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Replication of Mitochondrial DNA. Circular Replicative Intermediates in Mouse L Cells

(electron microscopy/separated strands/duplex synthesis/displacement synthesis)

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ABSTRACT The frequency, composition, and structure of circular replicating forms of mitochondrial DNA, including two new forms described here, suggest a scheme for the mode of replication of this DNA.

The closed-circular mitochondrial DNA (mtDNA) isolated from exponentially growing mouse L cells contains a substantial fraction of displacement-loop molecules (1). These molecules, formed by displacement synthesis in the first discrete stage of DNA replication, contain a short progeny strand hydrogen-bonded at a unique site to the light parental strand. This communication describes the structure of larger circular replicative intermediates that appear to have been formed upon the continuation of displacement synthesis and the subsequent asynchronous synthesis of light strand on the displaced heavy strand. A sequential arrangement of the replicative structures has suggested a scheme for certain aspects of the replication of mtDNA (Fig. 1). This scheme is given here so as to simplify the presentation of the results on which it is based.

MATERIALS AND METHODS

The radioactive mtDNA and the separate mtDNA complements were prepared as described (1). The upper and lower bands from the final ethidium bromide–CsCl density gradient were fractionated as shown in Fig. 2 of ref. 1, except that the central four fractions, rich in catenanes, were not included in the upper-band sample. The hybridizations were performed at 70°C in 3 M CsCl, pH 8.5, for 4 hr.

The electron-microscope experiments were performed as described (1), except that the formamide concentration in the hypophase was 17% instead of 10% so as to enhance the difference in appearance between duplex and single-stranded DNA. The solution corresponding to T_m −25°C contained 50% formamide–0.01 M Na_2EDTA–0.1 M (Tris base, Tris·HCl) (pH 8.5), $\mu \simeq 0.05$. Single-stranded ϕX DNA was present in all experiments in which discrimination between duplex and single-stranded regions was important. Duplex RF ϕX DNA was also present as a standard to normalize the length of single-stranded ϕX DNA. The factor was usually close to unity. Long duplex and single-stranded DNA regions were readily distinguished from each other by the criteria of thickness and contour uniformity (2). The position of the junction between a single-stranded and a duplex part of a linear DNA segment was located by comparison of short contiguous lengths with each other. The minimum length that led to a consistent location of a junction was about 0.1 μm of DNA, 300 nucleotide pairs, or 1 cm on a 50-fold enlarged image on the Nikon 6 projection comparator. Multiple independent tracings of the same molecules demonstrated that junctions were indeed specified with a precision of ± 0.05 μm or ± 0.01 genome lengths (G). The recognition of duplex regions with lengths in this size range was difficult, and in some molecules was nonreproducible (Fig. 4*B*). Small gaps, on the other hand, were more reliably detected because they were perceived as thin irregularities in an otherwise smooth contour of continuous duplex.

RESULTS

The mtDNA from exponentially growing mouse LA9 cells contains several distinguishable structures that migrate into either the upper or the lower band of an eithidium bromide–CsCl buoyant density gradient. The lower band contains the main closed-circular DNAs: D-loop DNA and mature mtDNA (1). The upper band contains linear DNA, clean circular DNA, nicked D-loop DNA, expanded D-loop DNA (Exp-D DNA), and gapped circular DNA (Gpc DNA). The Gpc DNAs are of mitochondrial size and contain varying proportions of a duplex and a single-stranded region. Because there were about equal amounts of DNA in the two bands, the frequencies of the various structures (Table 1) in the unfractionated preparations may be obtained by dividing each value by two.

