In Vivo Determination of Replication Origins of Human Mitochondrial DNA by Ligation-mediated Polymerase Chain Reaction*

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A large part of replication is aborted in human mitochondria, the result being a D-loop. As few attempts have been made to distinguish free 5' ends of true replicate from those of abortive ones, we examined the 5' ends of true replicate of human mitochondrial DNA at one nucleotide resolution in vivo by making use of ligation-mediated polymerase chain reaction. The distribution and relative amounts of origins of the true replicate are exactly the same as those of total newly synthesized heavy strands, which means that the abortion of replication is independent of 5' ends. Treatment of DNA with RNase H frees 5' ends on both heavy and light strands. This is the first in vivo evidence for covalently attached primer RNA to nascent strand in human mitochondrial DNA.

Replication of mitochondrial DNA (mtDNA) begins with synthesis of the heavy strand (H strand)1 from the replication origin O$_{H}$, following transcription of the light strand (L strand). Transcription of the L strand is regulated by the L strand promoter (LSP) sequence. When synthesis of the H strand has proceeded about two-thirds, synthesis of the L strand begins from replication origin O$_{L}$. Thus, the total replication rate of mtDNA is determined by H strand synthesis (for reviews, see Refs. 1–3). The multiple replication origins of human mitochondrial H and L strands have been determined as free 5' ends of mtDNA extracted from prepared mitochondria. The molecular mechanism and physiological significance of these events are poorly understood.

A large part of the synthesis of H strand is aborted, leaving a displacement loop (D-loop) or 7 S DNA. For example, over 95% of the newly synthesized H strand is a D-loop in the case of mouse L cells (4). This makes it difficult to precisely determine origins of the true replication form (nascent H strand) (5). Few investigators have distinguished the free 5' end of the true replicate form from that of the D-loop even though selective detection of the free 5' end of the true replicate form is critical to determine replication origin. Although the 5' ends of the nascent H strand are suggested to be the same as those of D-loop in human mitochondria (5), it is uncertain whether the distribution and relative amount of the 5' ends of nascent H strand are exactly the same as those of the D-loop. In addition, the free 5' ends of human mtDNA are not set at one nucleotide resolution (6). To precisely determine the replication origin but not the simple free 5' end, it is required to selectively detect the replication origin at a higher resolution.

We made use of the ligation-mediated polymerase chain reaction (LMPCR) to detect free 5' ends (7, 8), an approach which makes feasible use of total DNA extracted directly from whole cells. By selective amplification of nascent H (not D-loop), we determined precise and comprehensive replication origins of the H strand in vivo. Here, we describe sites of free 5' ends and the transition sites of RNA to DNA for both H and L strands at one nucleotide resolution.

EXPERIMENTAL PROCEDURES

Materials

BamHI, RNase inhibitor, and T4 DNA ligase were purchased from Takara (Seta, Japan). RNase A and diethylpyrocarbonate were from Sigma. Proteinase K was from Boehringer Mannheim (Mannheim, Germany). Long Ranger® was from FMC® BioProducts (Rockland, ME). Vent DNA polymerase was from New England Biolabs (Beverly, MA). Other reagents were of analytical grade.

Preparation of DNA

HeLa MRV11 and Jurkat (human T cell leukemia line) cells were cultured as described (9) and were harvested in logarithmic proliferation phase. The cells (about 10$^{8}$ cells) were centrifuged, and the pellets were rapidly denatured and solubilized in 100 μl of denaturing buffer containing 50 mM Tris-HCl, pH 7.4, 1 mM EDTA, 1% SDS, and 0.1 mg/ml proteinase K at 50 °C for 1 h. Total DNA was isolated by two extractions with phenol/chloroform (1/1), and then DNA was ethanol precipitated. The pellets were dried, solubilized in 100 μl of distilled water, and treated with RNase A (0.1 μg/μl) and BamHI (0.1 unit/μl). DNA was extracted with phenol/chloroform (1/1), ethanol precipitated, and solubilized in water. The amount of the total DNA was determined by measuring A$_{260}$. In some cases, the initially extracted DNA was solubilized in diethylpyrocarbonate-treated water containing RNase inhibitor (0.7 units/μl) and digested with BamHI alone. After extracting DNA with phenol/chloroform (1/1), precipitating with ethanol, and solubilization in water, DNA was treated with either RNase A (0.1 μg/μl) alone or RNase A plus RNase H (6 units/μl) before LMPCR.

