DOUBLE-FORKED CIRCULAR MOLECULES

ABSTRACT

INTRODUCTION

Mitochondrial DNAs (mtDNA) of all metazoans so far studied comprise circular molecules with species-specific contour lengths of 5–6 µm (see recent reviews of Rabinowit and Swilt, 1971; Wolstenholme et al., 1971; and Borst, 1972). A variety of apparent replicative forms of circular mtDNA from rat and chick tissues (Kirschner et al., 1968; Wolstenholme et al., 1971 and 1973; Arenberg et al., 1971) and mouse L cells (Kasamatsu et al., 1971; Robberson et al., 1972; Robberson and Clayton, 1972; Kasamatsu and Vinograd, 1973) have been described.

From a consideration of the replicative forms found in mtDNA of rat tissues, we proposed (Wolstenholme et al., 1973) that this DNA can replicate by one of two alternative modes. In the
first, sections of the molecule are completely replicated sequentially by synthesis first along a section of one nucleotide strand followed by synthesis along the equivalent portion of the complementary nucleotide strand. In the alternative mode, synthesis on one nucleotide strand of the molecule can be at least 80% complete before synthesis on the second nucleotide strand is initiated.

From a study of the frequency and structure of replicative forms in mouse L-cell mtDNA, Robberson et al. (1972) have suggested that this DNA replicates by a single mode, which is basically similar to the second mode suggested by us. The main difference is that initiation of synthesis on the second strand occurs only after synthesis on the first strand is at least 60% completed.

In this report we present evidence obtained from a study of the lengths of daughter segments of replicative intermediates of mtDNA from Novikoff rat ascites hepatoma cells which indicates that replication of molecules of this DNA is a discontinuous process.

MATERIALS AND METHODS

Novikoff rat ascites hepatomas (Novikoff, 1957) were obtained from Drs. Carl F. Tesmer and Jeffrey P. Chang, M. D. Anderson Hospital and Tumor Institute, Houston, Tex. The methods of hepatoma growth and harvesting, isolation of mitochondria, purification of mtDNA, preparation of mtDNA for electron microscopy, and the conditions for electron microscopy were exactly as described previously (Wolstenholme et al., 1973).

Double-stranded and single-stranded DNA were distinguished from each other by the differences in contrast and filament conformation characteristic for the respective protein monolayer preparations (Wolstenholme et al., 1973). As before (Wolstenholme et al., 1973), when crossovers of different segments of replicating molecules occurred, the following rules were observed in defining the paths of these segments: when the angles formed by the crossing filaments were close to 90°, the filament paths were considered unambiguous; when the filament crossover angles were sufficiently different from 90° to create ambiguity, the molecule was not used.

Measurements of molecules were made on positive prints at magnifications of approximately X 160,000. Exact magnifications were calibrated for each set of prints by use of a diffraction grating replica (2,160 lines/mm, Ernest F. Fullam, Inc., Schenectady, N. Y.). Percentage lengths of the daughter segments of replicating molecules were estimated by the following procedure. Single photographic enlargements of each of at least 30 replicating molecules were prepared. The three segments of each molecule were measured once and the values in centimeters recorded on the back of the respective prints. Percentage values of the daughter segments were calculated only after measurements of all molecules of a group had been completed. This procedure was adopted in order to eliminate the possibility of bias by the measurer towards values which would either artificially create groups or which fell within previously observed high frequency ranges.

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

RESULTS

mtDNA from Novikoff rat ascites hepatoma cells prepared for electron microscopy by both the aqueous and the formamide protein monolayer techniques was found as before (Wolstenholme et al., 1973) to include double-forked circular molecules with form (Figs. 1-6) and size (Fig. 7) suggesting they were replicative intermediates. These molecules were of two structurally distinct kinds. In approximately 11% of such molecules, both daughter segments, as well as the unreplicated segment, appeared to be totally double stranded (Figs. 1 and 2). In the remaining 89%, one and only one of the daughter segments was either totally or partially single stranded (Figs. 3-6). On the basis of observations using formamide preparations, the latter class could in turn be subdivided. In about 19% of the molecules of this class, one daughter was single stranded at one fork and double stranded at the other (Fig. 3); in 72% of this class, one daughter segment appeared to be totally single stranded (Fig. 4); and the remaining 9% of this class was of a form not previously demonstrated in Novikoff rat ascites hepatoma mtDNA. In this 9% a daughter segment was single stranded at both forks but the central region of this segment was double stranded (Figs. 5 and 6). The lengths of the daughter segments of this latter kind of molecule fell within a range equal to 12-80% of the circular contour length. Also, as in our previous study, simple (nonforked) circular molecules were observed in which a single region measuring up to 40% of the circular contour length appeared to be single stranded.