Expanded displacement loop mtDNA

Circular mtDNA with displaced strands that vary in size from 3% G to the full genome size account for 8–12% of the circular molecules in the upper band (Table 1). Examples of Exp-D DNA with expansions of 0.36, 0.80, and 0.96 G are shown in Fig. 2. The displaced segment is partially duplex in two of the three examples shown. The distribution of the relative lengths of 82 expansions in the upper-band DNA of preparation 2 contains a maximum in the interval of 0.0–0.1 G (Fig. 3). Since the mean expansion, 0.035 ± 0.014 G, of the molecules in this interval is the same as in closed D-loop DNA, it is probable that some of the molecules in this interval arose by adventitious nicking of closed D-loop DNA during isolation and that the frequency of upper-band Exp-D molecules

Abbreviations: mtDNA, mitochondrial DNA; G, genome unit; Exp-D DNA, expanded D-loop DNA; Gpc DNA, gapped circular DNA.

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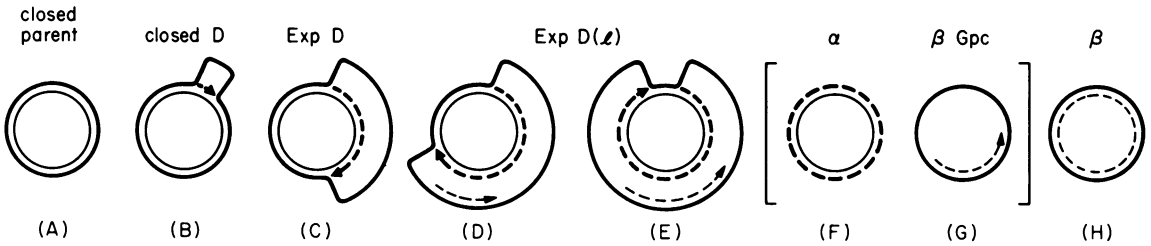

FIG. 1. The circular replicating structures of mtDNA arranged in order of increasing degree of replication reveal certain aspects of the displacement loop model for the replication of circular mtDNA. Parental strands, *solid lines;* progeny strands, *dashed lines;* heavy strands, *thick lines;* light strands, *thin lines.* Nicks and small gaps are not indicated. Daughter molecules (*F* and *G*) are represented as having formed after separation occurred in a completely (or almost completely) expanded D-loop molecule. *Exp-D (l)*, an Exp-D molecule in which light-strand synthesis has occurred; *Gpc*, gapped circular molecule.

should be about 4–6%. The otherwise flat distribution indicates that the rate of displacement synthesis is uniform. The zero value in the 0.1–0.2 G interval may be a statistical fluctuation. Expansions of this size were seen in other experiments. Catenanes were excluded from the 0.9–1.0 G interval with the requirement that some single-strandedness be observable close to at least one of the two junctions between the two circles. This requirement may have reduced the frequency and obscured a maximum value in the latter interval.

Duplex synthesis in Exp-D molecules. The initiation sites for light-strand synthesis

About one-third of the Exp-D molecules examined for Fig. 3 contained a duplex region in the displaced segment. The duplex regions are regarded as having been formed by the synthesis of light strands on the displaced heavy single strand—a process we refer to as *duplex synthesis.* The size and the location of the duplex regions in the displaced segments were measured in this sample and in a fast-sedimenting fraction, which contained 37% larger Exp-D DNA (fraction 18, Fig. 6). The relative duplex synthesis on the displaced strand, $d/(a + b)$, is presented as a function of the relative expansion, $a/(a + b)$, in Fig. 4*A*. It is clear from the results that duplex synthesis does not occur until the replicating molecule has expanded through 0.55 of the genome, and that the amount of duplex synthesis at any particular expansion is highly variable. About one third of the Exp-D molecules >0.55 G contained no detectable duplex regions in the displaced segment. All data points, with one exception, fall below the diagonal line that gives the position for fully duplex expansions or Cairns' forms. Such forms have been reported to occur at a low frequency in mtDNA (3).

