

# SINGLE STRAND-CONTAINING REPLICATING MOLECULES OF CIRCULAR MITOCHONDRIAL DNA

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## ABSTRACT

Mitochondrial DNAs (mtDNAs) from Chang rat solid hepatomas and Novikoff rat ascites hepatomas were examined in the electron microscope after preparation by the aqueous and by the formamide protein monolayer techniques. MtDNAs from both tumors were found to include double-forked circular molecules with a form and size suggesting they were replicative intermediates. These molecules were of two classes. In molecules of one class, all three segments were apparently totally double stranded. Molecules of the second class were distinguished by the fact that one of the segments spanning the region between the forks in which replication had occurred (the daughter segments) was either totally single stranded, or contained a single-stranded region associated with one of the forks. Daughter segments of both totally double-stranded and single strand-containing replicating molecules varied in length from about 3 to about 80% of the circular contour length of the molecule. Similar classes of replicating molecules were found in mtDNA from regenerating rat liver and chick embryos, indicating them to be normal intermediates in the replication of mtDNA. All of the mtDNAs examined included partially single-stranded simple (nonforked) circular molecules. A possible scheme for the replication of mtDNA is presented, based on the different molecular forms observed.

## INTRODUCTION

Mitochondrial DNA (mtDNA) of all metazoan animals so far studied, which range from nematode worms to man, has been shown by electron microscopy to comprise circular molecules with contour lengths of about 5  $\mu\text{m}$  (for recent reviews see Swift and Wolstenholme, 1969; Wolstenholme et al., 1971). Evidence has been presented, from the results of density labeling experiments (Reich and Luck, 1966; Gross and Rabinowitz, 1969), autoradiography studies (Stone and Miller, 1965; Parsons and Rustad, 1968), and in vivo and in vitro studies involving incorporation of radioactively labeled DNA precursors (Neubert, 1966;

Schneider and Kuff, 1965; Wintersberger, 1966; Schneider and Neubert, 1966; Parsons and Simpson, 1967), that the mtDNA of a variety of organisms is synthesized *in situ* rather than being manufactured in the nucleus and transferred to the mitochondria. An indication of *in situ* mtDNA synthesis in higher animals was our finding by electron microscopy of some circular DNA molecules isolated from mitochondria of normal rat livers, which had structural properties and size consistent with the interpretation that they had been stopped during replication (Kirschner et al., 1968). Each such molecule contained two forks.

Two of the segments delimited by the forks (the daughter segments) were equal in length, while the third segment was either longer or shorter. All three segments of these DNA molecules appear to be essentially totally double stranded.

Recently, Kasamatsu et al (1971) demonstrated the presence in mouse L cell mtDNA of covalently closed circular molecules which contained a small segment of uniform length (about 3.5% of the molecule contour length) in which replication of only one strand had occurred. Similar molecules have been found in mtDNA from chick liver (Ter Schegget and Borst, 1971; Arenberg et al., 1971).

The purpose of the present paper is to report the finding in mtDNA from cells of two different kinds of rat tumors, regenerating rat liver, and chick embryos of double-forked circular molecules in which one of the daughter segments, varying from 3 to 80% of the contour length, is either totally or partially single stranded.

## MATERIALS AND METHODS

### *Tumors*

Chang rat solid hepatomas (Chang et al., 1967) and Novikoff rat ascites hepatomas (Novikoff, 1957) were obtained from Drs. Jeffrey P. Chang and Carl F. Tessmer, M. D. Anderson Hospital and Tumor Institute, Houston, Texas. All of the rats used in the present study were female albinos weighing 100–200 g, and obtained from Sasco Company, Omaha, Nebraska.

The Chang solid hepatomas used in the present experiments were grown intraperitoneally. Intraperitoneal solid tumors were initiated by transplantation of a Chang solid hepatoma maintained in subcutaneous pockets on the backs of rats (Chang et al., 1967). Subsequently, the intraperitoneal solid tumor, was maintained as follows. A piece of solid tumor was excised from a rat, placed in 10 vol of isotonic saline, and broken up in a Waring Blendor (5 s, slow speed, Waring Products Div., Dynamics Corp. of America, New Hartford, Conn.). The cell suspension so produced was squeezed through two layers of muslin, and 10 ml was injected into the peritoneal cavity of a rat. Transplants were made at about 20-day intervals, when the rat had gained between 50 and 70 g in weight. Solid tumors were harvested for the preparation of mtDNA when the rat showed a weight gain of approximately 70 g.

Novikoff ascites hepatoma cells were grown in the peritoneal cavity of rats and maintained by syringe passage of 10 ml of ascites fluid every 7 days. Cells used for the preparation of mtDNA were harvested 6, 7, and 8 days after infection.

### *Partial Hepatectomy of Rats*

The median lobe and left lateral lobe of the liver (representing about 70% of the organ) were removed from rats under ether anesthesia and aseptic conditions (Higgins and Anderson, 1931). The hypertrophied remaining caudate and right lateral lobes of the liver were harvested from rats 24 and 42 h after surgery.

### *Chick Embryos*

Fertilized chick eggs were incubated at 37.5°C, 60% relative humidity, and turned once every 90 min for 6 days in a Robbins incubator (Robbins Incubator Co., Denver, Colo.) in a commercial hatchery.

### *Preparation of Mitochondria*

All rats were sacrificed by cranial fracture. Regenerating rat livers were excised and immediately immersed in 10 vol of a buffer (homogenizing medium) containing 0.3 M sucrose, 1 mM disodium ethylenediaminetetraacetate (EDTA) and 0.1 M Tris (tris[hydroxymethyl]aminoethane)-HCl, pH 7.4 at 0–4°C, and homogenized first in a Waring Blendor (top speed for 10 s) and then in a Potter-Elvehjem homogenizer (top speed, four strokes). Mitochondria were then prepared as described by Kirschner et al. (1968). Mitochondria were similarly prepared from the other tissues, but with the following modifications.

Chick embryos were taken from eggs immediately after removal from the incubator at the hatchery and collected in homogenizing medium at 0–4°C. The embryos were homogenized within 30 min of removal from the eggs.

Homogenization of Chang solid tumor cells did not involve the Waring Blendor, but was accomplished by only two strokes at top speed in the Potter-Elvehjem homogenizer.

Novikoff ascites hepatomas were collected from the abdominal cavities of three to five rats, pooled, and the cells pelleted by centrifugation at 480 g for 10 min. The cells were suspended in 10 vol/vol packed cells of isotonic saline, pelleted again by centrifugation, and suspended in 10 vol/vol packed cells of the homogenizing medium. 100 mg/ml boiled kieselguhr saturated with homogenizing medium was added and the cells were broken open in a Potter-Elvehjem homogenizer using 20 strokes at top speed.

### *DNA Extraction and Purification*

DNA was extracted from mitochondria as described previously (Kirschner et al., 1968). Each mtDNA was finally purified by elution from a column of methylated albumin kieselguhr (Sueoka and Cheng, 1962).

