

## Replication of Mitochondrial DNA in Mouse L Cells and Their Thymidine Kinase<sup>-</sup> Derivatives: Displacement Replication on a Covalently-Closed Circular Template

(electron microscopy/mouse DNA/density gradient centrifugation)

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**ABSTRACT** Thymidine kinase<sup>-</sup> lines of mouse L cells incorporate thymidine exclusively into mitochondrial DNA. This fact permits assessment of labeled mitochondrial DNA components in ethidium bromide-cesium chloride gradients. Contaminating nuclear DNA is unlabeled and need not be removed. Elimination of the DNase treatment of purified mitochondria reveals that the replicative forms that exhibit displacement replication up to full genome size are covalently-closed circular molecules. Denaturation followed by brief renaturation of these larger replicating molecules produces closed-circular DNA with a deficiency of Watson-Crick turns, appearing as single-strand loops. This result suggests that displacement replication proceeds with nicking and rapid closure of the covalently-closed circular template.

Thymidine kinase<sup>-</sup> lines of mouse L cells incorporate exogenous thymidine exclusively into the mitochondrial DNA (mtDNA) of the cells (1), and they contain thymidine kinase only in their mitochondria (Berk, A. J. & Clayton, D. A., submitted). These aspects of thymidine kinase<sup>-</sup> cells permit detection of thymidine-labeled mature mtDNA and replicative intermediates (2-4) in ethidium bromide-CsCl gradients in the presence of unlabeled nuclear DNA.

### MATERIALS AND METHODS

**Cells.** The thymidine kinase<sup>-</sup> counterpart of LA9, LMTK<sup>-</sup>, and the thymidine kinase<sup>-</sup> derivative of the circular dimer containing line LD, LDTK<sup>-</sup>, were isolated by growth in increasing amounts of BrdU (5). LMTK<sup>-</sup> has been used in earlier studies (1). The LDTK<sup>-</sup> line is introduced here. The cells were grown as described (1).

**Preparation of mtDNA.** Cells labeled with [<sup>3</sup>H]thymidine (0.5  $\mu$ Ci/ml, 3 Ci/mmol) were harvested in log phase at densities of 3 to 7  $\times 10^5$ /ml. Stationary phase is reached under these growth conditions at 1.3  $\times 10^6$  cells per ml. The cells were washed with 30 volumes of reticulocyte standard buffer (RSB) (10 mM NaCl-1.5 mM MgCl<sub>2</sub>-10 mM Tris·HCl, pH 7.5) and then suspended in RSB at a ratio of 20 ml/0.5 ml of wet packed unswollen cells. Swelling was allowed to continue for 5 min, after which the cells were broken by 7-15 passages in a Dounce homogenizer. 2.0 M sucrose in RSB was then added to bring the final concentration to 0.28 M, and nuclei were removed by centrifugation at 2500 rpm in a Sorvall GLC-1 for 5 min. Mitochondria were then purified by one-step (3) or two-step (1) sucrose gradients, collected from

the interface of these gradients, suspended to 1 ml in 0.5 M NaCl-50 mM Tris-10 mM EDTA, pH 8.5, and lysed by addition of 1 ml of 2% sodium dodecyl sulfate dissolved in the same buffer warmed to 37°. There is no apparent difference in results obtained from the one-step or two-step sucrose gradients. Incubation at 37° for 3 min completed the lysis. Solid CsCl was added to a final density of 1.55 g/ml, and ethidium bromide was added to a final concentration of 350  $\mu$ g/ml. The samples were centrifuged in a polyallomer tube in an SW50.1 rotor for 18 hr at 38,000 rpm at 20°, after which the contents of the tube were poured into a second polyallomer tube. The volume was brought to 3.3 ml by addition of 4.5 M CsCl containing 350  $\mu$ g/ml of ethidium bromide and the mixture was centrifuged at 30,000 rpm for 44 hr at 20°. In some gradients, radioactive simian virus 40 (SV40) DNA was used as a position marker (gift from M. DePamphilis). The gradient was collected into 70- $\mu$ l fractions and ethidium bromide was removed by dialysis against Dowex 50 resin in STE (0.1 M NaCl-10 mM EDTA-50 mM Tris, pH 8.5) as described (3).