If we assume that the fork at origin *O* (Fig. 4*A*) remains stationary during replication, we find that there are two ways to explain the restriction against the initiation of light-strand synthesis before the expansion has reached 0.55 G. Release from some regulatory mechanism occurs after the expansion has reached the critical value and permits initiation anywhere on the displaced heavy strand. Alternatively, initiation of duplex synthesis is limited to the 0.55–1.0 G region on the displaced strand. Initiation of light-strand displacement synthesis on a duplex part of an Exp-D molecules is regarded as unlikely because second displacement loops, easily visualized if over 100 nucleotides long, were absent. In order to examine the above alternatives, we measured the normalized lengths $(c + d)/(a + b)$ and $(e + d)/(a + b)$, as defined in Fig. 4*A*, on each Exp-D molecule observed in fraction 18 and constructed a histogram of all the lengths (Fig. 4*C*). The maximum in the interval of 0.6–0.7 G indicates that the latter explanation is more likely and that the first initiation occurs mainly in this interval.

In order to test this premise further, we arranged the displaced segments in order of size and selected the orientation of each segment so that the distance between the fork (at the left side of the diagram) and the distal end of the duplex region was closest to 0.6 G (Fig. 4*B*). Eleven displaced segments were free of duplex. Five contained short duplex regions, that were nonreproducible (see *Materials and Methods*), and are indicated as *dashed lines.* 28 contain duplex regions

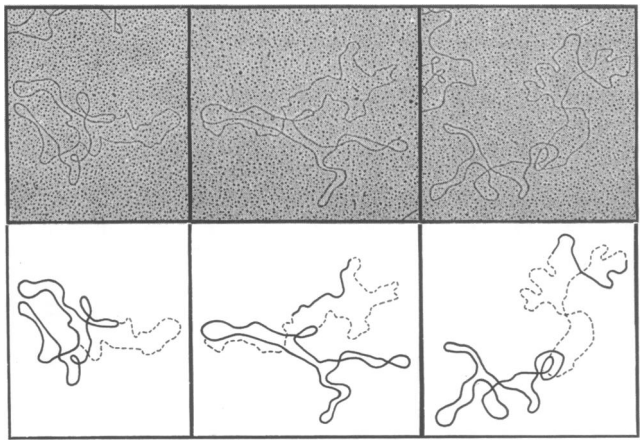

FIG. 2. Exp-D mtDNA with expansions of (*left* to *right*) 0.36, 0.80, and 0.96 G. The expansions in the latter two molecules contain duplex regions, 0.15 and 0.21 G in size. *Bottom: solid lines,* duplex regions; *dashed lines,* single-stranded regions.

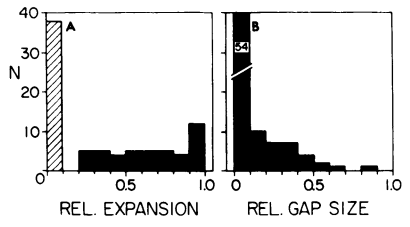

FIG. 3. (*A*) Frequency distribution of the relative size of the expansion in Exp-D molecules photographed on a specimen grid prepared with the DNA from the upper band of preparation 2 (Table 1). The molecules in the first interval may be adventitiously nicked D-loop mtDNA. (*B*) Frequency distribution of the relative gap size in Gpc molecules in the above sample.