## Denaturation of DNA

Thermal denaturation of open circular mtDNA was accomplished by heating a DNA solution containing 75 mM sodium chloride and 7.5 mM sodium citrate (pH 8.0) at 97°C for 5 min, followed by rapid cooling in an ice bath. Denatured mtDNA was stored at 0°–4°C and used for electron microscopy within 20 h of preparation.

## Electron Microscopy

MtDNAs were prepared for electron microscopy by the aqueous protein monolayer technique of Freifelder and Kleinschmidt (1965), and rotary shadowed with platinum-palladium, exactly as described by Wolstenholme and Gross (1968). Each mtDNA was also prepared for electron microscopy by the formamide protein monolayer technique following the procedure of Davis et al. (1971). 20  $\mu$ l of 0.8 M NaCl containing 0.1  $\mu$ g DNA was added to 10  $\mu$ l of 1 M Tris-HCl (pH 8.5), 1  $\mu$ l of 1 M EDTA (pH 8.5), 1  $\mu$ l of 0.5% cytochrome c, and 70  $\mu$ l formamide. This solution was allowed to flow down an inclined glass slide onto a hypophase of freshly prepared 10% formamide containing 10 mM Tris-HCl (pH 8.5). The surface film was picked up on freshly prepared parlodion membranes supported on 200-mesh copper grids, immersed for 30 s in 95% ethanol containing  $5 \times 10^{-5}$  M uranyl acetate and  $5 \times 10^{-4}$  M HCl, then in 2-methyl butane for 10 s, and air dried. The dried grids were rotary shadowed with platinum-palladium (Wolstenholme and Gross, 1968). Finally,

carbon was evaporated onto the grids to ensure stability of the membranes in the electron beam.

All grids were examined in a Hitachi HU-11B electron microscope or in a Siemens Elmiskop 101 electron microscope at a magnification of approximately  $\times 6000$ . Shadowed molecules were photographed (using projector pole piece 2 in the Hitachi, and projector pole piece 1 in the Siemens) at an original magnification of  $\times 12,000$ . Exact calibrations were made for each microscope using a diffraction grating replica (Ernest F. Fullam, Inc., Schenectady, N. Y., 2160 lines/mm). Measurements of molecules were made on positive prints using a map measure, at a magnification of  $\times 150,000$ .

Many of the open circular molecules observed in this study had one or a low number of crossovers of the filament. After Inman (1966), the following rules were observed in defining the paths of the segments delimited by the forks when two of the filaments delimited by the forks crossed over each other at a wide angle, their paths were considered unambiguous (see Figs. 1 and 4). When such filaments crossed at a narrow angle or ran parallel to each other, then the molecule was not used.

The relative lengths of simple circular molecules and double-forked molecules were determined using simple circular molecules found on the same negatives as the double-forked molecules. This minimized contour length differences resulting from systematic technical variations.

Probabilities ( $P$ ) mentioned in the text resulted from analysis of variance.

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FIGURES 1–8 Electron micrographs of rotary shadowed molecules of mtDNA, prepared by the aqueous protein monolayer technique. All micrographs  $\times 57,900$ . Figs. 1, 2, 4, 6, and 8 are from Novikoff rat ascites hepatoma cells. Figs. 3, 5, and 7 are from Chang rat solid hepatoma cells.

FIGURES 1 and 2 Double-forked circular molecules in which all three segments delimited by the forks (arrows) appear to be totally double stranded. Two of the segments are equal in length ( $A$  and  $B$ ) while the third ( $C$ ) is longer. The sum of the lengths of  $A$  or  $B$  plus  $C$  is within the range of lengths of simple circular molecules (see Fig. 10). Fig. 1. An open circular molecule.  $A$  or  $B = 0.87 \mu\text{m}$ ,  $C = 4.03 \mu\text{m}$ ,  $A + C = 4.90 \mu\text{m}$ . Fig. 2. A highly twisted circular molecule.  $A$  or  $B = 0.30 \mu\text{m}$ ,  $C = 4.77 \mu\text{m}$ ,  $A + C = 5.07 \mu\text{m}$ .

FIGURES 3–6. Open (Figs. 3–5) and highly twisted (Fig. 6) double-forked circular molecules in which all or part of one of the segments delimited by the forks (arrows) has the kinky, low contrast appearance characteristic of single-stranded DNA ( $S$ ) prepared for electron microscopy under the present conditions. In each of the molecules shown this segment is shorter than the other two. The sum of the lengths of the two apparently totally double-stranded segments ( $A$  and  $C$ ) is within the range of lengths of simple circular molecules (see Fig. 10). Fig. 3.  $A = 1.66 \mu\text{m}$ ,  $C = 3.26 \mu\text{m}$ ,  $A + C = 4.92 \mu\text{m}$ . Fig. 4.  $A = 1.92 \mu\text{m}$ ,  $C = 2.98 \mu\text{m}$ ,  $A + C = 4.90 \mu\text{m}$ . Fig. 5.  $A = 0.79 \mu\text{m}$ ,  $C = 4.14 \mu\text{m}$ ,  $A + C = 4.93 \mu\text{m}$ . Fig. 6.  $A = 0.71 \mu\text{m}$ ,  $C = 4.10 \mu\text{m}$ ,  $A + C = 4.71 \mu\text{m}$ .

FIGURE 7 A simple circular molecule in which a part of the filament has the kinky appearance characteristic of single-stranded DNA (arrow).

FIGURE 8 Single-stranded mtDNA produced by heat denaturation of open circular native mtDNA.