Preparation of Mitochondria

Mitochondria of HeLa cells were prepared by differential centrifugation (9). Briefly, the cells suspended in buffer (TES) containing 0.25 M sucrose, 10 mM Tris-HCl, pH 7.4, and 0.1 mM EDTA were homogenized with a Potter-Elvehjem homogenizer and centrifuged at 600 × g for 10 min at 4 °C. The supernatant was centrifuged at 7,000 × g for 10 min. The pellets were washed three times with TES. The mitochondrial fraction was allowed to proceed in in vitro replication at 37 °C for 1 h, as described by Koike et al. (10, 11) prior to DNA extraction.

Ligation-mediated PCR

The primer sets used in this study are shown in Tables I and II. A unidirectional linker was prepared by hybridizing LMPRI (5'-aggtgac-
chloroform (1/1), ethanol precipitated, dried, and finally solubilized in pH 8.9, 5 mM MgSO4, 0.01% gelatin, 0.3 pmol primer 1, 0.2 mM of each dNTP, 0.5 units of Vent DNA polymerase, and DNA (0.2 μg) for sequence ladder and 0.4 μg for detection of 5' ends). DNA was denatured at 95 °C for 5 min, and the primer was annealed at 54–60 °C for 30 min, after which polymerase reaction was performed at 76 °C for 10 min.

**Ligation**—After cooling on ice, 45 ml of ice-cold ligation mix was added, and the ligation reaction was performed at 16 °C for more than 6 h. The ligation mix consisted of 77 mM Tris- HCl, pH 7.5, 13.3 mM MgCl2, 33.3 mM dithiothreitol, 8.3 mg/ml bovine serum albumin, 1.7 mM ATP, 100 pmol of unidirectional linker, and 3 Weiss units of T4 DNA ligase. DNA was precipitated with 9.4 ml of ethanol, and then the entire mixture was kept at −20 °C for 2 h.

**PCR Amplification**—DNA pellets were solubilized in 66.5 μl of water, and then 33.5 μl of amplification mix (123 mM NaCl, 61.5 mM Tris-HCl, pH 8.9, 15 mM MgSO4, 0.03% gelatin, 0.3% Triton X-100, 10 pmol LMPR1, 20 pmol primer 2, 0.67 μM of each dNTP, and 1 unit of Vent DNA polymerase) was added to the DNA solution. DNA was denatured initially at 95 °C for 3 min, and then the reaction underwent 20 PCR cycles of 95 °C for 1 min, 60–65 °C for 2 min, and 76 °C for 3 min plus an extra 5 s for each cycle. Final extension was allowed to proceed at 76 °C for 10 min. Usually, the 5’ end of primer 2 was fluorescent isothiocyanate-labeled, and this step was followed by the DNA extraction and analysis as described below.

To distinguish the nascent H strand from the total newly synthesized H strand, a three-primers system was adopted. H1 and H2 were used as primers 1 and 2, respectively, for the nascent H strand. For the total H strand, a three-primers system was adopted. H1 and H2 were used as Primers for PCR

| Set | Primer 1 | Primer 2 | Primer 3 |
|-----|----------|----------|----------|
| D-6 | D6       | FD6      |          |
| D-7 | D7       | FD7      |          |
| D-8 | D8       | FD8      |          |
| H   | H1       | H2       | FD6      |
| D   | D1       | D2       | FD6      |
| O1  | RFL5870L | RFL58150L|          |

* Prescripts L and H denote L and H strands, respectively.