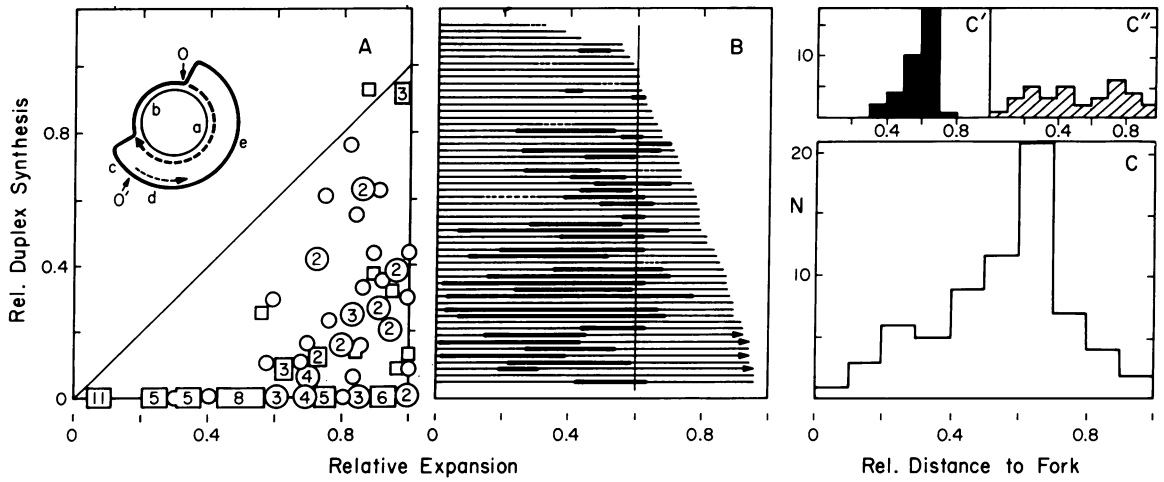

FIG. 4. (*A*) The relative size of the duplex region in the expanded segment of Exp-D DNA as a function of the relative expansion. O, data obtained with the Exp-D molecules from fraction 18 (Fig. 6); □, data obtained on measurement of the molecules used for construction of Fig. 3*A*. The numbers within the symbols represent the number of overlapping data points. The origin for displacement synthesis and the first origin for duplex synthesis are labeled *O* and *O'*, respectively. (*B*) An array of the 55 expansions in Exp-D molecules listed with the symbol O in *A*. *Thick* and *thin lines*, duplex and single-stranded regions oriented as explained in the text: *left side* of the diagram, the origin *O; dashed lines*, segments classified as duplex in only one of three independent tracings of the films. The expansions marked with an *arrow* are discussed in the text. The *vertical line* represents the mean position for the first initiation of duplex synthesis as explained in the text. (*C*) A histogram of all normalized distances from a fork to a distal end of the duplex, (*C'*) after selection, as described in the text; (*C''*) the alternative normalized distances.

(*heavy lines*) that begin between 0.5 and 0.7 G from the origin, and two begin between 0.4 and 0.5 G. The mean starting position in these 30 displaced segments is 0.60 ± 0.07 G. Initiation apparently occurred at 0.80 G in one large displaced segment containing 0.84 genome units of duplex, if we assume that synthesis takes place only in the conventional 5'-to-3' direction. Four displaced segments, marked with an *arrow*, appear to have been misoriented. If oppositely oriented, the starting positions would be between 0.77 and 0.95 G from the origin. The combined results are taken to indicate that the first initiation site exposed is at 0.6 G, and that further initiation sites also exist between 0.6 and 1.0 G. The orientations in Fig. 4*B* result in the formation of a much narrower distribution of starting positions (Fig. 4*C'*) than is indicated in Fig. 4*C*. The alternative orientations result in a distribution (Fig. 4*C''*) free of any significant maxima or minima.

TABLE 1. *Frequencies of replicating forms in mtDNA isolated from exponentially growing mouse LA9 cells and purified in ethidium bromide–CsCl gradients*

	Upper band			Lower band	
	Preparation				
	1	2	3*	1	2
Duplex circles, %	(72)†	(69)†	(69)†	(99)	(98)
With D-loops	8‡	12‡	8‡	34	41
With single-stranded tails	3	2	2	3	0
With gaps	16	18	22	0	0
Clean	72	66	66	59	59
Unidentified	1	3	2	3	0
Molecules classified	457	381	442	146	334

* Preparation 3 is a pool of three different upper-band DNA samples from LA9 cells harvested at cell densities of 1.5–2.5 × 10^5 cells/ml.

† About half of the linear molecules were of mtDNA size. The remainder were smaller and may be nuclear DNA. Linear molecules with lengths less than 0.1 G, 6% of the mass of the linears, were not included. The frequency of each form in the upper band relative to the mtDNA forms in this band can be obtained by multiplying by 0.82.

