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Cleavage of Replicating Forms of Mitochondrial DNA by *Eco*RI Endonuclease

(restriction enzyme/electron microscopy/unidirectional DNA synthesis)

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ABSTRACT **Digestion of mouse L cell mitochondrial DNA with *Eco*RI restriction endonuclease produces two linear duplex fragments comprising 86.3 ± 2.0% and 14.2 ± 1.0% of the circular genome length (16,000 ± 470 nucleotide pairs). Digestion of human HeLa cell mitochondrial DNA with *Eco*RI produces three linear duplex fragments comprising 49.2 ± 1.0%, 44.4 ± 0.9%, and 6.4 ± 0.4% of the circular genome length (16,590 ± 710 nucleotide pairs). These fragments are shown to be generated by cleavage in unique regions of the mouse and human mitochondrial DNAs. An electron microscopic analysis of partially replicated molecules cleaved by *Eco*RI establishes a unidirectional mode of DNA replication for L cell mitochondrial DNA. The origin for DNA replication is located on the larger *Eco*RI fragment at a position that is 1,890 ± 250 nucleotide pairs (11.8 ± 1.2% of the circular genome length) from the proximal restriction site. Replication proceeds unidirectionally away from this restriction site throughout the length of the larger *Eco*RI fragment. Analysis of L cell, D-loop mitochondrial DNA cleaved by *Eco*RI indicates that a unique sequence is synthesized in formation of the D-loop in these nonreplicating molecules. The origin of D-loop synthesis is located on the larger *Eco*RI fragment at a position 1,760 ± 180 nucleotide pairs (11.0 ± 1.1% of the circular genome length) from the proximal restriction site and is, therefore, the origin for unidirectional displacement replication.**

Restriction endonuclease *Eco*RI introduces double-strand staggered cleavages at unique sites in a variety of DNAs (1, 2). Cleavage at a unique position on the genomes of simian virus 40 (3, 4) and polyoma virus (5) has provided an electron microscopic basis for assessing bidirectional replication from a unique initiation site on these DNAs (6, 7).

Although the chromosomal DNA of mammalian cells also replicates in a bidirectional mode (8, 9), the mtDNA purified from mouse LD cells appears to replicate unidirectionally (10, 11). There are two possible modes of unidirectional replication in which a growing fork can move either to the left or the right of a particular origin at which initiation has occurred. The mode of unidirectional replication that is operative in circular dimeric mtDNA was not defined by earlier studies (10–12) and may not be equivalent to the mode of replication in monomeric mtDNA. The replication of monomeric mtDNA has previously been assumed to proceed unidirectionally (10, 12). The existence of unique *Eco*RI cleavage fragments of mouse L mtDNA has now permitted examination of unidirectional DNA synthesis from a fixed origin on monomeric mtDNA.

Abbreviations: D-mtDNA, mtDNA that contains D-loops; Exp-D mtDNA, mtDNA that contains expanded D-loops.

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MATERIALS AND METHODS

Preparation of Mitochondria and mtDNA. Mitochondria and mtDNA were purified (10) from mouse L cells (LA9, LMTK$^-$) and human HeLa cells growing exponentially in suspension cultures. The closed circular mtDNA was fractionated from ethidium bromide-CsCl gradients (10). After removal of ethidium bromide (10), the separate fractions were dialyzed against a buffer composed of 0.1 M NaCl, 0.01 M Na_2EDTA, 0.05 Tris, pH 8.5 (buffer A) and stored at 4°.

EcoRI Cleavage of mtDNA. Samples of mtDNA were dialyzed at 4° against a solution of 0.1 M Tris·HCl, pH 7.7, at 25°, in which $MgCl_2$ had been dissolved to a final concentration of 0.01 M. One microliter of *Eco*RI was added to 70 μl of the dialyzed sample (containing 0.05–0.1 μg of mtDNA) and subsequently incubated for 15 min at 37°. An additional 1 μl of *Eco*RI was then added and the incubation continued for 15 min at 37°. Approximately 10 μl of 0.5 M Na_2EDTA, adjusted to pH 8 with NaOH, was added and the samples dialyzed against buffer A at 4°. One microliter of this sample of *Eco*RI, prepared by the method of Yoshimori (13), has been observed to cleave approximately 1 μg of simian virus 40 DNA in 15 min under similar reaction conditions (3).