**Replication Origins of Human mtDNA**

**TABLE I**

| Combination of primers for LMPCR |
|-------------------------------|
| Set | Primer 1 | Primer 2 | Primer 3 |
| D-6 | D6 | FD6 |          |
| D-7 | D7 | FD7 |          |
| D-8 | D8 | FD8 |          |
| H   | H1 | H2 | FD6      |
| D   | D1 | D2 | FD6      |
| O1  | RFL5870L | RFL58150L|          |

**TABLE II**

| Primers for PCR | Position on mitochondrial DNA | Length | 5'-FITC | Annealing |
|-----------------|-------------------------------|--------|---------|-----------|
| D6              | L58–76                        | 19     | −       | 60        |
| FD6             | L76–100                       | 25     | +       | 65        |
| D7              | L167–186                      | 20     | −       | 54        |
| FD7             | L182–239                      | 26     | +       | 64        |
| D8              | L312–329                      | 18     | −       | 60        |
| FD8             | L322–349                      | 28     | +       | 65        |
| H1              | L16036–16055                  | 20     | −       | 54        |
| H2              | L16055–16079                  | 25     | −       | 60        |
| D1              | L16108–16128                  | 21     | −       | 54        |
| D2              | L16128–16153                  | 26     | −       | 60        |
| RFL5870L        | H5818–5837                    | 20     | −       | 60        |
| RFL58150L       | H5791–5815                    | 25     | +       | 65        |
| RL285D6         | H249–268                      | 20     | −       | 54        |
| RFL291D6        | H227–251                      | 25     | +       | 60        |

**RESULTS**

**Origins for H Strand Replication**—Free 5’ ends of the H strand were determined at one nucleotide resolution. A signal at a particular site indicates that a free 5’ end is located at the 3’ side of the signal by one base (e.g. a signal at nucleotide 192...
indicates that nucleotide 191 is a 5’ end on the H strand). The free 5’ ends were clustered from nucleotides 100 to 200 (Fig. 1). Although the signal at nucleotide 152 was weaker in Jurkat cells than in HeLa cells, distribution of the signals was essentially the same between the two cell lines (Fig. 1). The sequence at nucleotide 150 on the H strand is G in HeLa MRV11 cells and A in Jurkat cells. This difference might affect usage frequency of a replication origin at nucleotide 151. The major signals were grouped into 4 regions around nucleotides 110, 150, 170, and 190. The locations were essentially the same as those reported by Chang and Clayton (6).

These same authors (6) have reported two bands of 5’ ends (around nucleotides 220 and 310) that almost correspond to the 3’ ends of possible primer RNAs and another relative strong band at nucleotide 440. We did not detect the three bands by using primer sets D-7 and D-8 (results not shown).

Selective Detection of the Nascent H Strand—As a large part of the synthesis of H strand is aborted as D-loop, there is the possibility that the 5’ ends described above do not reflect origins of the true replicate (nascent H strand). The 3’ termini of the human D-loop have been mapped to nucleotides 16104–16106 (14). This indicates that the newly synthesized H strand exceeding this region is the nascent H strand. Hence, we amplified the nascent H strand using primers set outside of the D-loop. We designed the other primers on the inside of the D-loop to amplify the total newly synthesized H strand (i.e., D-loop and nascent H strand). Intensity of the signals for the nascent H strand was about 40% of that for the total newly synthesized H strand (results not shown). When the conditions were selected to match the apparent signal intensity between the nascent H strand and total newly synthesized H strand, the distribution and relative amounts of signals for the nascent H strand were much the same as those of the total newly synthesized H strand (Fig. 2).

Detection of RNA Covalently Attached to the Newly Synthesized Strand—It is considered that replication of the H strand is initiated by cleavage of the L strand transcript by RNase H-like activity. In human cells, RNA covalently attached to the newly synthesized H strand is not detectable in vivo (5, 15).
performed LMPCR. This treatment led to new free 5'-ends at the sites mapped (6). Considering the possibility that RNA is not cleaved in vivo, we treated DNA with RNase H (Fig. 3), suggesting that the primer RNA attached to DNA can be processed at these sites by an endogenous RNase H-like activity.