‡ About half of these molecules have expansions of 0.035 ± 0.015 G, and may be adventitiously nicked, closed D-loop mtDNA.

Gapped circular molecules

The gapped circles account for 16–22% of the circular forms in the upper-band preparations (Table 1). Fig. 5 presents Gpc molecules with gaps of 0.42, 0.10, and 0.04 G. The frequency of gapped circles in DNA from the upper band of preparation 2 decreases steadily as a function of relative gap size (Fig. 3*B*). The molecules in the interval of 0.1–0.9 G are regarded as

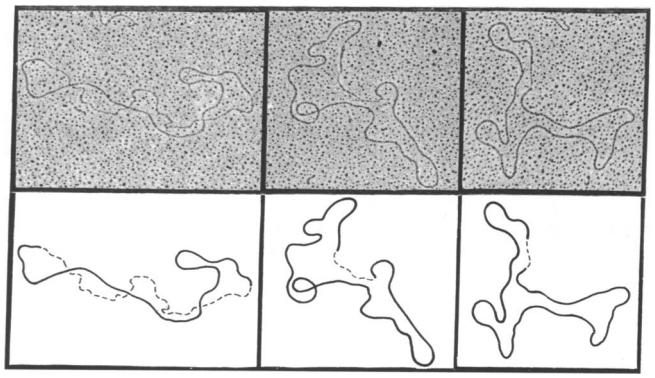

FIG. 5. Gpc molecules with relative gap sizes of (*left* to *right*) 0.42, 0.10, and 0.04 G. The *solid* and *dashed lines* in the drawing correspond to duplex and single-stranded regions, respectively.

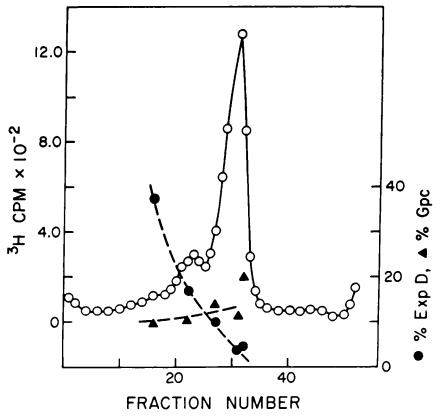

FIG. 6. A sucrose gradient fractionation of 5 μg of upper-band mtDNA from preparation 2 (Table 1) after 130 min at 36,000 rpm, 20°C, in an SW 50 rotor. The 5–20% sucrose gradient contained 0.5 M NaCl. Selected fractions were analyzed at the electron microscope for the relative frequency of Exp-D (○) and Gpc (△) molecules, catenanes, and clean circles.

incomplete β daughters that formed upon separation of the displaced β circles from the completely or almost completely expanded D-loop DNA (Fig. 1). The Gpc molecules containing small gaps between 0.0 and 0.1 G, with a mean length of 0.03 ± 0.02 G, account for 8% of the circular forms and for about one-half of the Gpc molecules. The frequency of these molecules suggests that separation has occurred just before displacement synthesis was completed and that small-gapped α Gpc molecules accumulate before the gap-filling synthesis occurs (Fig. 1*E*). Alternatively, if separation had occurred after completion of displacement synthesis but just before the completion of duplex synthesis, small β Gpc molecules would have formed.

Fractionation of Exp-D and Gpc molecules by velocity sedimentation

The distribution of radioactivity in a sucrose gradient after centrifugation of a sample of upper-band mtDNA has a maximum that corresponds to the major species, nicked monomeric DNA, and a small maximum at the calculated position for doubly open catenanes (Fig. 6). Selected fractions were examined in the electron microscope, and about 150 molecules in each were classified. The relative size of the expansion and the relative gap size were also estimated. The frequency of Exp-D DNA (ignoring linears and oligomers) increased steadily from 5 to 37% in the gradient. The relative expansion also increased. Fractions 16 and 22 contained Exp-D molecules of 0.2–1.0 G, while the slower fractions 27, 31, and 32 contained Exp-D molecules of 0.03–0.4 G. Gapped circles were fractionated by gap size, with small gaps absent in fractions 16 and 22.