Early in this work we discovered a pronounced effect of the DNase/RNase treatment of the purified mitochondria used previously to remove contaminating nuclear DNA (2, 3). Incubation of the purified mitochondria from LMTK<sup>-</sup>, LDTK<sup>-</sup>, or LA9 cells in the DNase/RNase buffer alone causes a loss of radioactivity from an equal quantity of mitochondria as well as a reduction in the ratio of the radioactivity of the lower and upper band (LMTK<sup>-</sup>, LDTK<sup>-</sup>). We have accordingly eliminated this step in the purification procedure.

**Samples for Electron Microscopy** were prepared and analyzed by the formamide modification (6) of the Kleinschmidt technique with the minor modifications described (2). For this work, the subunits of catenated forms were scored as monomers (LMTK<sup>-</sup>) or circular dimers (LDTK<sup>-</sup>). Elimination of DNase treatment leads to an increase in linear nuclear DNA in the upper band. It therefore requires more time to score the circular population in such mixes, but we have not found that long linear DNA presents any special problems in identification.

**Autoradiography.** Cells were grown on glass slides in plates and incubated with 10 ml of medium containing 0.1  $\mu$ Ci/ml of [<sup>3</sup>H]thymidine. After 48 hr (about three cell divisions), the slides were fixed in acetic acid-methanol 1:3 and prepared for autoradiographic analysis. The nuclear TK<sup>-</sup> character of LMTK<sup>-</sup> and LDTK<sup>-</sup> is evident in autoradiograms of these

Abbreviations: mtDNA, mitochondrial DNA; SV40, simian virus 40; D-mtDNA, mtDNA containing D-loops; DL-mtDNA, mtDNA with denaturation loops; Exp-D DNA, expanded D-loop DNA; Gpc DNA, gapped circular DNA.

two lines. No grains over nuclei were detected in examination of 500 cells. In striking contrast to this, 494 of the 500 nuclei examined in LA9 and LD cells, which are nuclear TK<sup>+</sup>, contained many grains.

## RESULTS

### Distribution of monomer replicative forms in ethidium bromide-CsCl buoyant gradients

Ethidium bromide-CsCl buoyant density profile of [<sup>3</sup>H]thymidine incorporated into mtDNA of LA9 cells (Fig. 1A) shows that about 40% of the tritium is in the upper band and represents mostly nuclear DNA contaminant, as judged by comparison with the corresponding profile for mtDNA isolated from the LMTK<sup>-</sup> line (Fig. 1B). The positions of fractions pooled for each sample (lower, intermediate, and upper) is shown. MtDNA used for electron microscopic analysis was prepared from cells labeled for 36 hr by three separate additions of [<sup>3</sup>H]thymidine to insure that the radioactivity accurately reflected the mass of mtDNA. This procedure allows calculation of frequencies of each mtDNA form in the total population. Table 1 shows that about 80% of the mtDNA molecules in the lower band of this preparation contained D-loops (D-mtDNA). The average size (weight average distance between the forks) of the D-loop in these molecules was  $4.3 \pm 1.5\%$  of the monomer genome, which is larger than reported previously (3) because the majority of forks in our preparations contained single-strand regions. The lower band also contained about 2% of molecules with expanded D-loops,

