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Displacement-loop replication initiation sequence in animal mitochondrial DNA exists as a family of discrete lengths

(DNA replication/5′-terminal labeling/nucleotide sequence/RNA primer/*Hae* III endonuclease)

AMANDA M. GILLUM AND DAVID A. CLAYTON

Laboratory of Experimental Oncology, Department of Pathology, Stanford University School of Medicine, Stanford, California 94305

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ABSTRACT **The single-stranded mitochondrial DNA (mtDNA) displacement-loop initiation sequence (7S mtDNA) is hydrogen-bonded at the origin of replication in animal cell mtDNA. Analysis of 7S mtDNA from several cell sources indicates that this initiation sequence exists as a family of fragments of relatively discrete lengths. mtDNA from both mouse L cells and mouse liver has four major sizes of 7S mtDNA fragments, ranging from 500 to 580 nucleotides in length. The 5′-end region of each of these species is the same; thus, the size heterogeneity is due primarily to differences in length at the 3′-end of these molecules. By contrast, 7S mtDNA from both human KB cells and human liver exists in three major forms, ranging from 555 to 615 nucleotides in length, due to differences at both terminal regions. The mtDNA initiation sequence from *Xenopus laevis* oocytes also exists in at least two forms, 1350 and 1510 nucleotides in length. Thus, the maintenance of multiple forms of mtDNA initiation sequence appears to be a general phenomenon of animal cells, although the precise mechanism of synthesis or processing of these forms is variable.**

The sequence of 42 nucleotides at the 5′-end of 7S mtDNA from mouse L cells has been determined and found to be rich in dGuo and dThd residues, with no apparent palindromes or potential secondary structures. We thus present sequence information on the replication origin of mtDNA, as defined by the naturally occurring 7S mtDNA.

A novel feature of animal cell mitochondrial DNA (mtDNA) is the maintenance of a short primer fragment hydrogen-bonded at a unique location on the closed circular genome, which results in a localized displacement of the opposite parental strand (1–3). Such molecules have been termed displacement-loop (D-loop) mtDNA. For mouse L cells it has been shown that this primer segment is a heavy-strand sequence (1), as defined by the alkaline buoyant densities of the separate strands of mtDNA, and is located at the origin of mtDNA replication (2, 3). More recent labeling data from L cells *in vivo* have indicated that this segment is rapidly lost and resynthesized on a substantial number of mtDNA molecules (4, 5).

In prior studies utilizing centrifugation and electron microscopic techniques, it had been assumed that this primer fragment was of unique length, or perhaps consisted of a class of lengths with one modal value. In fact, the isolated primer fragment from mammalian tissues is termed "7S mtDNA" on the basis of its sedimentation coefficient (1). We report here that the 7S mtDNA initiation sequences of mouse, human, and *Xenopus laevis* mtDNA are actually families of polynucleotides of discrete lengths. *In vitro* ^{32}P-labeling of the 5′-ends of these polynucleotides has allowed us to determine the basis for these length differences, both with restriction endonucleases and with direct chemical determination of the nucleotide sequence of the 5′-end regions of several individual family members. We present more refined sizing of these naturally occurring primer segments in mouse, human, and *Xenopus laevis* mtDNA, as well as the nucleotide sequence information on the origin of replication of mtDNA.

MATERIALS AND METHODS

Growth of Cells and *In Vivo* Labeling. Mouse LA9 cells and human KB cells were grown in suspension cultures as described (6, 7). Cells were grown in the presence of 0.2–1 mCi of [5-*methyl*-^{3}H]thymidine per liter (46 Ci/mmol, Amersham Corp.) during logarithmic phase for 1 hr before cell harvest.