Electron Microscopy. Before treatment with *Eco*RI, samples of mtDNA were examined by the formamide modification (14) of the Kleinschmidt technique (12). Samples of mtDNA treated with *Eco*RI were examined either by the aqueous Kleinschmidt technique (14) performed at 23° (15) or at 6.5° (1) or dialyzed against 50% formamide, 0.01 M Na_2EDTA, 0.1 M Tris·HCl, pH 8.5 at 4°. Cytochrome *c* (1 mg/ml) was added to the dialyzed sample to a final concentration of 0.05 mg/ml and the sample spread (12). At all stages of the latter procedure the sample was kept at <4° prior to spreading. In experiments in which mtDNA that contains D-loops (D-mtDNA) or mtDNA that contains expanded D-loops (Exp-D mtDNA) was analyzed, the total time for mounting the DNA sample did not exceed 30 sec.

Homoduplexes of *Eco*RI treated mtDNA were constructed (5, 14) and the renaturation conditions were chosen to permit reassociation of approximately 40% of the largest single-strands in a particular sample (5). The samples were then prepared for electron microscopy as described above.

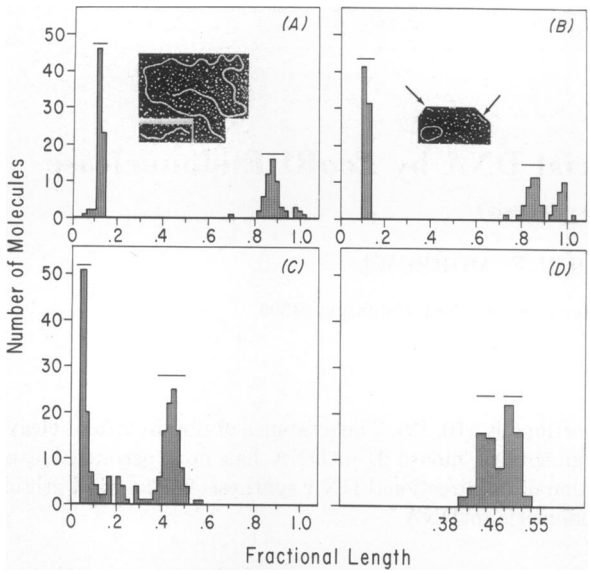

FIG. 1. Frequency distributions of *Eco*RI fragment lengths. The lengths of fragments, expressed as a fraction of the circular monomer genome, were determined for LMTK$^-$ (*A*), LA9 (*B*) and HeLa (*C*) mtDNAs. A portion of the plot in *C* has been expanded for presentation in (*D*). The bars indicate the intervals over which average lengths have been calculated. Micrographs inset in (*A*) illustrate the appearance of linear fragments derived by *Eco*RI digestion of LMTK$^-$ mtDNA. Micrographs inset in (*B*) illustrate the appearance of the smaller fragment upon cyclization and of fragments 150–300 nucleotide pairs in length which have not cyclized (indicated by arrows). Contrast has been reversed in printing.

Grids were rotary shadowed with Pt-Pd and examined in a Philips 300 electron microscope. Length measurements of the molecules were performed either with a map measure or with the aid of the Hewlett-Packard 9864A digitizing tablet and 9810A computer.

Glutaraldehyde Treatment of mtDNA. Samples of D-mtDNA were dialyzed against 50% formamide, 0.1 M KH_2PO_4, previously adjusted to pH 7.3 by the addition of KOH. Glutaraldehyde was then added to the solution to a final concentration of 0.01 M and the sample incubated at 23° for 15 min. The solution was then dialyzed exhaustively against 0.01 M $MgCl_2$, 0.1 M Tris·HCl, pH 7.6, at 4° in preparation for treatment with *Eco*RI as described above.