In the present study we examined the distribution of daughter segment lengths in samples of replicating molecules in which the replicated region measured between 2% and 44% of the
total circular contour length. We limited our present study to this spectrum of daughter segment lengths because with increased length of the daughter segments it is more difficult to find molecules which are displayed in such a way as to allow unambiguous determination of the paths of the various segments.

Molecules in which one daughter segment was totally or partially single-stranded from a single aqueous protein monolayer preparation were first considered. The lengths of the totally double-stranded daughter segment of each of 173 such molecules, expressed as percentage of total circular contour length, are shown in Fig. 8. It appeared that these daughter segment lengths fell into eight distinct groups with mean values separated by 4.1–7.6% of the circular contour length.

In order to test the reproducibility of the groupings, we examined the distribution of lengths of the totally double-stranded daughter segment of single-strand-containing replicating molecules from the same mtDNA sample as that represented in Fig. 8, but in a formamide protein monolayer preparation. The results are summarized in Fig. 9. Eight groups of length values are again evident and their mean values correspond closely to those found for the daughter segments of single strand-containing replicating molecules from the aqueous protein monolayer preparation of this DNA. The differences between the mean values of six of the eight apparently corresponding groups in Figs. 8 and 9 (the corresponding groups at approximately 4%, 11%, 16%, 20%, 30%, and 42% contour length) were not significant ($P = >0.05$ for each comparison). The probability that each of the differences between the mean values of the other two corresponding groups in the two histograms was due to chance alone was, however, less (for the corresponding groups at approximately 26% and 36% contour length, $P = <0.01$, >0.001, and $P = <0.05$, >0.01, respectively).

The sample in Fig. 9 comprised the three different kinds of single strand-containing double-forked molecules described above. The single-stranded regions of daughter segments which also contained double-stranded DNA varied from about 3–15% of the circular contour length. However, there were not sufficient data to determine

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**Figures 1–6** Electron micrographs of rotary-shadowed double-forked circular molecules of mtDNA from Novikoff rat ascites hepatoma cells. In each molecule two of the segments (A and B, the daughter segments) delimited by the forks (arrows) appear to be approximately equal in length while the third (C) is longer. The mean of the lengths of A or B plus the length of C is within the range of lengths of simple circular molecules in the respective protein monolayer preparations (see Fig. 7).

**Figure 1** A molecule prepared for electron microscopy by the aqueous protein monolayer technique. All three segments appear to be totally double stranded. $A + C = 4.90$ μm, $A + B = 1.19$ μm.

**Figures 2–6** Molecules prepared for electron microscopy by the formamide protein monolayer technique. All five micrographs × 66,500.

**Figure 2** All three segments appear to be totally double stranded. $A + C = 4.90$ μm, $A + B = 1.05$ μm.

**Figure 3** A region, accounting for about one-half of one of the daughter segments (B), and associated with the lower fork, has the thinner, low contrast appearance of single-stranded DNA (S). $A + C = 4.77$ μm, $A + B = 0.90$ μm, $C = 3.87$ μm.

**Figure 4** All of one of the daughter segments (B) appears to be single stranded. $A + C = 4.47$ μm, $B = 0.53$ μm.

**Figure 5** All of one of the daughter segments (B) appears to be single stranded. $A + C = 4.91$ μm, $B = 0.71$ μm. $A = 0.75$ μm, $B = 0.83$ μm, $C = 4.21$ μm, $A + C = 4.96$ μm. $A + C = 4.96$ μm.
In Fig. 9, single-stranded DNA accounted for approximately 16%, 19%, 37%, and 47% of the segments.

The distribution of lengths of the daughter segments of totally double-stranded replicating molecules in aqueous protein monolayer preparations was next examined. Owing to the lower frequency of replicating molecules of this class, data regarding them were accumulated from six different preparations. The results are shown in Fig. 10. The lengths of the daughter segments of these molecules were found to fall into seven groups with mean values corresponding closely to those of seven of the groups observed for single-strand-containing replicating molecules. A group of molecules with daughter segment lengths around a mean value of 26% contour length was not apparent. The difference between the mean values of only two of the seven apparently corresponding groups (at approximately 36% and 41% contour length) in Figs. 8 and 10 was not significant ($P = >0.05$ for both comparisons). The probability that each of the differences between the mean values of the other five corresponding groups (at approximately $4\%$, $11\%$, $16\%$, $21\%$, and $30\%$ contour length) was due to something other than chance alone was greater than 1 in 100. Whether these latter differences represent real differences in the mean daughter segment lengths in the two classes of replicating molecules, or result from technical variations in assessing these values in the two classes cannot be decided from the data. What is clear, however, is that all of these differences as well as those noted from similar comparisons made for molecules prepared for electron microscopy under different conditions (Figs. 8 and 9) are small compared to the differences between the means of adjacent groups in each of the histograms presented in Figs. 8, 9 and 10.