Isolation of mtDNA. Mitochondria from tissue culture cells were isolated without sucrose gradient purification as described (5, 6). Mitochondria were isolated from the livers of four CxB/J mice and from ~10 g of one human liver obtained at autopsy. The tissue was finely minced in cold buffer (0.21 M mannitol/0.07 M sucrose/5 mM EDTA/5 mM Tris·HCl, pH 7.5), cells were dispersed and ruptured by three to five strokes in a Dounce homogenizer, and mitochondria were isolated without sucrose gradient purification (6). mtDNA was isolated from the oocytes of one female *Xenopus laevis* by J. Rubenstein as described (8). Closed circular mtDNA was purified by at least two cycles of ethidium bromide/CsCl buoyant density centrifugation (2, 4, 9).

***In Vitro* 5′-End Labeling of 7S mtDNA.** The termini of 7S mtDNA strands were dephosphorylated while hybridized to closed circular mtDNA. The 50-μl reaction mixture, containing 5–10 μg of mtDNA at pH 8.5 and 0.05 μg of calf intestinal alkaline phosphatase (~0.02 unit, Boehringer Mannheim Corp.) was incubated at 57° for 1 hr. The phosphatase was inactivated and the 7S mtDNA denatured from duplex DNA in the presence of spermidine (10) as follows. The reaction mixture was cooled to 25° and 5 μl of 50 mM nitrilotriacetic acid was added (11). After 15 min, 5 μl of a solution of 50 mM Na-glycine (pH 9.5)/1 mM spermidine/0.1 mM EDTA (12) was added. The mixture was heated at 100° for 2 min and quickly chilled in ice water. To the mixture were added: 10 μl of a solution of 500 mM Na-glycine (pH 9.5)/100 mM $MgCl_2$/50 mM dithiothreitol/50% (vol/vol) glycerol; 5 μl of 50 mM $MgCl_2$ (to counteract the nitrilotriacetic acid); 500 pmol of [γ-^{32}P]ATP (1000–2000 Ci/mmol), prepared by exchange synthesis (12, 13); and 1–2 units of T4 polynucleotide kinase (generously provided by J. Chien and I. R. Lehman) to a final volume of 100 μl. The reaction was incubated at 37° for 30 min. The kinase was inactivated by the addition of 150 μl of chilled 2 M NH_4OAc/20 mM EDTA, and the DNA was precipitated with 750 μl of ethanol in the presence of 50 μg of carrier yeast tRNA.

***In Vitro* 3′-End Labeling of 7S mtDNA.** This was per-

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Abbreviations: mtDNA, mitochondrial DNA; D-loop, displacement loop.

formed on denatured 7S mtDNA in the presence of total closed circular mtDNA, without previous dephosphorylation, with calf thymus deoxynucleotidyltransferase (14) (prepared by R. L. Ratliff and generously provided by T. S-F. Wang and D. Korn) and [α-^{32}P]GTP (250 Ci/mmol, Amersham Corp.) followed by alkali treatment, as described (12, 15).

Polyacrylamide Gel Electrophoresis and Recovery of DNA. The ethanol precipitate from the phosphorylation reaction was dissolved in 10 μl of water. Then 15 μl of loading solution (10 M urea/0.025% xylene cyanole FF/0.025% bromphenol blue/5% sucrose) was added and the mixture was heated at 100° for 1 min and chilled in ice water. The sample was analyzed by electrophoresis at 4–6 V/cm (constant voltage) for 14–20 hr at 25° through a 4% polyacrylamide gel (19 parts acrylamide, 1 part bisacrylamide) containing 7 M urea (16) (Ultrapure, Schwarz/Mann) in 90 mM Tris-borate, pH 8.3/2.5 mM EDTA (17). For recovery of DNA, excised gel bands were placed in small dialysis bags containing 150 μl of Tris-borate/EDTA at $\frac{1}{20}$ the normal concentration and 30 μg of yeast carrier tRNA. Bags were placed in an electroelution apparatus (18) containing 200 ml of $\frac{1}{20}$ concentration Tris-borate/EDTA, for 1–2 hr at 175 V (15–20 mA) at 25°. The buffer containing the eluted DNA was withdrawn from the bags and made 0.3 M in NaOAc, and the nucleic acid was precipitated with three volumes of ethanol.