RESULTS

Sizes of *Eco*RI mtDNA fragments

*Eco*RI digestion of mtDNA purified from mouse LMTK$^-$ and LA9 cells converts more than 95% of the circular molecules to linear duplex forms which possess no electron microscopic evidence of single-strand termini (Fig. 1*A*). Length distributions for the populations of linear fragments (Figs. 1*A* and *B*) display two major components which have average lengths comprising 86.3 ± 2.0% and 14.2 ± 1.0% of the mouse mtDNA genome (Table 1). The sum of these two fragment lengths is within the error of measurement for the average length determined for mouse mtDNA before *Eco*RI cleavage (Table 1). The standard deviations of measured lengths are close to those expected for fragments which have these average lengths and are homogeneous in size (14) and indicate that LMTK$^-$ and LA9 mtDNAs contain equimolar quantities of these two fragments. This was experimentally verified by scoring each molecular form on defined regions of an electron microscope grid.

Although the reaction conditions were established as being exhaustive for *Eco*RI cleavage of simian virus 40 DNA (3), a fraction of the mouse mtDNA appears to be incompletely digested and is present as linear molecules that are slightly shorter than the circular monomer genome (Figs. 1*A* and *B*). Very short duplex fragments were also present with lengths of 150–300 nucleotide pairs (Fig. 1*B*). These smaller fragments were infrequently seen in spreadings of *Eco*RI in the absence of mtDNA as well as spreadings of mtDNA in the absence of *Eco*RI. Although the major *Eco*RI fragments were observed to cyclize in aqueous spreadings at 23° and 6.5° (Fig. 1*B*) (1), these very small fragments do not form circles under these conditions (Fig. 1*B*) and, therefore, do not have both ends generated by *Eco*RI cleavage. It is possible that there is a cleavage site(s) for a minor nuclease contaminant in the *Eco*RI preparation that reacts near one or more of the *Eco*RI endonuclease cleavage sites.

*Eco*RI digestion of mtDNA purified from HeLa cells converts the circular molecules to duplex linear fragments which have average lengths representing approximately 47% and 6.4 ± 0.4% of the human mitochondrial genome (Fig. 1*C*). The length distribution for the largest fragments from this digestion (Fig. 1*C*) corresponds to two populations of fragments (Fig. 1*D*) with average lengths comprising 49.2 ± 1.0% and 44.4 ± 0.9% of the mitochondrial genome (Table 1). The sum of these three fragment lengths is within the error of measurement for the average length determined for human mtDNA prior to *Eco*RI cleavage (Table 1). These results indicate that HeLa mtDNA contains equimolar quantities of these three fragments. Although digestion of HeLa mtDNA appears to be complete (Fig. 1*C*), very short duplex fragments were also present with lengths of 150–300 nucelotide pairs. In analogy with the short fragments in *Eco*RI treated mouse mtDNA, these duplex segments were not observed to cyclize at 23° or 6.5°.

*Eco*RI cleavage sites occur in unique regions of mtDNA

Cleavage of mouse mtDNA in two unique regions of the genome is demonstrable by the formation of denatured-renatured duplex segments equivalent in size to the undenatured *Eco*RI fragments. The length distribution of reassociated *Eco*RI fragments of LMTK$^-$ mtDNA (Fig. 2*A*) displays two major components with average lengths of 13,700 ± 620 and 1,890 ± 230 nucleotide pairs. These fragments thus comprise 85.6 ± 3.8% and 11.8 ± 1.4% of the circular genome length (Table 1). The sizes of these renatured segments correspond to the lengths of *Eco*RI fragments of LMTK$^-$mtDNA (Table 1) within the error of our measurements and require that the cleavages introduced by the enzyme digestion occur in two unique regions of this mitochondrial genome within a space of approximately 600 nucleotide pairs. Furthermore, the majority of renatured strands possess no evidence of long single-strand termini. Infrequently, a renatured duplex will have one clean end and one single-strand end (Fig. 2*A*). A single-strand segment at a terminus is generally detectable if the length of this segment is greater than 200 nucleotide pairs.