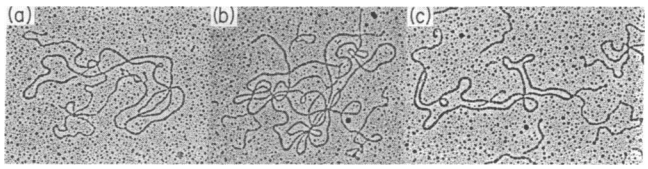

FIG. 7. (*a* and *b*) Hybrid molecules formed by annealing larger Exp-D molecules with long (about 0.5 G) mtDNA light strands. (*a*) An example of a simple hybrid; (*b*) a complex multinucleated hybrid; (*c*) a hybrid molecule formed upon annealing of a large-gapped Gpc molecule with at least two light strands.

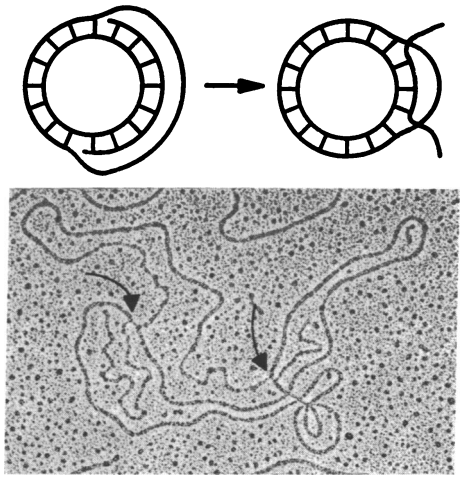

FIG. 8. An Exp-D molecule with a tail formed by branch migration at each fork, as indicated by the *arrows*. The displaced strand is in the upper part of the molecule and contains a duplex region. The appearance of such molecules indicates noncovalent attachment of the displacing strand, as shown in the drawing.

Strand identification in Exp-D and Gpc molecules

To determine whether strand specificity is involved in the formation of Exp-D molecules, we annealed the DNA in fraction 16 (Fig. 6), enriched in larger Exp-D molecules, in separate experiments with a 50-fold excess of heavy and light mtDNA strands. The annealing with heavy strands did not alter the appearance of Exp-D molecules, which declined in frequency from 37 to 22% of 160 molecules. The loss is at least in part due to scission(s) in expanded segments, which results in molecules indistinguishable from hybridized Gpc molecules. Annealing with light strands formed simple Exp-D hybrids (Fig. 7*a*), as well as complex structures (Fig. 7*b*). The hybrid frequency was 19% of 133 molecules, which included only one unhybridized Exp-D molecule. These results indicate that it is the heavy strand that is displaced in D-loop expansion.

The DNA in the combined slowly sedimenting fractions 31 and 32 (Fig. 6) contained 15% Gpc molecules with about half the gaps larger than 0.1 G and 4% Exp-D molecules with 0.03-G expansions. This DNA was similarly hybridized with a 25-fold excess of heavy and light strands at a concentration of 5 μg/ml. Hybridization of a single-strand segment in a Gpc molecule with one long complementary strand generally leads to a molecule with two forks. Branch migration (4) at a fork results in the appearance there of two single-stranded tails. A hybrid formed by reaction of a Gpc molecule with at least two strands is shown in Fig. 7*c*.

The frequency of hybrid molecules that formed after annealing the Gpc molecules with light strands was 7% of 525 molecules, as compared with 0.8% of 393 molecules after annealing with heavy strands. The frequencies of the remaining large-gapped molecules were 4 and 9%, respectively. The frequency of the remaining small-gapped Gpc molecules was 7% in both experiments. The above results indicate that the larger single-strand regions in Gpc molecules are segments of the heavy strand. The results also indicate that the hybridiza-

tion conditions, while adequate for the reaction with the larger gaps, were inadequate for reaction with the smaller ones.