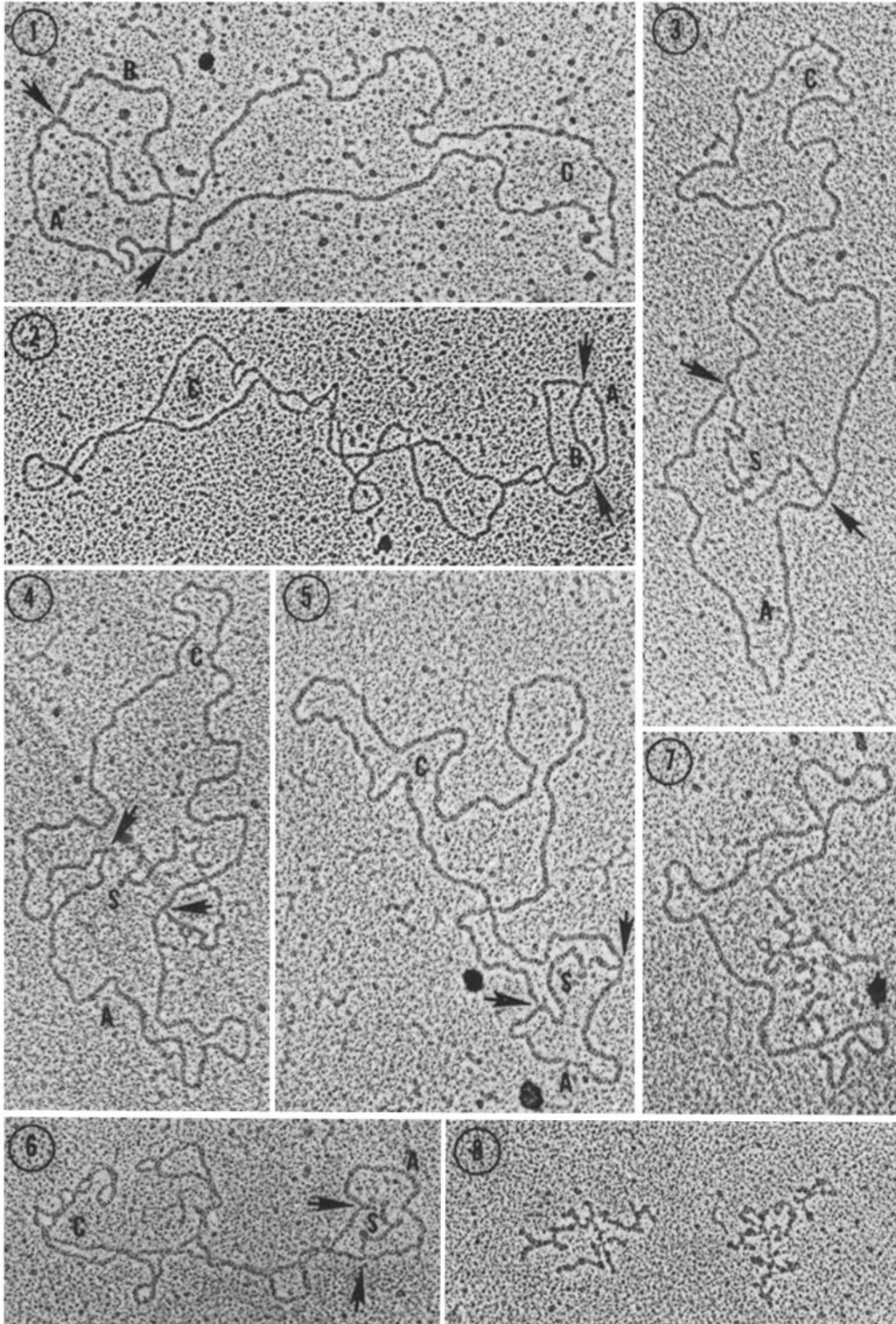


TABLE I

The Frequencies of Certain Molecular Forms in Two Preparations of Chang Rat Solid Hepatoma mtDNA and in Three Preparations of Novikoff Rat Ascites Hepatoma mtDNA, Prepared for Electron Microscopy by the Aqueous and the Formamide Protein Monolayer Techniques

	Type of protein monolayer preparation	Number of circular molecules examined	Double-forked molecules				Simple circular molecules containing a single-strand region	
			Total	Totally double stranded	Single strand-containing			
					Daughter segment 3-4% of contour length	Daughter segment 5-80% of contour length		
			%	%	%	%	%	
Chang solid hepatoma								
I	Aqueous	1196	9.7 ± 1.7	2.4 ± 0.9	0.1 ± 0.2	7.2 ± 1.5	0.9 ± 0.5	
II	Aqueous	1147	15.9 ± 2.1	2.0 ± 0.8	0.4 ± 0.4	13.5 ± 2.0	1.1 ± 0.6	
II	Formamide	1031	25.9 ± 2.7	2.2 ± 1.0	9.2 ± 1.8	14.5 ± 2.2	1.2 ± 0.7	
Novikoff ascites hepatoma								
8 day	Aqueous	1123	2.2 ± 0.8	0.8 ± 0.5	0	1.4 ± 0.7	0.3 ± 0.3	
7 day	Aqueous	1085	4.1 ± 1.2	1.8 ± 0.8	0.1 ± 0.2	2.2 ± 0.9	0.5 ± 0.4	
6 day	Aqueous	1046	5.8 ± 1.4	1.5 ± 0.7	0.1 ± 0.2	4.2 ± 1.2	0.9 ± 0.6	
6 day	Formamide	1012	31.6 ± 2.9	1.1 ± 0.6	25.1 ± 2.7	5.4 ± 1.4	0.6 ± 0.5	

95% confidence limits, calculated according to the procedures used by Clayton et al. (1968) are given.

## RESULTS

It was determined by electron microscopy that between 90 and 97% of each mtDNA preparation from the different types of cells used in this study was in the form of circular molecules. Each of the mtDNAs comprised highly twisted or supercoiled circular molecules as well as open circular molecules, as reported before for mtDNAs from a variety of metazoan animals (see reviews of Swift and Wolstenholme, 1969, Wolstenholme et al., 1971).

MtDNAs from both Chang rat solid hepatoma cells and from Novikoff rat ascites hepatoma cells prepared for electron microscopy by the aqueous protein monolayer technique were found to include molecules containing two forks (Figs. 1-6). The frequency of double-forked molecules (Table I)<sup>1</sup> was 9.7 and 15.9% in the two Chang hepatoma mtDNAs examined and varied from 2.2 to 5.8% in the Novikoff hepatoma mtDNAs. Two classes of double-formed molecules were found in each preparation. The first class comprised molecules in

which all three segments had a rigid appearance and high contrast similar to the double-stranded simple (nonforked) circular DNA molecules lying near to them on the grid squares (Figs. 1 and 2 Table I). For each such molecule, two of the segments delimited by the forks were equal in length (Fig. 9) while the third was either longer or shorter. The sum of the length of one of the similar segments and the length of the odd segment of these molecules was found not to differ significantly from the length of simple circular molecules in the same preparation ( $P = >0.2$  for each comparison of means, Fig. 10). Following the earlier interpretation of these molecules as replicative intermediates (Kirschner et al., 1968), the regions of similar length are taken to be the products of the portion of the molecule which has undergone replication (the daughter segments) and the odd segment the unreplicated portion. Open circular (Fig. 1) and highly twisted circular molecules (Fig. 2) of this class were found in the relative proportion of approximately 9:1. In the open circular molecules, the daughter segments varied from about 3 to about 80% of the circular contour length (Fig. 9). In the highly twisted molecules, the daughter seg-

<sup>1</sup> Evidence concerning the possibility of selective loss of one or more of the molecular forms of mtDNA during isolation was not obtained.