Analysis of 5′-End Groups of 7S mtDNA. Aliquots of 5′-end-labeled 7S mtDNA were digested to completion with endonuclease P_1 from *Pencillium citrinum* (19). The reaction mixture (4 μl), containing about 6000 cpm of the 5′-end-labeled DNA, 50 mM NH_4OAc (pH 5.3), and 5–20 μg of carrier yeast tRNA, was incubated at 37° for 1 hr. The resultant mixture of 5′-mononucleotides was spotted onto two cellulose thin-layer plates along with a solution containing 0.05 A_{260} unit each of dAMP, dGMP, dCMP, and dTMP (the digested carrier tRNA provided ribonucleotide monophosphate markers), and developed in two solvent systems: (*i*) isobutyric acid/concentrated. NH_4OH/water (66:1:33, vol/vol/vol, pH 3.7) and (*ii*) 0.1 M sodium phosphate (pH 6.8)/$(NH_4)_2SO_4$/*n*-propanol (100:60:2, vol/wt/vol). The nonradioactive markers were visualized under a short-wave UV lamp, and the ^{32}P-labeled products were located by autoradiography. For quantitation of radioactivity, the cellulose spots were removed from the thin-layer plate by the method of Turchinsky and Shershneva (20) and assayed in Omnifluor scintillant (New England Nuclear Corp.).

Nucleotide Sequence Analysis of 7S mtDNA. The chemical procedure for sequence determination developed by Maxam and Gilbert (12) for end-labeled DNA was used, with the following modifications: 2 μg of carrier calf thymus DNA was used during methylation of purines or hydrazinolysis of pyrimidines; 25 μg of carrier tRNA was used and $Mg(OAc)_2$ was omitted during the subsequent ethanol precipitation steps.

Digestion of 7S mtDNA with *Hae* III Restriction Enzyme. The reaction mixture (10 μl), containing about 1000 cpm of end-labeled 7S mtDNA in the presence of 1–10 μg of carrier tRNA, 6 mM Tris·HCl (pH 7.5), 6 mM $MgCl_2$, 6 mM NaCl, 6 mM 2-mercaptoethanol, and 0.5–4 units of *Hae* III enzyme (21, 22) (New England Biolabs), was incubated at 37° for 2 hr, with a second addition of enzyme after 1 hr. The reaction was terminated by addition of EDTA to 25 mM and analyzed by electrophoresis as described above. Autoradiography was for 1–3 days with an intensifying screen, as described below.

Autoradiography. Preparative gels were exposed directly on Kodak X-Omat R film at 4°. Thin-layer plates, analytical gels, and sequencing gels were exposed on Kodak X-Omat R film at −70° with the aid of a Dupont Cronex Lightning-Plus intensifying screen. Exposure enhancement over regular autoradiography was 10- to 15-fold. The analytical 4% polyacrylamide gels were fixed in 10% trichloroacetic acid, neutralized, and dried under reduced pressure before autoradiography.

RESULTS

Labeling of 7S mtDNA *In Vitro*. Our *in vitro* labeling procedure was designed to eliminate preliminary purification of the trace quantities of single-stranded 7S mtDNA (~0.1 μg of 7S mtDNA/ml of packed L cells). The only 5′-end substrates available in D-loop mtDNA for phosphorylation by polynucleotide kinase are 7S mtDNA and occasional nicked parental strands. Fig. 1*A* shows an autoradiograph typical of a preparative gel containing end-labeled products from one such kinase reaction of 7S mtDNA in the presence of total L cell mtDNA. Some large parental duplex strands were labeled, and remain at the gel origin. Within the gel is a family of several discrete end-labeled DNA fragments. The two larger, predominant species, $a + b$, and two shorter species, $c + d$, were each isolated from the gel for analysis. Fig. 1*B* shows that each 7S mtDNA species retains its initial mobility upon re-electrophoresis. The length of each segment was calculated by comparison of mobility with those of several denatured λc1857 *Hpa* I fragments (23). The four major mouse 7S mtDNAs vary in length from 580 to 500 nucleotides (Table 1). Two other minor species of 7S mtDNA, 630 and 560 nucleotides in length, were discernible only after longer autoradiographic exposure and were not analyzed in detail.