DISCUSSION

A sequential arrangement of the circular replicative structures suggests a model (Fig. 1) for certain aspects of the semiconservative replication of mtDNA. The closed D-loop mtDNA (Fig. 1*B*), formed from the parental molecule by displacement replication of a hydrogen-bonded, unique 450-nucleotide segment of heavy strand (1), accumulates and accounts for about one-fourth of the mtDNA in exponentially growing mouse L cells. Unidirectional displacement replication of heavy strand apparently then continues in the presence of a nicking system with expansion of the D-loop (Fig. 1*C*). After at least 60% expansion has occurred, the synthesis of light strand begins at about 0.6 G from the origin and proceeds counterclockwise (Fig. 1*D* and *E*). The replication of the two daughter strands in a single molecule is thus highly asynchronous, with a large distance, 8×10^3 nucleotides or 3 μm, between the site for the initiation of heavy-strand synthesis and the first site for the initiation of light-strand synthesis. Further initiations occur at sites between 0.6 and 1.0 G from the origin. The separation of the daughter molecules from the Exp-D replicative structure is presented as occurring (Fig. 1*F* and *G*) before light-strand synthesis is completed. The products are α daughters and large-gapped β Gpc molecules. The latter are then finished to form β daughter molecules (Fig. 1*H*).

As was noted earlier, we have made the assumption in the interpretation of our results that displacement synthesis is unidirectional and that the fork at the origin *0* (Fig. 4*A*) is stationary in the course of displacement synthesis. This assumption is being tested experimentally by examining double-length mtDNA replicative intermediates (1) that contain a small D-loop, as well as an expanded D-loop. The results of this still incomplete study indicate that one fork in the expansion grows unidirectionally while the other remains fixed, as is shown in Fig. 1.

We have also assumed in the above displacement-loop model for replication that the progeny and parental strands are not covalently connected. In our initial attempts to examine this question experimentally, we exposed fraction 16 (Fig. 6), enriched in Exp-D molecules, to submelting conditions, $T_m - 25°C$, in order to induce branch migrations at the forks (4). 6 and 9% of the 90 molecules examined in each of two experiments developed single-strand tails at both forks (Fig. 8), and 12 and 29% did so at one fork. While these preliminary results demonstrated that a small fraction of the larger displacing strands are noncovalently attached, they are not taken to mean that the remainder are covalently attached. The factors that govern the rates and the equilibria for the process of branch migration in Exp-D molecules are inadequately understood. These studies are continuing.

The data in Table 1 and in Fig. 3 have been combined to provide an estimate of the fractional number of circular molecules that are replicating in the exponentially growing mouse L cells at any one time. The larger (>0.1 G) Exp-D and the larger-gapped (>0.1 G) Gpc molecules account for approximately 5% of the mtDNA in preparation 2. If we assume that this frequency is an index of the fractional number of molecules replicating at any one time and that each molecule replicates once per division during the 12-hr S and G_2 periods (5, 6), the estimated minimum average rate of duplex formation is about 0.1 μm/min, compared with 0.5–1 μm/min for the nuclear DNA (7) and 50 μm/min for bacterial DNA. The contribution of the closed D-loop DNA was excluded from the calculation because these molecules represent a holding point in the process (1). The small-gapped Gpc and the small Exp-D molecules may similarly be holding points.

Several studies of the mode of replication of circular DNA in animal cells have appeared recently. Arnberg *et al.* (8) have shown that closed D-loop molecules also occur in chick liver mtDNA. Replicative intermediates in SV40-infected monkey cells appear to be nicked and rapidly reclosed in the course of a Cairns-type replication with incomplete progeny strands noncovalently attached to parental strands (9, 10). This same Cairns mode of replication was also inferred in studies of the replication of polyoma DNA in infected mouse cells (11, 12).

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