In order to test further whether it was likely that our observations could be explained by random groupings of daughter segment lengths, histograms were constructed for three sets of 173 random numbers ranging from 2 to 44 (taken from Fisher and Yates, 1963). The sample size and the range of values correspond, respectively, to the largest samples and the range of daughter segment lengths examined. Groups of numbers did occur within each set (Fig. 11) but the same groupings were clearly not repeated from one set to another.
**DISCUSSION**

Each form of replicating molecule described from Novikoff hepatoma mtDNA has also been found in mtDNA from normal chick embryos (Wolstenholme et al., 1973) and normal rat liver (Wolstenholme and Cochran-Fouts, unpublished observations). This supports the contention that the molecules in the hepatoma-derived sample are representative of normal mtDNA replication.

A general scheme for the replication of circular mtDNA, based upon the different molecular forms observed and modified from the scheme previously presented by us (Wolstenholme et al., 1973), is given in Fig. 12. Following evidence presented by Kasamatsu and Vinograd (1973) for the replication of double-sized circular molecules of mouse L-cell mtDNA, replication as a whole is indicated to be unidirectional (arrow by Fig. 12 a). Also, DNA synthesis is assumed to proceed locally only in the 5'-3' direction along a given strand (Okazaki et al., 1968; Mitra et al., 1967; Richardson, 1969; Inman and Schnös, 1971).

One sequence of events by which replication can occur entails a stepwise process (Fig. 12 a-e). A portion of one strand replicates and this is followed by replication of the equivalent portion of the complementary strand. A second mode of replication is represented in Fig. 12 a-k-l-e. At least 80% of one strand can replicate before replication of the complementary strand begins. Further, the daughter molecules can separate before replication of the strands is complete (Fig. 12 e-m and n).

Our present documentation of molecules of the form shown in Figs. 5 and 6 in which the daughter segment lengths are as small as 12% of the circular contour length indicates that replication does not always proceed strictly by the schemes outlined in Fig. 12 a-e or a-k-l-e. A possible scheme for replication which would include this form is given in Fig. 12 a-i-j-e.

Robberson et al. (1972) have examined the replicative forms of mtDNA from mouse L-cells. They found double-forked molecules in which one daughter segment was totally single stranded and varied from 3% to 100% of the circular contour length. They also found double-forked molecules in which a daughter segment was single stranded at both forks and double stranded in a central region. The latter molecules were only found, however, when the daughter segment length exceeded 55% of the circular contour length. Simple circular molecules containing a single stranded

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**Figure 8** Frequency distribution of the length of the totally double-stranded daughter segment (expressed as a percentage of the circular contour length) of 173 replicating circular mtDNA molecules in which the second daughter segment was totally or partially single stranded. The molecules were from a sample of Novikoff hepatoma mtDNA prepared for electron microscopy by the aqueous protein monolayer technique. The arrows in the top portion of the histogram indicate the means of the apparent groups. The standard errors of the means of the eight groups are from left to right: ±0.23, ±0.26, ±0.18, ±0.20, ±0.23, ±0.27, ±0.32 and ±0.31, respectively.
region were found, but totally double-stranded, double-forked molecules were rare (1 in 106 molecules examined) and double-forked molecules in which one daughter segment was single stranded at one fork only were apparently not observed. They suggested a scheme for a single mode of replication of mouse L-cell mtDNA which is basically similar to the scheme outline in Fig. 12 a-k-l-e-m and n-g and h. However, they interpret their observation to indicate that initiation of replication on the second strand does not begin before replication of one strand is at least 60% completed. The alternative modes of replication apparent from our data for mtDNA from rat and chick tissues must be used very rarely or not at all in mouse L-cell mtDNA.

The means of the groups of lengths of daughter segments of the different classes of replicating molecules, and of the same classes of replicating molecules in preparations made using different techniques closely correspond. These findings, together with the distributions of random numbers, support the argument that daughter segment lengths of replicating molecules of this mtDNA are distributed in groups.