Although such RNA is noted in mouse cells (16). We did not detect the 5'-ends at areas where the 3'-ends of free RNA are mapped (6). Considering the possibility that RNA is not cleaved at the sites in vivo, we treated DNA with RNase H and then performed LMPCR. This treatment led to new free 5'-ends at nucleotides 297 and 302–309 (Fig. 3, A and B) but not around nucleotide 220, corresponding to conserved sequence block (CSB) I (Fig. 3A). The appearance of new 5'-ends after treatment with RNase H indicates the covalent attachment of RNA to DNA. This is apparently the first demonstration of the covalent attachment of RNA to a newly synthesized H strand in human cells.

The free 5'-ends on the L strand were also detected at nucleotides 5772, 5775, 5776, 5778, 5779, 5780, and 5762 by LMPCR (Fig. 3C). Although covalent attachment of RNA to the L strand is noted in an in vitro system (15), such has not been demonstrated in vivo. We obtained new free 5'-ends at nucleotide 5770 and to a lesser extent at 5768, 5769, and 5774 after treatment with RNase H (Fig. 3C). These new 5'-ends are located at the base of the stem portion in the proposed stem-loop structure near O_L (15). This is the first example of the existence of covalently attached RNA to a human nascent L strand in vivo.

**DISCUSSION**

We selectively detected free 5'-ends of the true nascent H strand by LMPCR. We found no difference in the distribution and relative amounts of 5'-ends between the nascent H strand and the total newly synthesized H strand. The same distribution pattern of 5'-ends of nascent H strand as that of D-loop suggests that the abortion of replication is not affected by the origin.

**A transcript must form a persistent RNA-DNA hybrid to prime replication.** We observed new free 5'-ends only near the CSB II region after RNase H treatment of DNA (Fig. 3). This suggests that the primer RNAs extend to CSB II and covalently attach to the nascent H strand. Chang and Clayton (6) have reported that the 3'-termini of free RNA are mapped to three CSB regions (CSBs I-III). Our observations suggest that the RNA-DNA hybrid at CSB II is more stable than those at the other CSBs in vivo. Consistent with this, a persistent RNA-DNA hybrid is reported to be formed under an in vitro system containing the GC-rich CSB II region downstream of the primer.

**FIG. 5.** Location of free 5'-ends. The sites of free 5'-ends in HeLa MRV11 cell are summarized. The site of the nucleotide with the free 5'-end is corrected by one base from the signal located on L strand. The sequence and numbering of nucleotides are accorded to Anderson et al. (20). The actual sequence in CSB II is 5'-TTTGGGGGGGGAGGGGGG-3' in HeLa MRV11 cells. The large circles indicate major nucleotides with free 5'-ends, and the small circles indicate minor nucleotides with free 5'-ends. The arrows indicate nucleotides of which the 5'-ends appear only after treatment with RNase H. Three CSBs are marked as same as in panel A.
of a promoter (17, 18).

Although we noted a small amount of covalently attached RNA to nascent H strand near the CSB II region, almost all free 5′ ends were confined downstream of the CSB I or the region between nucleotides 100 and 200. These 5′ ends were free of RNA. Because the 5′ ends in the region of nucleotides 100 to 200 should have been primed with RNA, the RNA-DNA hybrid should extend to nucleotides 100–200 from the LSP region (around nucleotide 400) and be processed at the region. A large part of free RNA should have free 3′ ends at nucleotides 100 to 200, while most of 3′ ends of free RNA are clustered in three CSBs (6). The reason for the discrepancy remains to be explained.

We extensively determined the 5′ ends of the true replicate of H strand by LMPCR (Fig. 5). This approach is sensitive, rapid, facilitated, and precise. Detection of a free 5′ end equals to detection of the strand with a free 5′ end (i.e. nascent strand), therefore, the signal intensity reflects the amount of nascent strand. Hence, it is possible to estimate the steady-state level of replication in cells using LMPCR (19).

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