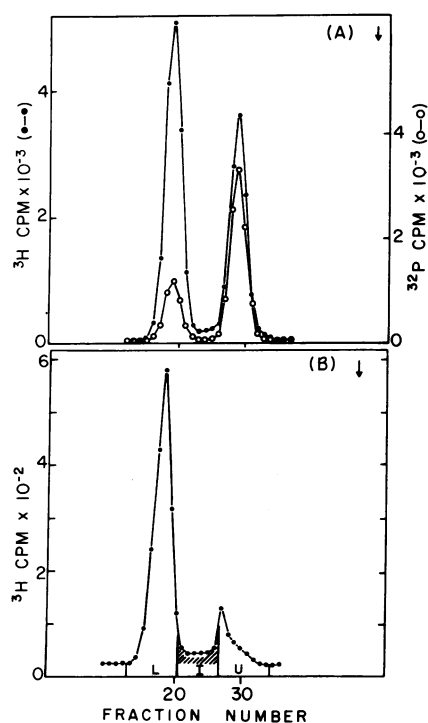


FIG. 1. (A) Ethidium bromide-CsCl density gradient profile of mtDNA from LA9 cells labeled with [<sup>3</sup>H]thymidine (0.17  $\mu$ Ci/ml added at 36, 24, and 12 hr before harvest), ( $\bullet$ — $\bullet$ ). <sup>32</sup>P-labeled SV40 DNA ( $\circ$ — $\circ$ ) was added before banding. (B) Gradient profile of mtDNA from LMTK<sup>-</sup> cells labeled for 9 hr. This profile illustrates the typical positions of lower (L), intermediate (I), and upper (U) regions of the gradient of mtDNA used for electron microscopy. Arrow indicates meniscus of each gradient.

(Exp-D DNA), which were absent at this position in the gradient in the previous study (2). The intermediate region was substantially enriched for these expanded D-loop molecules (Table 1), with length distributions representing expansions up to a full genome complement. Confinement of these molecules to lower and intermediate regions of the gradients suggests that expanded D-loop molecules are covalently-closed circles. A similar distribution has been reported for partially replicated SV40 DNA (8, 9), and kinetoplast DNA (10). The intermediate region is also enriched for gapped circular molecules (Gpc DNA). The length distribution for these gapped circular molecules demonstrates that a substantial fraction contains a large single-strand region of  $>0.1$  of a genome. These molecules have been previously found in the upper band (2).

The upper band from this same preparation contains very few of the larger expanded D-loop molecules (Table 1). The predominant replicative form in this band is a gapped circular molecule containing a small region of single-stranded DNA. The length distribution for these gapped circular molecules in the upper band demonstrates that the gap of single-stranded DNA is generally  $<0.1$  of a genome. The average gap size in molecules containing one single-strand gap of  $<0.1$  of a genome is 2.7% of the monomer length. Ten molecules in this interval possessed two gaps; the combined lengths of these gaps were, on the average, 3.5% of the monomer length. If these molecules were not simply damaged by nonspecific nuclease action, they indicate that duplex synthesis on the complementary strand is discontinuous.

In the previous study (2, 3) the large expanded D-loop molecules were in the upper band of such gradients. This probably resulted from the method of purification of mitochondria used in that study. Similar frequencies and partitioning of the replicative forms are observed in these three regions of the ethidium bromide-CsCl gradient for mtDNA isolated from LA9 cells (Table 1). With the exception of D-mtDNA, the frequencies and length distributions of the

TABLE 1. Frequencies of replicating forms in lower, intermediate, and upper bands of ethidium bromide-CsCl gradients containing LMTK<sup>-</sup> or LA9 mtDNA

Region*	Lower (L) [70]†	Intermediate (I) [6]	Upper (U) [24]			
	% Duplex circles					
Cell line:	LMTK-	LA9	LMTK-	LA9	LMTK-	LA9
With D-loops	82	71	35	36	9	6
With single-strand tails	1	1	0	0	0	2
With expanded D-loops	2	2	24	14	1	1
With gaps	0	0	21	10	33	39
Clean	15	26	20	40	57	52
No. of molecules classified	138	136	120	127	121	211

\* See Fig. 1B.

† Percent of total [<sup>3</sup>H]thymidine counts in indicated region of gradient determined for two preparations of mtDNA isolated from LMTK<sup>-</sup>. 0.17  $\mu$ Ci/ml of [<sup>3</sup>H]thymidine was added to the cells at 36, 24, and 12 hr before harvest.