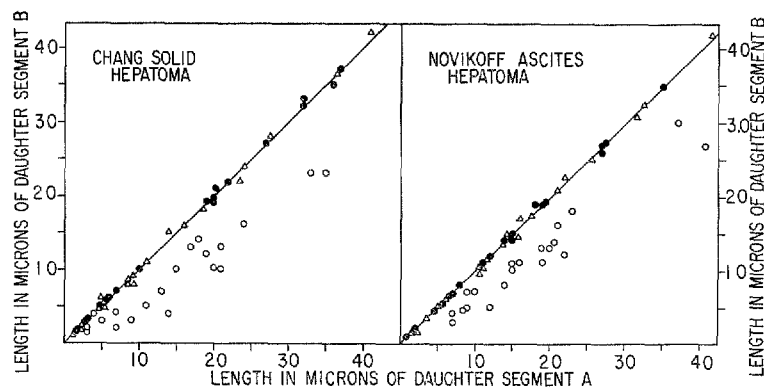


FIGURE 9 Comparative lengths of the daughter segments of double-forked open circular molecules of mtDNA from Chang solid hepatoma cells (preparation II, Table I) and from Novikoff ascites hepatoma cells (6 day preparation, Table I). The solid circles and the open circles represent molecules prepared for electron microscopy by the aqueous protein monolayer technique. The solid circles represent molecules in which all three segments appeared to be totally double stranded. Two of the segments were approximately equal in length, and the first of these to be measured in each molecule was designated the *A* daughter segment, and the second, the *B* daughter segment. The open circles represent molecules in which all or part of one, and only one of the segments appeared kinky, suggesting it was fully or partially single stranded. In such molecules, the kinky segment was designated the *B* daughter segment. When it was the shortest segment of the molecule, the shorter of the remaining two segments was designated the *A* daughter segment. When the kinky segment was intermediate in length between the remaining segments, the larger nonkinky segment was taken to be the *A* daughter segment. In no case was the kinky segment the longest in the molecule. The triangles represent double-forked molecules from samples prepared for electron microscopy by the formamide protein monolayer technique. The molecules represented were of the class defined by the following characteristics: two of the segments were approximately equal in length; one of these segments appeared to be fully double stranded (designated the *A* segment), while the whole or part of the other segment had the thinner, lower contrast characteristic of single-stranded DNA prepared under these conditions (designated the *B* segment).

ments measured up to 10% of the circular contour length.

The second class of double-forked molecules found in each tumor mtDNA preparation comprised forms in which all or part of one of the segments was kinky and low in contrast relative to the remainder of the molecule (Figs. 3-6, Table I). This kinky, low contrast appearance is characteristic of single-stranded DNA prepared for electron microscopy by the aqueous protein monolayer technique (Fig. 8, see also Dawid and Wolstenholme, 1968; Wolstenholme et al., 1968; Davis et al., 1971). As the combined lengths of the other two, apparently totally double-stranded segments of such molecules did not differ significantly from the length of the simple circular molecules in the same preparation ( $P = >0.05$  for each comparison of means, Fig. 10), the segment containing a kinky region is indicated to be one of the daughter segments. Molecules were never found in which a kinky, low contrast region was apparent in more than one of the segments.

Measurements of the segments containing kinky regions were made, which included measuring around the periphery of each kink. It was found that in 90% of these double-forked molecules, the segment containing a kinky region was the shortest segment of the molecule (Figs. 3-5). This is also in agreement with the interpretation of the kinky regions as single-stranded DNA. Under the conditions of preparation used, nonspecific intrastrand base interactions (Lee et al., 1970; Davis et al., 1971) occur, resulting in the characteristic kinky appearance and in apparent shortening of the molecule. In these molecules, the shorter of the two apparently double-stranded segments was taken to be the other daughter segment. In the remaining 10% of these double-forked molecules, the segment containing a kinky region was intermediate in length between the remaining two segments. In this case, the longer totally double-stranded segment was taken to be the second daughter segment (Fig. 9). In no case was the segment containing a kinky region the longest in the molecule.

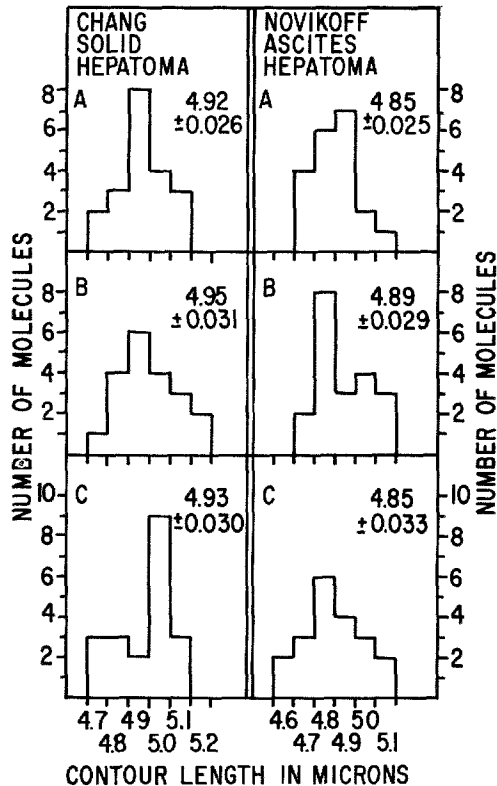


FIGURE 10 Frequency distributions of the contour lengths of circular molecules of mtDNA from Chang solid hepatoma cells (preparation II, Table I) and from Novikoff ascites hepatoma cells (6 day preparation, Table I) prepared for electron microscopy by the aqueous protein monolayer technique. (A) Simple circular molecules. (B) Double-forked circular molecules in which one of the segments appeared to be totally or partially single stranded. The contour lengths represent the sum of the lengths of the two totally double-stranded segments. (C) Double-forked circular molecules in which all three segments appeared to be totally double stranded. Two of the segments were approximately equal in length (the daughter segments). The contour lengths represent the sum of the mean length of the daughter segments plus the length of the odd segment. The mean and standard error of each sample are given.

Single strand-containing, double-forked molecules of both open circular and highly twisted circular forms were found, again in the relative proportions of approximately 9:1. Also, as observed for totally double-stranded open circular molecules, the daughter segments of open circular, single strand-containing molecules varied from about 3 to about 80% of the circular contour

length (Fig. 9). The lengths of the daughter segments of highly twisted, single strand-containing molecules measured up to 35%. This is in contrast to the maximum value of 10% found for the daughter segments of highly twisted, totally double-stranded molecules.

Single-stranded RNA might be expected to have a similar appearance to single-stranded DNA when prepared for electron microscopy under the present conditions. However, as the preparation of all mtDNAs included incubation for 30 min at 37°C with approximately 200  $\mu$ g of ribonuclease A (Sigma Chemical Co, St Louis, Mo., freed from deoxyribonuclease activity by heating at 90°C for 10 min at pH 5.0; Hotta and Bassel, 1965) per  $\mu$ g/mtDNA, it seems unlikely that regions interpreted as single-stranded DNA are in fact single-stranded RNA.

Molecules which were apparently simple circles, but in which a part of the filament had the kinky, low contrast appearance characteristic of single-stranded DNA (Fig. 7) were found in mtDNA preparations from both types of hepatoma (Table I).