In a similar manner, the denatured-renatured product of *Eco*RI treated HeLa mtDNA displays a length distribution of duplex segments (Fig. 2*B*) compatible with the double-strand cleavages in three unique regions of this mitochondrial genome. The average length of the largest duplex segments

TABLE 1. *Sizes of mitochondrial DNAs before and after treatment with EcoRI**

Source of mtDNA	Length of circular genome†	Length of fragment 1	2	3
LMTK$^-$	16,000 ± 470	13,810 ± 320	2,270 ± 160	—
LA9	16,040 ± 550	13,880 ± 400	1,950 ± 120	—
HeLa	16,590 ± 710	8,170 ± 170	7,370 ± 150	1,070 ± 70

* Expressed as the number of nucleotide pairs determined relative to PM2 viral DNA (9900 nucleotide pairs).
† Determined for measurement of 25–35 molecules.

(7,400 ± 450 nucleotide pairs) is to be identified with the mean length of the two largest HeLa mtDNA fragments (Table 1) prior to denaturation–renaturation (Fig. 1*C*). The average length of the smallest renatured fragment (1,220 ± 220 nucleotide pairs) corresponds to the length of the smallest *Eco*RI fragment of HeLa mtDNA (Table 1). These two classes of denatured-renatured fragments comprise 44.6 ± 2.7% and 7.4 ± 1.3% of the HeLa mitochondrial genome.

In contrast to LMTK$^-$ mtDNA, a substantial portion of these renatured duplexes have lengths of approximately 25% of the genome. This length is unexpected for the three unique double-strand cleavages in the HeLa mitochondrial genome (Fig. 2*B*). Approximately 70% of the larger renatured duplexes (130 molecules examined) possess clean ends (Fig. 2*B*). The remaining 30% of the renatured duplexes contain one clean end and a single-strand segment as the other terminus (Fig. 2*B*). The length of the single-strand region in such molecules was variable. The combined results indicate that a few additional single-strand cleavages have occurred in HeLa and mouse mtDNAs, although their presence is more apparent in the renatured *Eco*RI fragments of human mtDNA.

Unidirectional replication from a unique origin in mouse mtDNA

*Eco*RI cleavage in two unique regions of mouse mtDNA provides a basis for examination of the mode of DNA replication. Before enzyme digestion, replicative forms that contain D-loops and expanded D-loops are prevalent among the population of closed circular molecules (10) (Table 2). Upon cleavage with *Eco*RI and subsequent examination by the formamide technique for electron microscopy (14), the frequency of resulting duplex linear molecules that contain a D-loop is substantially lower than the initial frequency of closed circular D-mtDNA (Table 2). The frequency of linear molecules that contain an expanded D-loop (Fig. 3) is similar to the frequency of closed circular Exp-D mtDNA before enzyme cleavage (Table 2). The smaller displacing strand in the linear form of D-mtDNA is probably lost through the process of branch migration (16) when the linear or nicked circular forms are incubated during the *Eco*RI digestion or in the spreading medium for electron microscopy (17). Branch migration of the longer displacing strands of linear Exp-D mtDNA appears to be slower.