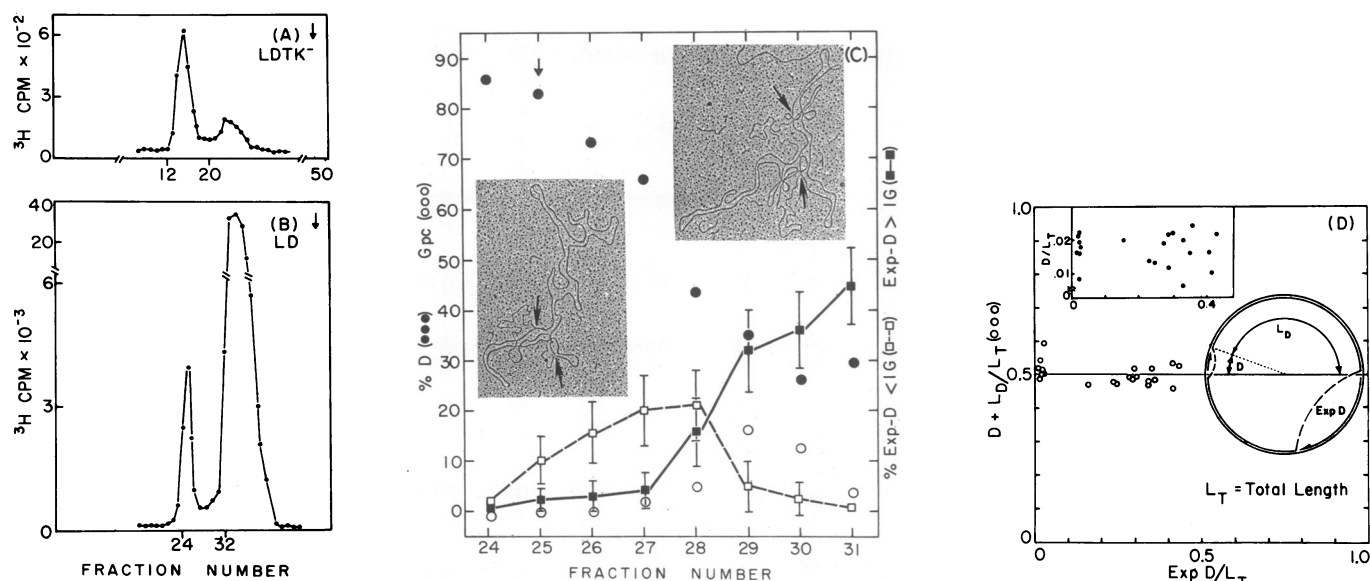


FIG. 2. Ethidium bromide-CsCl density gradient profile of tritium incorporated in LDTK<sup>-</sup> (A) and LD (B) mtDNA preparations. Arrow indicates meniscus of gradient. (C) Frequencies for circular dimer D-mtDNA. (D) (●), gapped circular mtDNA (*Gpc*) (○), expanded D-mtDNA <1 genome (*Exp-D* <1 G) (□), and expanded D-mtDNA >1 genome (*Exp-D* >1 G) (■) in fractions 24–31 of LD gradient. About 130 molecules were analyzed in each fraction. Error bars indicate the interval that contains the true mean at a level of confidence of 95%. Grids were prepared to contain about 20 circular forms per grid square. Fractions 27–31 are more heavily contaminated with linear DNA, as expected, since these fractions are nearer the upper band. The peak fraction of the lower band is indicated by the arrow. The insert on the left displays an expanded D-loop molecule with expansion <1 genome. The insert on the right displays an expanded D-loop molecule with expansion >1 genome. Arrows in the inserts indicate the expanding D-loops. (D) Directional mode of displacement synthesis in circular dimer expanded D-loop molecules selected from fractions 25, 26, and 27 of the LD gradient. Circular dimer expanded D-loop molecules with one expanding D-loop and one nonexpanding D-loop contain two unreplicated duplex segments. If the D-loop expands unidirectionally, then the fractional length of the longer unreplicated duplex segment ( $L_D$ ) plus the replicated segment of the nonexpanding D-loop ( $D$ ) will be equal to one-half the total length of the parental molecule ( $L_T$ ) independent of the degree of expansion. The fractional lengths for 21 molecules are plotted as a function of the fractional length of the expanding D-loop. Inset contains a plot of the fractional lengths of the corresponding smaller nonexpanding D-loops on these same circular dimer expanded D-loop molecules. These molecules satisfy the additional requirement that the length of the displaced strand in the expanding D-loop (dashed) be about equal to the duplex segment in expanding D-loop.

monomer replicative forms are substantially the same as previously described (2, 3), although their position in the ethidium bromide-CsCl gradient is different.