To provide a further test for our interpretation of molecules prepared by the aqueous protein monolayer technique, mtDNAs from tumor cells were prepared for electron microscopy by the formamide protein monolayer technique. Formamide, under the specific conditions used by us, prevents nonspecific intrastrand base interactions, and single-stranded DNA appears extended in the electron microscope, but thinner and lower in contrast compared to double-stranded DNA (Westmoreland et al., 1969; Lee et al., 1970; Davis et al., 1971). The relative lengths of double-stranded and single-stranded mtDNA in our formamide protein monolayer preparations were determined as follows. Approximately equal parts of heat denatured and native Novikoff hepatoma mtDNA were mixed and prepared for electron microscopy in the presence of formamide (only denaturation of noncovalently closed circles, which represented about 50% of the DNA in the preparation used results under the conditions employed; see for example Wolstenholme et al., 1972). Two classes of open circular molecules distinguished by a distinct difference in thickness and contrast were observed (Fig. 11). As such a difference between molecules was not apparent when native mtDNA alone was prepared for electron microscopy under identical conditions, the circular molecules of lesser thickness and lower

contrast were assumed to comprise single-stranded DNA. The mean length of the double-stranded circles ( $5.03 \mu\text{m}$ ,  $\text{SE} = \pm 0.023$ ;  $n = 20$ ) was found to be only 3% greater than the mean length of the single-stranded circles ( $4.89 \mu\text{m}$ ;  $\text{SE} = \pm 0.034$ ;  $n = 20$ ).

In each formamide preparation of native tumor mtDNA, two classes of double-forked molecules were again observed. As in the aqueous preparations, the first class comprised molecules in which all three segments had a rigid appearance and high contrast similar to the simple circular molecules lying near to them (Fig. 12, Table I). Not a single molecule containing a kinky region was found in any of the formamide preparations. However, in these preparations a second class of double-forked molecules was defined by either the whole or part of one of the segments being thinner and much lower in contrast than the remainder of the molecule (Figs. 13–17, Table I). The length of this segment was always similar to that of one of the remaining, apparently totally double-stranded segments (Fig. 9). These findings are, therefore, again in agreement with the interpretation that one of the daughter segments of some double-forked molecules includes single-stranded DNA. Also, the relative lengths of the three segments were again in agreement with these molecules being replicative intermediates (Fig. 22).

In about 30% of the single strand-containing molecules, it appeared that in one daughter segment single-stranded DNA was associated with one of the forks and double-stranded DNA was associated with the other fork (Figs. 13 and 14). Although the actual location of a junction between single-stranded and double-stranded DNA was rarely determinable in our preparations (see also Lee et al., 1970) it was clear that single-stranded DNA accounted for considerable portions of these daughter segments. Three examples are given in Figs. 13–15. In Fig. 13, almost all of one of the daughter segments is single stranded. In Fig. 14, the single-stranded DNA seems to account for at least 46% of one daughter segment  $2.31 \mu\text{m}$  in length.

In the remaining 70% of this class of molecules, the appearance of one daughter segment was consistent with it being totally single stranded (Figs. 16 and 17). In these cases, it was not always possible to exclude the possibility that a portion of the central region of this segment was double stranded.

Partially single-stranded daughter segments and apparently totally single-stranded daughter seg-

ments both measuring up to  $4.0 \mu\text{m}$  (about 80% of the circular contour length) were found (Fig. 9).

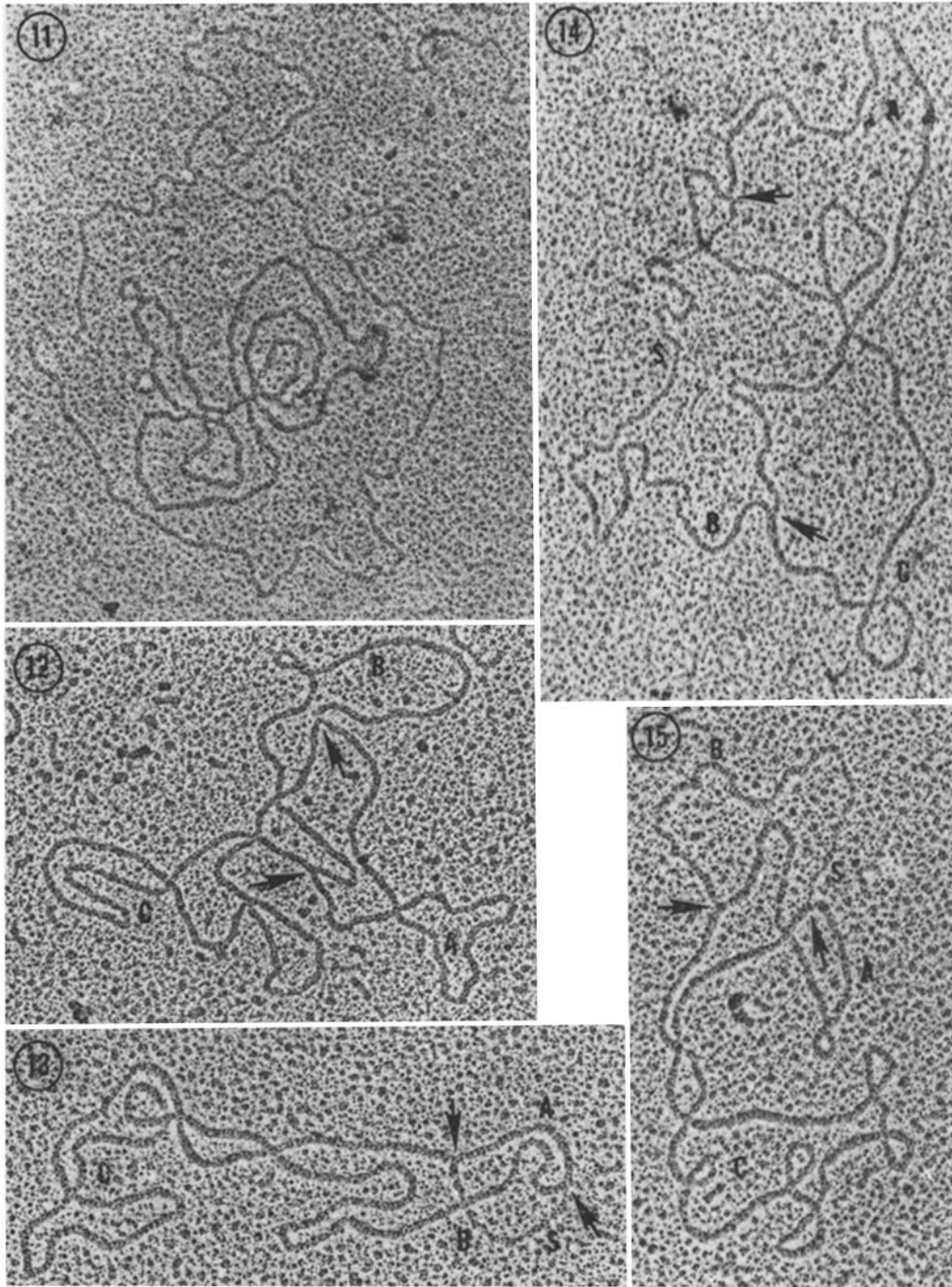
At approximately 5% of the forks with which single-stranded daughter segment DNA was associated, a short free-ended single-stranded piece of DNA was found which measured between 0.05 and  $0.3 \mu\text{m}$  (Fig. 17). These could be displaced short sections of the newly synthesized strand of the double-stranded daughter segment. The displacement is presumed to be brought about by progressive pairing of the homologous parental strands and has been termed branch migration (Lee et al., 1970; Kasamatsu et al., 1971).