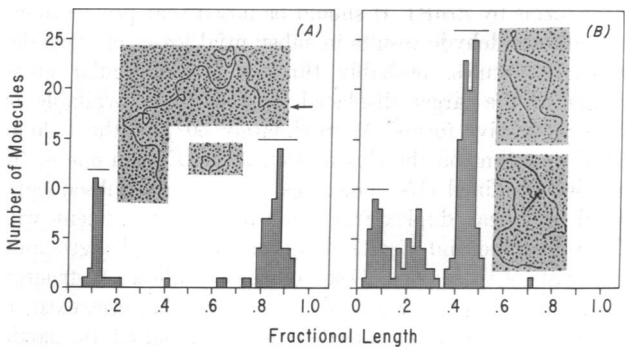

FIG. 2. Frequency distributions of *Eco*RI fragment lengths after denaturation–renaturation of LMTK$^-$ (*A*) and HeLa (*B*) mtDNA restriction fragments. Micrograph inset in (*A*) illustrates the appearance of the larger *Eco*RI fragment of LMTK$^-$ mtDNA after denaturation–renaturation. A small single-strand segment occurs at one end, indicated by the arrow. The small micrograph inset in (*A*) is a single-strand fragment for comparative purposes. Micrographs inset in (*B*) illustrate the appearance of the larger *Eco*RI fragments of HeLa mtDNA after denaturation–renaturation which have two clean ends (at top) or one clean end (bottom). The junction between double and single strands is indicated by the arrow.

Length measurements of linear replicative forms derived by *Eco*RI cleavage provide an array of molecules with increasing degrees of replication (Fig. 3). Alignment of these forms (Fig. 3) indicates that initiation of displacement replication occurs on the larger *Eco*RI fragment of LMTK$^-$ mtDNA (Table 1) at a position on the genome 1,890 ± 250 nucleotide pairs from the proximal restriction site. DNA replication proceeds unidirectionally away from this restriction site throughout the length of the larger *Eco*RI fragment (Fig. 3). The opposing mode of unidirectional replication initiated at this origin would at first proceed through a region of the genome that contains the *Eco*RI cleavage sites (Fig. 4). Cleavage of these replicative forms by *Eco*RI (Fig. 4*B*) and subsequent dissociation of the smaller *Eco*RI fragment (Fig. 4*C*) will produce a gapped circular molecule with double-strand tails at the junctions of the gap (Fig. 4*C*). Branch migration at these forks (Fig. 4*C*) will

TABLE 2. *Frequencies of replicative forms after cleavage with EcoRI and incubation at T_m–25°*

Replicative form	Frequency (%)*	Molecules classified (no.)
Circular D-mtDNA	1.5 (55.0)†	12
Linear D-mtDNA	1.3 (0.0)	10
Circular Exp-D mtDNA	0.0 (2.0)	—
Linear Exp-D mtDNA	2.4 (0.0)	19
Gapped circular mtDNA	0.6 (0.5)	15
Clean circular mtDNA	2.4 (42.5)	19
Clean linear mtDNA	93.0 (0.0)	726

* It should be noted that glutaraldehyde fixation was not employed in this experiment.
† Numbers in parentheses refer to frequencies of the indicated replicative forms prior to cleavage with *Eco*RI. Approximately 150 molecules were classified in this predigest analysis.

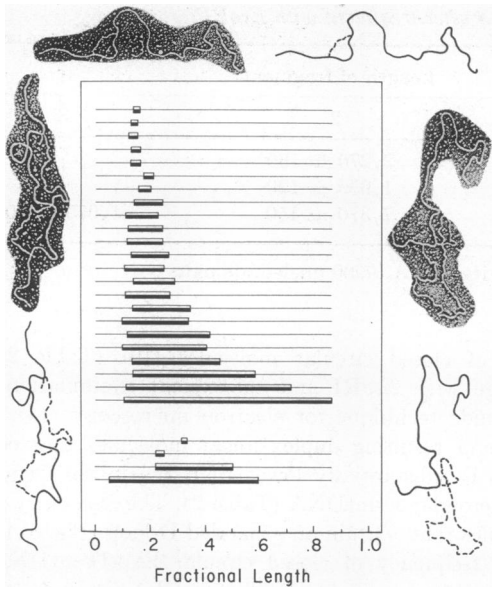

FIG. 3. Array of linear displacement replicative forms derived by *Eco*RI cleavage of LMTK$^-$ mtDNA. The extent of replication increases from top to bottom. Synthesis of a displacement loop is initiated on the larger *Eco*RI fragment at 11.8 ± 1.2% of the circular genome length from the proximal restriction site (at the left) and proceeds to the right throughout the length of this *Eco*RI fragment. The group of four molecules in the lower part of this array have initiation sites which are greater than ±1 standard deviation for measurements of the entire population. Micrographs and accompanying line drawings (with single-strand segments as dashed lines and duplex segments as solid lines) illustrate linear replicative forms with synthesis of 16% (top), 74% (left), and 96% (right) of the circular genome length. Branch migration is evident at one fork of the linear replicative form shown at the left.