Several discrete sizes of 7S mtDNA are also present in mtDNA from other cell sources. mtDNA from human KB cells (Fig. 1*C*, lane 1) has three major 7S mtDNA species that are slightly larger than those from L cells (Table 1). Also, 7S mtDNAs isolated from mouse and human liver tissue display patterns identical to those seen in cultured cells, although relative amounts vary somewhat (Fig. 1*D*). A major fraction of mtDNA from oocytes of the amphibian *Xenopus laevis* contains large D-loops (24). The 14S single-stranded initiation segments from *Xenopus* mtDNA labeled *in vitro* consist of at least two poorly resolved species, 1510 and 1350 nucleotides in length (Fig. 1C, lane 2; Table 1).

***Hae* III Digestion of 7S mtDNAs.** In order to investigate the basis for observed size heterogeneity, we digested the four forms of 5′-end-labeled single-strand 7S mtDNA from L cells with endonuclease *Hae* III and analyzed the products by gel electrophoresis (Fig. 2A). Identical results were obtained for 7S mtDNA from mouse liver. *Hae* III digestion of single-stranded DNA is often incomplete (21, 22). Undigested 7S mtDNA is seen in lanes b–f, as well as three 5′-end-labeled partial digestion products about 260, 180, and 60 nucleotides in length. The striking feature is that the various sizes of L cell 7S mtDNA generate 5′-end-labeled digestion products of equal lengths; thus the distance from the 5′-end to each of the *Hae* III cleavage sites is the same in all four species of 7S mtDNA. Therefore, the size heterogeneity of these forms of DNA must be due to length differences at the 3′-end of the molecules. This result has been confirmed by similar analyses of 3′-end-labeled 7S mtDNAs (Fig. 2*B*). Again, undigested 7S mtDNA is seen in all lanes. The lengths of the final 3′-end digestion products of forms *a–d* are about 300, 245, 220, and 200 nucleotides, respectively which account well for the differences in length between whole molecules.

By contrast, when the three 5′-labeled 7S mtDNAs from human KB cells or liver are analyzed by *Hae* III digestion, the distances from the 5′-ends to the *Hae* III cleavage sites are