#### Distribution of closed-circular expanded D-loop molecules in ethidium bromide-CsCl buoyant gradients

In both cell lines the qualitative observation was made that expanded D-loop molecules in the intermediate region had expanded through a larger portion of the genome than those in the lower region. This observation suggests that expansion of the D-loop leads to greater dye uptake. Rather than measuring the amount of expansion on many molecules from each fraction, we developed a simpler method of assessing the amount of expansion, using LD cells.

MtDNA from LD cells contains more than 90% circular dimer molecules (1–3). In two separate preparations of LD mtDNA from the lower band, 91 and 86% of the dimer molecules contained D-loops, and most of these D-loop molecules (67% and 81%) possessed two D-loops diametrically opposed on the 10- $\mu\text{m}$  circle. These frequencies are considerably higher than reported previously (3). Examination of the lower and intermediate regions of these preparations by electron microscopy showed that all but one of the 50 expanded D-loop molecules examined with expansions <1 genome possessed a second small D-loop. Expanded D-loop molecules with expansions >1 genome cannot have a second

small D-loop because the origin of displacement replication and the second D-loop are 1 genome apart, and displacement replication is unidirectional. Continued displacement replication on these circular dimer molecules proceeds by expansion of only one of the two D-loops. This selection may be mediated by enzyme(s) that are bound to membrane at the replication site. Thus, the presence or absence of the second D-loop serves as a marker for expansions <1 or >1 genome on the circular dimer expanded D-loop molecules and correlates with the apparent size of the expanded D-loop\*.

\* This analysis depends on the assumption that displacement replication is unidirectional. This has been established for circular dimer mtDNA prepared as described previously (ref. 2 and Kasamatsu, H. & Vinograd, J., *Nature*, in press). The substantial differences that exist between our preparations of mtDNA and those reported previously (2, 3) required a test for unidirectional growth of the expanding D-loop in these fractions. We selected at random 11 expanded D-loop molecules possessing one expanding D-loop and one nonexpanding D-loop from each of the three fractions 25, 26, and 27 of the LD gradient. About 1/3 of the molecules photographed were sufficiently twisted so as to preclude measurement. Two circular dimer expanded D-loop molecules possessed ambiguities in the path chosen for measurement of the expanding and nonexpanding portions of the molecule. The remaining 21 molecules were clearly replicating unidirectionally (Fig. 2D).

The distribution of tritium in the ethidium bromide-CsCl gradient of one preparation of LD mtDNA is presented in Fig. 2*B*. The large upper band is mainly nuclear DNA when compared with the corresponding distribution for the thymidine kinase<sup>-</sup> derivative, LDTK<sup>-</sup> (Fig. 2*A*). The masses of upper band DNA estimated by UV fluorescence before collection were comparable. The lower and intermediate fractions [24–31] of the gradient containing LD mtDNA were examined by electron microscopy. Fraction 25 is the peak fraction of the lower band, and fraction 33 is the peak fraction of the upper band. The frequency of D-mtDNA decreases with decreasing density (Fig. 2*C*, from left to right). Concomitant with this decrease in frequency of D-mtDNA there is an increased frequency of expanded D-loop molecules in these same fractions (Fig. 2*C*). Fractions 24–26 contain expanded D-loop, but not gapped circular, molecules. These latter forms appear in fractions 27–31 of this gradient (Fig. 2*C*) as observed for monomer mtDNA (Table 1). Furthermore, the frequency of expanded D-loop molecules with expansions <1 genome is maximum in fraction 28, while the frequency of such molecules with expansions >1 genome increases continuously from fraction 25–31 (Fig. 2*C*). Thus, expanded D-loop molecules with small expansions possess greater densities than such molecules with large expansions. This result is similar to the observation that molecules of SV40 and kinetoplast DNA that are more fully replicated are found in lighter regions of an ethidium bromide-CsCl gradient (8–10).