release two short single strands of DNA and result in the formation of a gapped circular molecule (Fig. 4*D*). The length of the single-strand gap will correspond to the length of the smaller *Eco*RI fragment of LMTK$^-$ mtDNA (approximately 14% of the circular genome length). Examination of the replicative forms of LMTK$^-$ mtDNA after cleavage with *Eco*RI revealed no structures of the type described in Fig. 4*C*. Furthermore, only one gapped circular molecule (about 5%) was found in which the length of the single-strand gap approximated the length of the smaller *Eco*RI fragment (Table 3). Thus, unidirectional replication that opposes the mode described in Fig. 3 must occur in less than 5% of these mtDNA replicative intermediates, if at all. The presence of gapped circular molecules containing one or more gap(s) (Table 3) probably reflects a small contaminant of these forms from

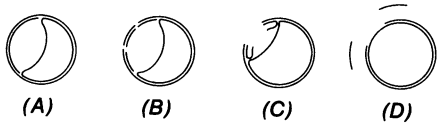

FIG. 4. Displacement replication (*A*) that proceeds through the *Eco*RI cleavage sites (*B*) of LMTK$^-$ mtDNA (to the left in Fig. 3) will produce circular forms with two duplex tails (*C*) upon cleavage with *Eco*RI. Branch migration (*C*) will result in the formation of two single-strands and a gapped circular molecule (*D*). The size of the single-strand gap in (*D*) will be equivalent to the size of the smaller *Eco*RI fragment of LMTK$^-$ mtDNA (*B*) which has been removed from the molecule upon cleavage and incubation at T_m–25°.

TABLE 3. *Gapped circular mtDNA apparent after cleavage with EcoRI and incubation at T_m–25°*

Number of circular molecules classified	Number of single-strand gaps detected	Length of single-strand gap‡
13	0†	0
11	1	0.014 0.061 0.127 0.851 0.036 0.067 0.285 0.932 0.052 0.070 0.317
3	2	(0.034, 0.059) (0.090, 0.213) (0.048, 0.163)
5	>2	—

* Prior to *Eco*RI treatment, 0.5% of the molecules in this sample occurred as gapped circular mtDNA (Table 2).

† These molecules either contain a single-strand gap(s) too small to have been detected or possess no sequences cleaved by *Eco*RI.

‡ Expressed as a fraction of the duplex circular contour length of mtDNA.

the intermediate or upper band portions of the ethidium bromide-CsCl gradient (10).

A unique initiation site for D-loop synthesis in monomer mtDNA

As previously noted, *Eco*RI cleavage of mtDNA and incubation at T_m–25° in formamide (spreading conditions for electron microscopy) results in a substantial loss of D-loop structures. This is presumably mediated through a branch migration process that is possible for the linear or nicked circular molecule (17) but prohibited in the covalently closed circular molecule. Chemical modification of the free bases on the displaced strand of the D-loop by reaction with glutaraldehyde is expected to inhibit the branch migration process after cleavage of D-mtDNA with *Eco*RI. This allows a larger proportion of D-mtDNA to be examined after conversion to linear forms by *Eco*RI. It should be noted that pretreatment with glutaraldehyde results in substantial losses of expanded D-loop molecules, probably through intermolecular cross-linking of the larger displaced single-strands available on these replicative forms. Approximately 50% of the D-loops initially present on the closed circular mtDNA in one of the samples examined (150 molecules scored) were subsequently found on linear duplex fragments after pretreatment with glutaraldehyde and *Eco*RI digestion (Fig. 5). Length measurements of these linear D-loop molecules allow construction of the array shown in Fig. 5. We have noted that pretreatment of the DNA with glutaraldehyde has resulted in partial *Eco*RI digestion of the LMTK$^-$ D-mtDNA (Fig. 5). This permits alignment of these forms at the two *Eco*RI cleavage sites of LMTK$^-$ mtDNA and provides a unique basis for positioning the origin of D-loop synthesis at 1,760 ± 180 nucleotide pairs from the proximal restriction site. This position on the mouse mitochondrial genome is thus identified with the origin for unidirectional displacement replication (Fig. 3) within these errors of length measurement.