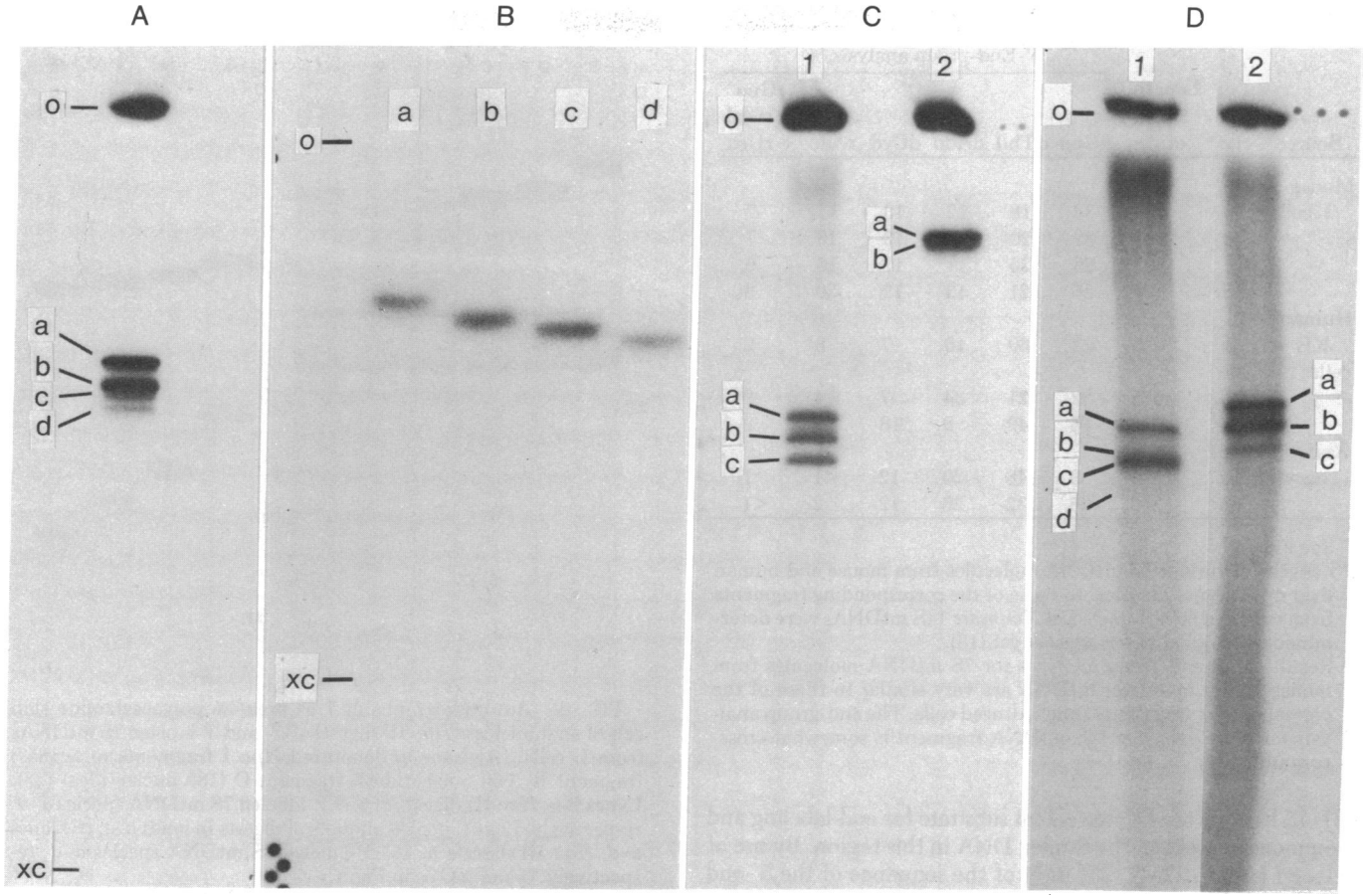

FIG. 1. Autoradiographs of 7 M urea/4% polyacrylamide slab gels of *in vitro* 5′-end-labeled 7S mtDNAs from various sources. (*A*) *In vitro* labeling reaction of 7S mtDNAs from mouse L cells; (*B*) purified 7S mtDNA species *a*–*d* from *A*; (*C*) *in vitro* labeling reactions of (*1*) 7S mtDNAs from human KB cells and (*2*) 14S mtDNAs from *Xenopus laevis* oocytes; (*D*) *in vitro* labeling reaction of 7S mtDNAs from (*1*) mouse liver and (*2*) human liver. O and XC indicate positions of the origin and xylene cyanole tracking dye, respectively.

found to be greater for the larger forms. However, the increase in length of the 5′-end regions does not appear to account for the overall length differences between the various whole molecules (data not shown). This suggests that both the 5′- and 3′-end regions can vary in length, although 3′-end-labeled 7S mtDNA has not been analyzed in this case.