#### Denaturation-renaturation of expanded D-loop molecules

The buoyant position of expanded D-loop molecules indicates that the parental strands of these molecules are covalently-closed circles. The two contiguous strands of closed-circular DNA are topologically linked and, thus, are not physically separated upon denaturation. Removal of the daughter strands of expanded D-loop DNA by denaturation will result in a closed-circular molecule. If replication of this mtDNA proceeds in the absence of nicking, then removal of progeny strands and renaturation of parental strands should lead to complete recovery of the fully duplex structure with the superhelix density of the unreplicated parental molecule. If the repeated process of nicking and closing relieves the topological constraint arising from displacement replication, removal of progeny strands of the expanded D-loop molecule should result in a closed-circular molecule with a large deficiency of Watson-Crick turns [e.g., 750 such turns for an expanded D-loop molecule replicated through 0.5 genome (Fig. 3*A*)]. A subsequent renaturation of this molecule could lead to complete recovery of the Watson-Crick turns with a concomitant introduction of many superhelical turns. Alternatively, if this introduction of a large number of superhelical turns is not possible, a combination of superhelical turns and denatured region(s) would result.

We thermally denatured and briefly renatured a portion of fraction 28 in the analyses of the gradient distribution of circular dimer replicative forms (Fig. 2). Before denaturation, this sample contained 15% clean circular mtDNA, 5% gapped circular mtDNA, 44% D-mtDNA, and 37% expanded D-loop molecules with about equal frequencies of expansions <1 and >1 genome (Fig. 2*C*). After denaturation-renaturation, the sample contained only two duplex forms, 62% clean circular DNA and 37% of a new form for the 131 duplex molecules scored. This form (Fig. 3*B*, *C*) we designate DL-

mtDNA (denaturation loop mtDNA). The clean circular DNA present after denaturation-renaturation is ascribed to D-mtDNA and clean circular DNA in the undenatured

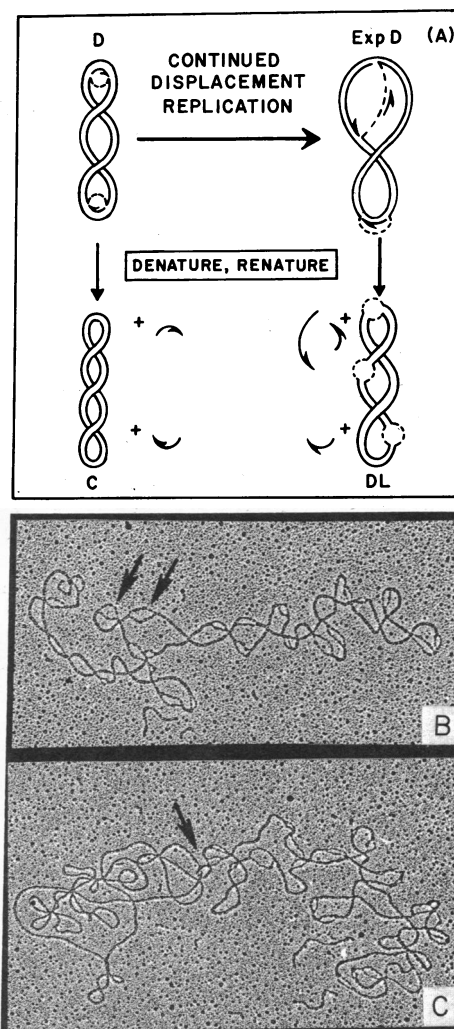


FIG. 3. (A) Diagram of the effect of denaturation-renaturation on circular dimer D-mtDNA with two D-loops (*D*), and on circular dimer expanded D-loop DNA with one small D-loop and one expanding D-loop, *Exp-D*. The former generates a clean circular molecule with a slightly greater number of superhelical turns, *C*, plus two small 7S displacing strands. The latter generates a DL-mtDNA molecule, containing both superhelical twists and regions of denaturation, plus three single-strand fragments: the 7S displacing strand, the newly replicated strand produced by displacement synthesis, and the newly replicated strand produced by duplex synthesis. In some expanded D-loop molecules duplex synthesis may not have been initiated at the time of isolation. Such molecules would generate only two single-strand fragments. (B) and (C) DL-mtDNA observed after thermal denaturation of a 10- $\mu$ l sample of fraction 28 (Fig. 2*B*) in STE buffer at 100° for 20 sec followed by quenching at 0° and subsequent renaturation in 50% formamide-STE buffer at 24° for 10 min at a final DNA concentration of about 0.2  $\mu$ g/ml. These conditions of renaturation would lead to less than 3% renaturation of the separated strands of LD mtDNA (11). Samples for electron microscopy were immediately prepared. The DL form in (B) contains at least 21 denaturation loops, with two of the larger loops indicated by the arrows. The DL form in (C) is catenated to a clean circular dimer molecule in the region indicated by the arrow.