A distinct class of double-forked molecules in which only one daughter segment was double stranded measured about 3.7% of the contour length was found in each tumor mtDNA prepared by the formamide technique. These molecules accounted for 25% of the 6 day Novikoff hepatoma mtDNA and 9% of the Chang hepatoma II (Table I) mtDNA. In such molecules from the Novikoff hepatoma, the double-stranded segment averaged 3.62% of the circular contour length ( $\text{SE} = \pm 0.084\%$ ;  $n = 50$ ) and the single-stranded segment, 3.87% ( $\text{SE} = \pm 0.095\%$ ;  $n = 50$ ). Such a small partial duplication was never found in molecules which contained a larger partial duplication.

Simple circular molecules were found in each mtDNA examined in which a single region measuring up to 40% of the circular contour length was thinner and lower in contrast than the remainder of the molecule (Fig. 18, Table I). These regions of low contrast could be the equivalent of the kinky regions observed in some simple circular molecules in mtDNA prepared by the aqueous protein monolayer technique (Fig. 7), and interpreted as single-stranded DNA.

The single strand-containing, double-forked molecules may be normal replicative intermediates of mtDNA or may, at least in part, be related to cell malignancy. In an attempt to test this, mtDNA has been examined from regenerating rat liver harvested 24 and 42 hr after partial hepatectomy when replication of mtDNA has been shown to be maximal (Nass, 1967), and from 6-day old whole chick embryos. MtDNA from the two tissues was prepared for electron microscopy by both the aqueous and the formamide protein monolayer techniques. Double-forked circular molecules were found to account for about 2 and 5% of the 24 and 42 h regenerating rat liver mtDNAs, respectively, and for 2.3% of the chick mtDNA. With respect to





the presence and position of single-stranded DNA, each of these mtDNAs included all of the classes of double-forked molecules found in mtDNAs of the two rat tumors (Figs 19–21). Simple circular molecules in which part of the filament was single-stranded were also observed in mtDNAs from both of these nonmalignant tissues

## DISCUSSION

The results presented are consistent with the interpretation that replicative intermediates of circular mtDNA from two rat hepatomas, regenerating rat liver, and chick embryos include double-forked molecules in which either all or a considerable part of one daughter segment is single-stranded. Double-forked circular molecules in which all of the segments appear to be totally double stranded were also found in each of these mtDNAs. The latter class of molecules have been reported previously (Kirschner et al., 1968) as the only observed form of double-forked molecules from normal adult rat liver mtDNA. As, however, only 21 molecules were found in the mtDNA from normal rat liver, it cannot be concluded that single strand-containing molecules are absent from this tissue.

Double-forked molecules which appeared in the electron microscope to be totally double stranded have been claimed as replicative intermediates of circular DNA molecules of *Mycoplasma hominis*

(Bode and Morowitz, 1967), bacteriophage lambda (Ogawa et al., 1968, Schnös and Inman 1970, Inman and Schnös, 1971), polyoma virus (Hirt, 1969, Meinke and Goldstein, 1971), colicin factor EI (Inselburg and Fuke, 1970, Fuke and Inselburg, 1971), and SV-40 virus (Jaenisch et al., 1971). Similar molecules, but in which a small single-stranded region (0.04–0.4  $\mu\text{m}$ ) was associated with one or both of the forks, were described by Inman and Schnös (1971) for circular DNA of bacteriophage lambda; also, Jaenisch et al. (1971) suggested that some of the SV-40 virus double-forked circular DNA molecules they observed contained a small single-stranded region associated with one or both of the forks.

Double-forked molecules in which only one daughter segment is double stranded and uniformly about 3.5% of the circular contour length have been reported previously in mtDNA from mouse tissue culture cells (Kasamatsu et al., 1971) and in mtDNA from chick liver cells (Ter Schegget and Borst, 1971; Arenberg et al., 1971). Evidence was presented that these molecules are covalently closed structures in the sense defined by Vinograd and Lebowitz (1966), in which replication of only one strand has occurred. The relatively high frequency of these molecules found in the mtDNA preparations in the present study and reported in the work cited above suggests that this small portion

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FIGURES 11–16 Electron micrographs of rotary shadowed circular molecules of mtDNA prepared by the formamide protein monolayer technique. Figs. 11, 12, and 14 are from Novikoff rat ascites hepatoma cells, Figs. 13 and 15 are from Chang rat solid hepatoma cells. Figs. 11–13 and 15,  $\times 56,000$ , Fig. 14,  $\times 58,500$ .

FIGURE 11 Two molecules from a mixture of native and heat denatured mtDNA. The outer molecule is thinner and of lower contrast than the centrally located molecule. As such a difference between molecules was not found when native mtDNA alone was prepared under similar conditions, the outer molecule is taken to be single stranded and the centrally located molecule, double stranded. The circular contour lengths of the centrally located molecule and the outer molecule are 5.34 and 5.31  $\mu\text{m}$ , respectively.