5′-End Group Analyses. Each form of end-labeled 7S mtDNA was digested to 5′-mononucleotides with nuclease P_1 (19). Products were analyzed by thin-layer chromatography (Table 1). For L cell mtDNA, there is extensive heterogeneity of 5′-end groups, including a significant amount of rAdo. The overall pattern is similar in the four forms, with dGuo as the major nucleoside. End-group analyses of each 7S mtDNA species isolated from mouse liver were very similar to those of L cell 7S mtDNA. The fraction of rAdo was not reduced by several efforts at further purification of mtDNA prior to end-labeling, including sucrose velocity gradients and additional ethidium bromide/CsCl gradients. Therefore, although the *Hae* III digestion pattern (Fig. 2*A*) indicates that the 5′-end regions are the same for the forms of L cell 7S mtDNA, there appears to be a microheterogeneity at the 5′-end of all forms when they are isolated and end-labeled by our procedure. A control experiment, in which an *Eco*RI DNA fragment was end-labeled, resulted in a 5′-end analysis of >90% dAdo. In one experiment, an aliquot of 7S mtDNA was phosphorylated with [γ-^{32}P]ATP without previous phosphatase treatment. Incorporation of label was about 10% that of the dephosphorylated control, and the same four forms of 7S mtDNA were discernible. However, end-group analyses of these species were quite unlike those presented in Table 1. For all forms, the major end group was rAdo (~40%) followed by dAdo (~20%). Thus, most of the rAdo end groups appear to exist *in vivo* with a free 5′-hydroxyl group, while, in general, the deoxynucleotides do not.

Results of the end-group analysis for each species of KB cell 7S mtDNA are different, and there is no significant ribonucleotide fraction (Table 1). Species *a* and *c* have predominately dThd, while species *b* has a much more heterogeneous pattern. The same was seen for the human liver 7S mtDNAs.

For *Xenopus* 14S mtDNA, although the two species, *a* and *b*, were only partially resolved from one another (Fig. 1*C*, lane 2), the end-group analysis for each species is somewhat different. Again, there is no significant amount of ribonucleotide end group in either fragment.

5′-End-Nucleotide Sequence Analysis of L Cell 7S mtDNA. The 5′-end-labeled 7S mtDNA species *a* and *b* were purified from three preparations of L cell mtDNA and each was analyzed by the chemical procedures for sequence determination of Maxam and Gilbert (12). The gel analysis was complicated by the heterogeneity of 5′-end groups, which produced a background pattern of cleavage products at most band positions. However, in all autoradiographs, the same predominating sequence pattern was clearly discernible for both species (Fig. 3). The nucleotide composition is biased: the 5′-half of the sequence is very rich in dGuo and dThd residues, while the other half has almost no dGuo residues. There is no extended symmetrical or repeated sequence. A *Hin*f cleavage site at position

Table 1. Properties of various forms of 7S mtDNA

Source	Species*	Length,† nucleotides	5′-End-group analysis, %‡ dGuo	dThd	dAdo	dCyd	rAdo	rGuo + rCyd + rUrd
Mouse								
L cells	*a*	580	33	18	12	13	17	7
	b	540	32	20	12	13	16	7
	c	525	29	26	11	12	16	6
	d	500	26	21	13	12	20	8
Human								
KB cells	*a*	615	18	60	10	7	3	2
	b	585	32	21	34	7	4	2
	c	555	18	49	9	18	4	2
Xenopus								
oocytes	*a*	1510	9	48	30	12	<1	1
	b	1350	28	32	26	12	2	<1

* See Fig. 1.

† Lengths of various 7S mtDNA molecules from mouse and human liver mtDNA are identical to those of the corresponding fragments from cultured cells. Lengths of *Xenopus* 14S mtDNAs were determined on an alkaline 2% agarose gel (18).

‡ Results of 5′-end-group analyses for 7S mtDNA molecules from mouse and human liver mtDNA are very similar to those of the corresponding fragments from cultured cells. The end-group analysis for each *Xenopus* 14S mtDNA fragment is somewhat cross-contaminated.

31–35 has provided a convenient substrate for end-labeling and sequence analysis of the duplex DNA in this region. By use of cloned LA9 mtDNA (25) most of the sequence of the 5′-end region of the 7S mtDNA synthesized *in vivo* has been confirmed, and is currently being extended (L. H. Robinson, A. M. Gillum, and D. A. Clayton, unpublished data).