sample. We thus ascribe DL-mtDNA to the denatured-renatured product of expanded D-loop mtDNA. (The frequency of single-stranded circles after denaturation-renaturation corresponds approximately to the initial frequency of gapped circular molecules.) These DL-mtDNA forms are duplex over a portion of their length and contain loops of unrenatured single-stranded DNA generally less than 1500 nucleotides in extent. 27 Molecules contained 1-5 loops, 8 molecules contained 5-10 loops, 9 molecules contained 10-20 loops, and 4 molecules contained 20-25 loops. Three molecules were found that contained one large loop comprising more than 70% of the circular dimer mass. The formation of DL-mtDNA from expanded D-loop mtDNA upon denaturation-renaturation establishes the closed circularity of expanded D-loop mtDNA and argues for nicking and reclosure of these forms during replication.

### DISCUSSION

The use of thymidine kinase<sup>-</sup> cells in studies of mtDNA replication has permitted detailed examination of the buoyant density distribution of tritium-labeled replicative forms. It is thus possible to determine the fraction of total mtDNA represented by each of the replicative forms of LMTK<sup>-</sup> cells growing exponentially in suspension culture.

The most abundant form of L-cell mtDNA is the replicative form, D-mtDNA. It constitutes 63% of all LMTK<sup>-</sup> mtDNA. A low frequency of D-mtDNA appears in the upper band of the ethidium bromide-CsCl gradient and probably represents adventitious nicking of about 3% of the total D-mtDNA population. These forms accumulate at the initial stage of displacement replication and represent a holding point in the replication of mtDNA. As proposed in the model for displacement replication (2), molecules are released from this holding point with expansion of the D-loop to form larger replicative forms, expanded D-loop molecules. The expanded D-loop molecules are shown in this study to contain covalently-closed circular parental strands. Displacement synthesis in the expanded D-loop molecules appears to proceed with repeated nicking and rapid closure of the parental molecule. Nicking-closing cycles in mtDNA in cells exposed to ethidium bromide are known to occur (12). These conclusions derive from analysis of the disposition of these replicative forms in ethidium bromide-CsCl gradients and from the formation of DL-mtDNA after denaturation-renaturation of circular dimer expanded D-loop molecules. This aspect of mtDNA replication is similar to the mode of DNA synthesis for SV40 and kinetoplast DNA (8-10). In the earlier study (2), expanded D-loop molecules were found in the upper band of the ethidium bromide-CsCl gradients. Their presence in the upper band most likely resulted from the method of mi-

tochondrial purification used in that study (see *Methods*). In previous studies lower frequencies of D-mtDNA were observed (2-4). The differences may reflect the purification procedures used or unknown factors in growth of the cells.

Gapped circular molecules, the  $\beta$  daughter molecules derived at completion of displacement synthesis (2), were found in this study to be fractionated by the ethidium bromide-CsCl gradients according to their content of single-stranded DNA. Gapped circular molecules with gaps <0.1 genome were enriched in the upper band (Table 1) and comprise 6% of LMTK<sup>-</sup> mtDNA. Gapped circular molecules with gaps >0.1 genome were enriched in the intermediate region of the ethidium bromide-CsCl gradient (Table 1) and comprise 3% of the LMTK<sup>-</sup> mtDNA, roughly equal to the quantity of expanded D-loop molecules in these cells. Clean circular mtDNA comprises 25% of LMTK<sup>-</sup> mtDNA. About half of these clean circles are in the upper band, which exceeds the quantity expected on the basis of random nicking of the mtDNA during preparation. These probably represent the daughters of displacement replication before closure.

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