Lehman, I. R. (1971). *J. Mol. Biol.* **60**, 131.
Brown, I. H. & Vinograd, J. (1971). *Biopolymers*, **10**, 2015.
Clayton, D. A., Davis, R. W. & Vinograd, J. (1970). *J. Mol. Biol.* **47**, 137.
Clayton, D. A., Smith, C. A., Jordan, J. M., Teplitz, M. & Vinograd, J. (1968). *Nature*, **220**, 976.
Gross, N. J. & Rabinowitz, M. (1969). *J. Biol. Chem.* **244**, 1563.
Hudson, B., Clayton, D. A. & Vinograd, J. (1968). *Cold Spr. Harb. Symp. Quant. Biol.* **33**, 435.
Jaenisch, R. & Levine, A. J. (1972). *Virology*, **48**, 373.
Karol, M. H. & Simpson, M. V. (1968). *Science*, **162**, 470.
Kasamatsu, H., Robberson, D. L. & Vinograd, J. (1971). *Proc. Nat. Acad. Sci., Wash.* **68**, 2252.
Kin, H. J., Karkas, J. D. & Chargaff, E. (1966). *Proc. Nat. Acad. Sci., Wash.* **56**, 954.
Reich, E. & Luck, D. J. L. (1966). *Proc. Nat. Acad. Sci., Wash.* **55**, 1600.
Rownd, R. (1969). *J. Mol. Biol.* **44**, 387.
Schildkraut, C. L., Marmur, J. & Doty, P. (1962). *J. Mol. Biol.* **4**, 430.
Schmid, C. W. & Hearst, J. E. (1971). *Biopolymers*, **10**, 1901.
Simon, E. H. (1961). *J. Mol. Biol.* **3**, 101.
Vinograd, J. & Hearst, J. E. (1962). *Fortschr. Chem. Org. Naturst.* **20**, 372.
Wake, R. G. & Baldwin, R. L. (1962). *J. Mol. Biol.* **5**, 201.
Watson, R., Bauer, W. & Vinograd, J. (1971). *Analyt. Biochem.* **44**, 200.
Yew, F. F. H. & Davidson, N. (1968). *Biopolymers*, **6**, 659.