DISCUSSION

Animal cell mtDNA provides a well-defined DNA replication origin for study due to the *in vivo* maintenance of a short initiation segment on a significant proporation of molecules (1–3, 24). Using *in vitro* end-labeling and gel electrophoresis, we have analyzed these 7S mtDNA initiation sequences from several cell sources. We have found that 7S mtDNA strands are actually a family of molecules of several discrete lengths. This phenomenon is likely to be a general one for vertebrate animal cell mtDNAs, since it has been demonstrated for mtDNAs isolated from mouse and human tissue culture cells, as well as from mouse, human, and *Xenopus laevis* whole tissues (Fig. 1).

The only 5′-end group available for labeling *in vitro* in closed circular D-loop mtDNA is that of the 7S mtDNA single strand. Therefore, we have phosphorylated these 5′-ends with ^{32}P without prior purification of 7S material from the bulk of duplex mtDNA. This should minimize degradation and adsorption losses possible during an extensive isolation procedure, and the information obtained should more accurately reflect the situation *in vivo*. In fact, 7S mtDNA isolated from mouse L cells ^{3}H-labeled *in vivo* also consists of a heterogeneous population of discrete sizes identical to those reported here for *in vitro* end-labeled 7S mtDNA (5).

Several lines of evidence indicate that the 5′-end region is the same for each species of mouse 7S mtDNA and that the major size differences are due to variation in length at the 3′-end. The sequences of the first 42 nucleotides at the 5′-end of the two larger species are identical (Fig. 3). The distances from ^{32}P-labeled 5′-ends to the various *Hae* III cleavage sites are the same

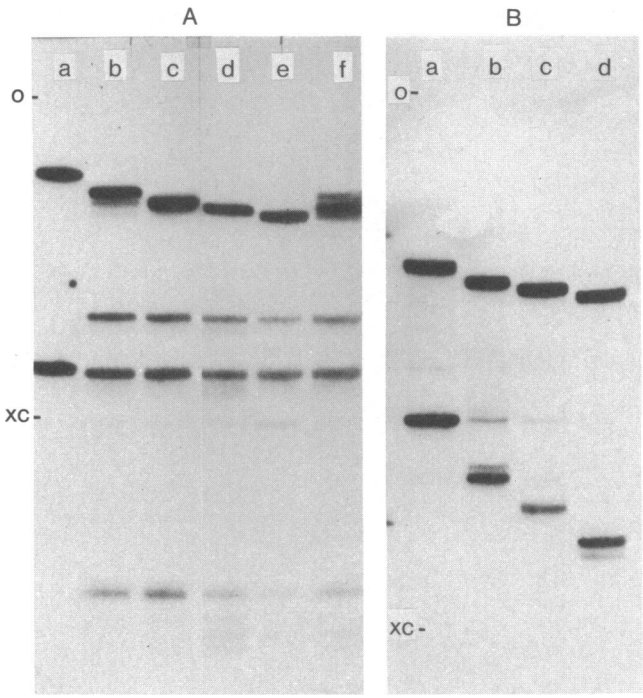

FIG. 2. Autoradiographs of 7 M urea/4% polyacrylamide slab gels of endonuclease *Hae* III digests of 5′- and 3′-labeled 7S mtDNAs from L cells. (*A*) Lane a, denatured *Hpa* I fragments of λc1857: fragment K (660 nucleotides); fragment O (185 nucleotides) (23). Lanes b–e, *Hae* III digests of 5′-^{32}P-labeled 7S mtDNA species *a*–*d*, respectively. Lane f, pooled aliquots of digests in lanes *b*–*e*; (*B*) lanes *a*–*d*, *Hae* III digests of 3′-^{32}P-labeled 7S mtDNA species *a*–*d*, respectively. O and XC as in Fig. 1.

for all forms (Fig. 2*A*). When the 3′-terminal region is examined with 3′-end labeled 7S mtDNAs, the distances from the 3′-terminus to the *Hae* III sites are different for each species and the differences correspond closely to the total length differences of the individual whole molecules (Fig. 2*B*). In contrast, similar analyses of human 7S mtDNA indicate that the length differences of these species are likely to be due to variation in length at both the 5′- and 3′-terminal regions (data not shown).