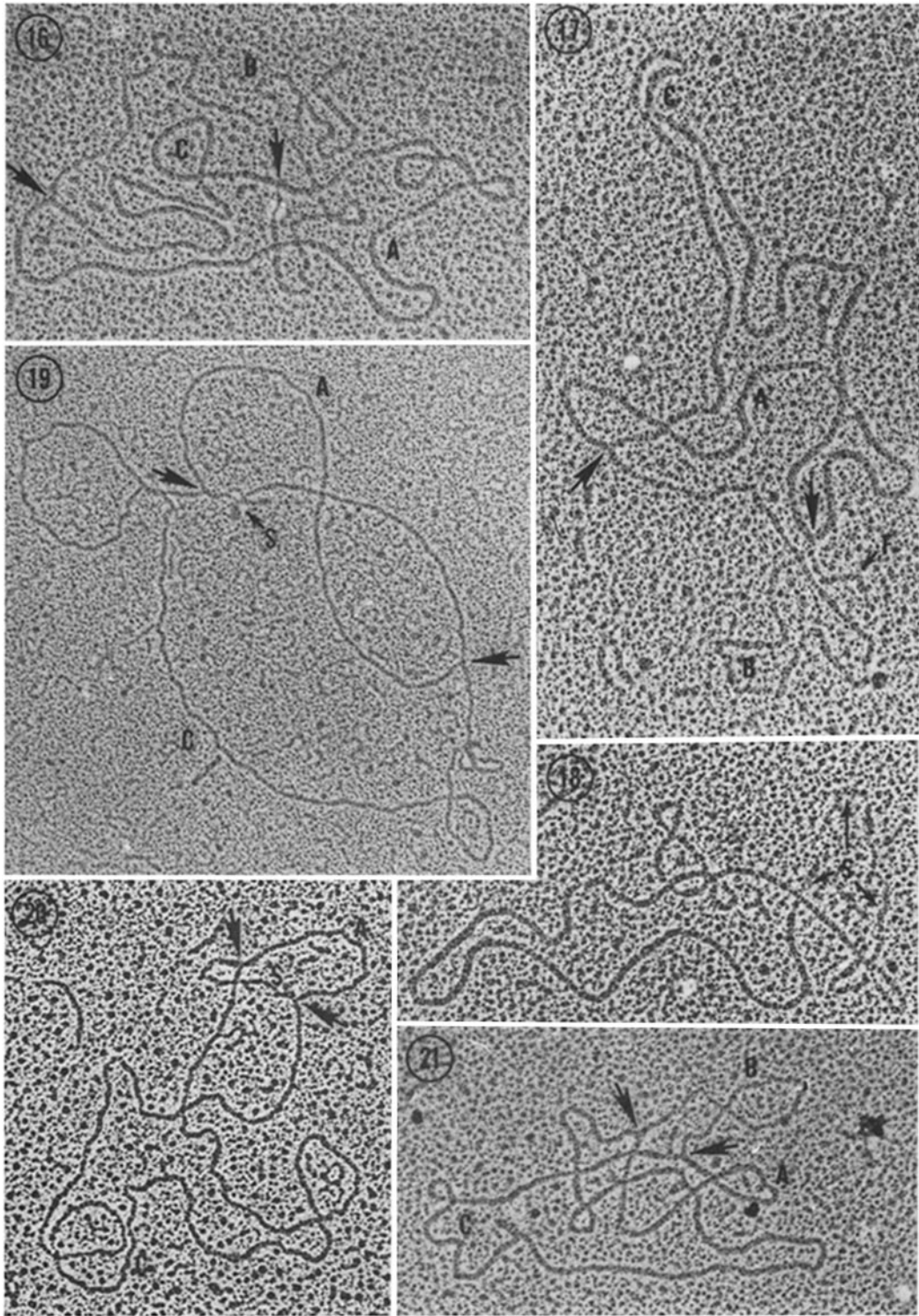
FIGURE 12 A double-forked molecule in which all three segments delimited by the forks (arrows) appear to be totally double stranded. Two of the segments (*A* and *B*) are equal in length while the third (*C*) is longer. *A* or *B* = 1.96  $\mu\text{m}$ , *C* = 3.21  $\mu\text{m}$ , *A* + *C* = 5.17  $\mu\text{m}$ .

FIGURES 13–15 Double-forked molecules in which a region of one daughter segment associated with one of the forks (arrows) has the thinner, lower contrast appearance of single-stranded DNA.

FIGURE 13 With the exception of the region close to the left-hand fork, most of segment *B* appears to be single stranded (*S*). *A* or *B* = 0.45  $\mu\text{m}$ , *C* = 4.91  $\mu\text{m}$ , *A* + *C* = 5.36  $\mu\text{m}$ .

FIGURE 14 About 1  $\mu\text{m}$  (46%) of segment *B* associated with the right-hand fork appears to be single stranded (*S*). *A* = 2.31  $\mu\text{m}$ , *B* = 2.22  $\mu\text{m}$ , *C* = 2.99  $\mu\text{m}$ , *A* + *C* = 5.30  $\mu\text{m}$ .

FIGURE 15 About 0.45  $\mu\text{m}$  (32%) of segment *B* associated with the right-hand fork appears to be single stranded (*S*). *A* = 1.43  $\mu\text{m}$ , *B* = 1.40  $\mu\text{m}$ , *C* = 3.66  $\mu\text{m}$ , *A* + *C* = 5.09  $\mu\text{m}$ .



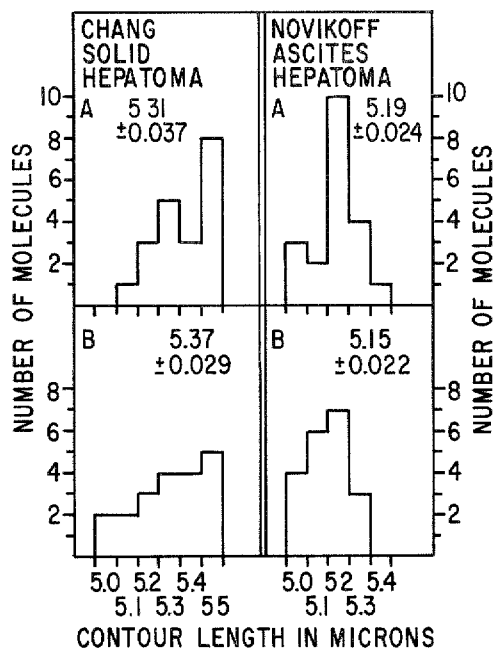


FIGURE 22 Frequency distribution of the contour lengths of circular molecules of mtDNA from Chang

of one strand replicates well in advance of the rest of the molecule. This step is shown as *a* in Fig 23, which is a possible scheme for the replication of circular mtDNA based upon the present observations. The present finding that the small partial duplication was never observed in the same molecules as a larger partial duplication is consistent with the interpretation that the larger duplications do, in fact, represent advanced stages of replication.

The observation (Fig 9) of molecules in which both daughter segments are apparently totally double stranded and represent only a 3-4% duplication suggests that the second step can be replica-

solid hepatoma cells (preparation II, Table I) and Novikoff ascites hepatoma cells (6 day preparation, Table I) prepared for electron microscopy by the formamide protein monolayer technique. (A) Simple circular molecules. (B) Double-forked molecules in which one of the segments appears to be totally or partially single stranded. The contour lengths represent the sum of the lengths of the two totally double-stranded segments. The mean and standard error of each sample are given.

FIGURES 16-21 Electron micrographs of rotary shadowed circular molecules of mtDNA.

FIGURES 16-18 MtDNA molecules from a Chang rat solid hepatoma prepared for electron microscopy by the formamide protein monolayer technique.

FIGURE 16 A double-forked molecule in which all of segment *B* appears to be single stranded. The arrows indicate the forks.  $A = 3.16 \mu\text{m}$ ,  $B = 3.25 \mu\text{m}$ ,  $C = 1.96 \mu\text{m}$ ,  $A + C = 5.12 \mu\text{m}$ .

FIGURE 17 A double-forked molecule in which all of segment *B* appears to be single stranded. The arrows indicate the forks. There is a free-ended piece of single-stranded DNA (*F*) measuring about  $0.26 \mu\text{m}$  associated with the right-hand fork.  $A = 1.97 \mu\text{m}$ ,  $B = 2.05 \mu\text{m}$ ,  $C = 3.41 \mu\text{m}$ ,  $A + C = 5.38 \mu\text{m}$ .

FIGURE 18 A simple circular molecule containing a region of at least  $0.85 \mu\text{m}$  which has the thinner, lower contrast appearance of single-stranded DNA (*S*). Contour length =  $5.30 \mu\text{m}$ .

FIGURES 19 and 20 Double-forked molecules prepared for electron microscopy by the aqueous protein monolayer technique. Both micrographs  $\times 59,000$ .