DISCUSSION

The existence of two unique *Eco*RI cleavage regions on the mouse mtDNA genome has provided unequivocal assign-

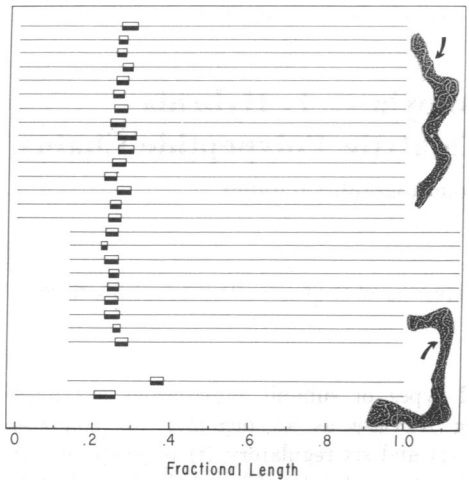

FIG. 5. Array of D-loop molecules of LMTK$^-$ mtDNA pretreated with glutaraldehyde and cleaved by *Eco*RI. Partial digestion results in molecules cleaved once at the *Eco*RI site which is distal (top part of the array) or proximal (center part of array) to the D-loop. Some molecules are cleaved at both *Eco*RI sites (lower part of array). The two molecules at the bottom have initiation sites for D-loop synthesis which are greater than ±1 standard deviation for measurements of the entire population. Synthesis of the D-loop is initiated 11.0 ± 1.1% of the circular genome length from the proximal restriction site. Micrographs illustrate the linear D-loop molecules with a single *Eco*RI cleavage at the restriction site proximal (top) or distal (bottom) to the D-loop (indicated by the arrows).

ment of the origin and direction of DNA synthesis. For mtDNA purified from LMTK$^-$ cells, the origin of displacement replication occurs on the larger *Eco*RI fragment (comprising 86.3 ± 2.0% of the mitochondrial genome) and is positioned 1,890 ± 250 nucleotide pairs from the proximal restriction site. Displacement replication proceeds unidirectionally away from this restriction site throughout the length of the larger *Eco*RI fragment (Fig. 3). D-mtDNA which has been pretreated with glutaraldehyde to prevent branch migration of the 7S DNA displacing strand and consequent loss of the D-loop structure, is partially sensitive to cleavage by *Eco*RI. The chemical basis for this partial sensitivity is not presently understood but has been used to advantage in orienting linear D-mtDNA. This analysis (Fig. 5) permits assignment of the origin for D-loop synthesis at 1,760 ± 180 nucleotide pairs from the proximal restriction site in correspondence with the position determined for the origin of continued displacement replication (Fig. 3). The three major *Eco*RI fragments of HeLa mtDNA comprise 49.2%, 44.4%, and 6.4% of the genome and are clearly distinguished from the pattern of fragmentation observed for mouse mtDNA.

In contrast to the complete *Eco*RI digestion of HeLa mtDNA, a fraction of LMTK$^-$ (Fig. 1*A*) and LA9 (Fig. 1*B*) mtDNA is cleaved in only one region of the genome to produce linear duplexes which are slightly shorter than the circular genome length. Since we have used large excesses of enzyme in these digestions, these latter mtDNAs appear resistant to further cleavage by *Eco*RI. This resistance to cleavage could reflect deletion or alteration of part of the *Eco*RI specific nucleotide sequence (2). Within the unique regions of *Eco*RI cleavage on mouse and human mtDNA, reiteration of the *Eco*RI sequence may occur, since we cannot define these regions with a resolution greater than the error inherent in length measurements of the fragments derived from exhaustive digestions (Table 1).

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