The finding of length groups suggests that along at least 44% of the molecular contour, there are discrete positions on the strands at which DNA synthesis tends to be arrested. Such positions will be referred to as stop points. Whether DNA synthesis actually stops at these positions during the normal course of replication in vivo is not clear. It might be that DNA synthesis does stop at discrete positions on a strand, pauses for a time considerably greater than the time taken for synthesis of the sections of a strand between stop points, and then starts again. However, as the mtDNA used in our studies was isolated from mitochondrial fractions, a second explanation of the length groups is also possible. In the living...
organisms replication of different sections of the molecule might in fact be temporally continuous, the continuity being dependent upon the constant availability of certain factors. In this case our data would be explained if, when mitochondria were isolated under the particular conditions of our experiments, synthesis of a section of a strand which was in progress could be completed, but synthesis beyond a stop point could not be continued for want of "continuation factors" ordinarily supplied from outside the mitochondrion in the intact cell. Either this or the preceding explanation would account for why molecules of the form diagramed in Fig. 12c, which could represent incomplete synthesis of a section of a strand in the direction away from the replicating fork in the scheme a-e in Fig. 12, were never observed (see Wolstenholme et al., 1973).
The length group data are summarized in Fig. 13. The groups observed for totally double-stranded replicating molecules indicate that there are seven discrete points at which DNA synthesis tends to be stopped with both strands totally replicated. This suggests that there are seven not necessarily independent stop points at corresponding positions on the two complementary nucleotide strands which, assuming unidirectional replication, lie within 44% of the circular contour length from the origin. If, as has been demonstrated for mtDNA molecules from mouse L cells (Robberson et al., 1972), the single-stranded regions of double-forked molecules always represent the same particular strand of the mtDNA molecule, then the groups observed for this class alone indicate that there are eight discrete points on that particular strand at which DNA synthesis tends to be independently arrested. That the positions of seven of these stop points are the same as those of the seven stop points on both strands indicated by the groups of totally double-stranded replicating...
Figure 12. A possible general scheme for the replication of circular mtDNA molecules of rat tissues, based on the different molecular forms observed in the electron microscope. The thin lines represent the two parental nucleotide strands, and the thick lines represent newly synthesized strands. It is assumed that replication is unidirectional (arrow by a) from a unique origin.

Figure 13. The positions of stop points (expressed as a percentage of the circular contour length) on 41% of the circular molecule of Novikoff ascites hepatoma mtDNA. It is assumed that replication of the molecule as a whole is unidirectional from a unique origin. The two horizontal lines represent the complementary nucleotide strands of the molecule. Seven stop points are indicated to be at corresponding positions on the complementary strands. The position of each of these is the mean of the means of each of the corresponding groups of the two classes of molecules from aqueous preparations represented in Figs. 8 and 10. The position of the stop point shown on only one strand is the mean (36.2) of the grouping apparently unique to single strand-containing double-forked molecules (see Figs. 8 and 10). Only data from aqueous preparations were used to calculate these mean stop point positions since formamide preparations were used to obtain daughter segment length distributions from only one class of replicating molecule.

Figure 14. A scheme (a–e) for partial replication of the circular mtDNA molecule which takes into account the finding of daughter segment length groups which include molecules in which one daughter segment is totally single stranded. The thin lines represent the two parental complementary nucleotide strands; the thick lines represent newly synthesized strands. The numbers at the top of the diagram are the positions of stop points expressed as percentages of the circular contour length and molecules is supported by the correspondence in mean values of the groups of the two classes of replicating molecules. As a group of replicating molecules with a mean daughter segment length equal to 26% of the contour length was found only for single strand-containing molecules, it remains possible that a stop point at this position is unique to one of the nucleotide strands. With this latter exception, the sections synthesized between stop points on a strand would be between 0.22 μm and 0.33 μm in length. Such sections would contain between 650 and 950 nucleotides. This compares with 1,000–2,000 nucleotides for the units of discontinuous synthesis reported for Escherichia coli DNA by Okazaki et al. (1968).

Double-forked molecules in which one daughter segment is totally single stranded and equal to up to 44% of the circular contour length may result simply by sequential replication of sections between stop points of one strand as shown in Fig. 14. Following the general stepwise process for mtDNA replication outlined above (Fig. 12 a–e), a suggested mode of replication which takes into account the length groups for totally double-stranded molecules is shown in Fig. 15. Consistent with this model is our finding of some molecules of the form diagramed in Fig. 15 d and f, in which the total length of a daughter segment was between 7% and 41% of the circular contour length and...
the single-stranded region was approximately equal to the average distance between adjacent stop points. The finding that the single-stranded region of some molecules having this form and daughter segment length had lengths up to values equal to the distances between two or three stop points, together with the observation of molecules such as those in Figs. 5 and 6, indicate other possible sequences of new starts than those proposed in Figs. 14 and 15.

In regard to the present findings, it is of interest that from measurements of daughter segment lengths of double-forked molecules of kinetoplast DNA of Trypanosoma cruzi, produced by treatment with the trypanocidal drug Berenil, Brack et al. (1972) suggested that a discontinuous process is involved in the replication of this DNA. They interpreted their results as indicating modes of lengths at approximately 15% intervals of the 0.4-μm circular contour length of the molecule, and suggested that this indicated that replication could be blocked at specific points.

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