Several mechanisms could generate these discrete heterogeneous populations of mtDNA initiation sequence. If all parental duplex mtDNAs were identical, 7S mtDNA size heterogeneity could result from initiation and/or termination of synthesis at various sites due to the presence of multiple functional control sequences or perhaps due to sequence duplication in these regions. The fact that mouse 7S mtDNA has major size heterogeneity at only the 3′-end while human 7S mtDNA can vary at both ends suggests that the termination sites could vary in both systems, but that the initiation site could be unique in the mouse system. Additionally, site-specific nucleolytic cleavage of 7S mtDNA could produce a family of initiation sequences at various stages of trimming. Alternatively, there could be size heterogeneity in the parental duplex molecules in the D-loop regions. Although restriction endonuclease patterns (26, 27) and electron microscopic analysis (28) have pro-

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5'          10          20          30          40  3'
GC(CT)TAGGTGATTGGGTTTTGCGGAC(T)AA(T)GATTCTTCACCG...
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FIG. 3. Nucleotide sequence of the 5′-end region of L cell 7S(s) mtDNA, species *a* and *b*. The residues in parentheses indicate tentative assignments. Numbers indicate position of nucleotides from the major dGuo 5′-end. Underlined residues are a cleavage site for endonuclease *Hin*f.

vided evidence against size heterogeneity of mtDNA molecules isolated from an individual mammal, we have observed 7S mtDNA size heterogeneity from the mtDNA isolated from the tissue of an individual frog (Fig. 1*C*, lane 2) and human (Fig. 1*D*, lane 2). However, using electron microscopic heteroduplex analysis of goat and sheep mtDNAs, Upholt and Dawid did observe a rapid rate of evolutionary sequence divergence at both ends of the mtDNA D-loop region, including a possible sequence duplication (28). Perhaps such sequence instability in this region is related to the size heterogeneity of the 7S mtDNAs that we have examined.

Heterogeneity of 5′-end groups of mouse L cell 7S mtDNA has been reported previously (29). We have demonstrated that this 5′-end heterogeneity is limited to the first few nucleotides of mouse 7S mtDNA and might be due to a staggering in the point of initiation of DNA synthesis, nucleolytic processing, or degradation of the 5′-end, or perhaps variation in position of an RNA·DNA junction, similar to that proposed for the colicin E1 plasmid DNA initiation sequence synthesized *in vitro* (30). A most intriguing observation is that ~17% of the 5′-end groups of mouse 7S mtDNA are rAdo. Many of the rAdo 5′-ends contain a free 5′-hydroxyl group *in vivo*, whereas the 5′-deoxynucleotides generally possess a phosphorylated 5′-end. Perhaps the rAdo is a residual portion of primer RNA. By contrast, there are essentially no ribonucleotides detected at the 5′-end of the human or *Xenopus* mtDNA initiation segments.

We note that the sequence of the first 42 nucleotides at the 5′-end of mouse L cell 7S mtDNA has no extended symmetry and provides no basis for a possible secondary structure arrangement such as those suggested by some of the other studies of DNA sequence at or near replication origins (31–34). Our preliminary examinations of the nucleotide sequence surrounding the mouse mtDNA replication origin with DNA fragments from cloned mtDNA (25) generated by the restriction enzyme *Hin*f, which has a cleavage site 31–35 nucleotides from the major 5′-end of the 7S mtDNA sequence (Fig. 3), has allowed alignment of the initiation sequence reported herein. Further examination should reveal information concerning regulatory sequences for DNA replication.

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