FIGURE 19 A molecule isolated from mitochondria of rat livers excised 42 h after partial hepatectomy. A part of one of the segments delimited by the forks (arrows) has the kinky, low contrast appearance characteristic of single-stranded DNA (*S*).  $A = 1.53 \mu\text{m}$ ,  $C = 3.72 \mu\text{m}$ ,  $A + C = 5.25 \mu\text{m}$ .

FIGURE 20 A molecule isolated from mitochondria of whole chick embryos, harvested after 6 days of incubation. A part of one of the segments delimited by the forks (arrows) appears to be single stranded (*S*).  $A = 0.59 \mu\text{m}$ ,  $C = 4.49 \mu\text{m}$ ,  $A + C = 5.08 \mu\text{m}$ .

FIGURE 21 A double-forked molecule prepared for electron microscopy by the formamide protein monolayer technique, isolated from mitochondria of rat liver excised 24 h after partial hepatectomy. The arrows indicate the forks. All of segment *B* appears to be single stranded.  $A$  or  $B = 1.08 \mu\text{m}$ ,  $C = 3.94 \mu\text{m}$ ,  $A + C = 5.02 \mu\text{m}$ .  $\times 55,800$ .

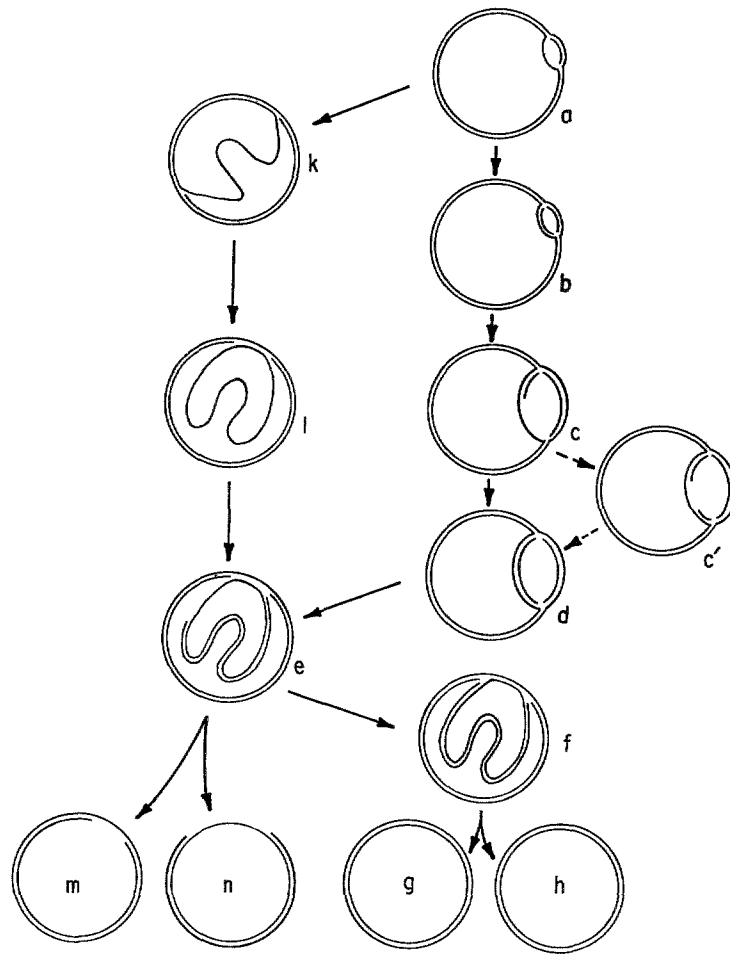


FIGURE 23 A possible scheme for the replication of circular mtDNA molecules, based on the different molecular forms observed in the electron microscope.

tion of the complementary strand (*b* in Fig. 23). Double-forked circular molecules in which both daughter segments were totally double stranded, and double-forked circles in which one daughter segment had a single-strand region associated with one of the forks were both observed throughout the spectrum defined by daughter segment lengths. This suggests that one sequence in which replication can occur entails a stepwise process (Fig. 23, *a-f*) in which a portion of one strand replicates, and then the equivalent portion of the complementary strand replicates until two totally double-stranded daughter circular molecules (Fig. 23, *g* and *h*) are produced. There is no direct indication from the present observations whether replication of one strand occurs in the opposite direction

chemically from that in the complementary strand. Such a model of replication has been suggested for bacteriophage lambda circular DNA (Inman and Schnös, 1971) based upon electron microscope observations and evidence described by Okazaki et al. (1968), Mitra et al. (1967), and Richardson (1969) which was interpreted to indicate that DNA synthesis proceeds only in the 5'-3' direction. As the double helix opens, synthesis occurs on one strand in the 5'-3' direction from the origin to the replicating fork for a specific portion of the molecule. This is followed by synthesis in the 5'-3' direction away from the replicating fork on the equivalent portion of the complementary strand. The single-stranded regions observed associated with one of the forks in some daughter segments in

the present study would be the strand which must be replicated second, and this fork would be the replicating fork. Following this scheme of replication, double-forked molecules might be expected in which all four regions of the daughter segments associated with the forks are double stranded, but in which a region of a daughter strand close to a fork is single stranded (Fig 23, *c'*). This would represent incomplete synthesis of a section of the second strand in the direction away from the replicating fork. Such molecules were not observed in the present study and were observed only rarely by Inman and Schnös (1971) for bacteriophage lambda circular DNA. This would be explained if this phase of synthesis were rapid compared to synthesis of the equivalent section of the complementary strand (Inman and Schnös, 1971), or if synthesis of both strands was rapid but separated by a temporal pause.

Following this model of replication, the finding that single-stranded DNA was never observed in both daughter strands is consistent with replication as a whole taking place in only one direction in an individual molecule.

The finding of double-forked molecules in which one daughter segment was 80% of the circular contour length and apparently totally single stranded, indicates that at least this portion of one of the strands can replicate before replication of the complementary strand begins (*a-k-l*, in Fig. 23). The molecule diagramed in *e* might then be an intermediate step in the formation of two totally double-stranded daughter molecules, either after the replication of the single-stranded daughter segment in *l* and continuing through *f* to *g* and *h*, or in the scheme represented by *a* to *g* and *h*. However, if the daughter segments of the *e* form separated before the completion of DNA synthesis in one or both strands, then the observation of simple circular molecules which contain a single-stranded segment (*m* and/or *n*) would be explained.

Highly twisted circular mtDNA molecules observed in aqueous protein monolayer preparation have been shown to be covalently closed (see for example Dawid and Wolstenholme, 1967). The finding of highly twisted double-forked molecules in such preparations, in which up to 35% of one strand has replicated, or in which up to 10% of both strands have replicated, suggests the possibility that replication of at least these amounts of the respective strands can occur with the parent molecule remaining in the covalently closed state.

Evidence that SV-40 virus DNA replicates as a covalently closed circle has been presented by Jaenisch et al, (1971), and Fuke and Inselburg (1971) reported finding highly twisted replicating molecules of colicin factor E1 circular DNA.

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