MATERIALS AND METHODS

Three different established human cell lines (HeLa, A549 and 143B.TK⁻) and several immortalized human lymphocyte cell lines were used in the present work. The detection in the total DNA preparations from a given cell line of the mtDNA nascent H-strand chains originating at different positions in the D-loop was carried out by primer extension, using VENT DNA polymerase (New England Biolabs) (1) and a purified [5'-32P]-labeled L-strand oligonucleotide corresponding to a sequence within the D-loop (internal primer), or to a sequence on the 3'-side of it (external primer). Unless differently specified in the Figure legends, the extended primers obtained with the internal primers were separated by 7M urea/10% polyacrylamide gel electrophoresis (PAGE) at 20 mA for 3 to 4 hr, whereas the extended primers obtained with the external primers were separated by 7M urea/5% PAGE at 10 mA for 16 or 20 hr.

The primers used and their sequences and positions in the mtDNA sequence (2) are specified below:

**Internal primers**

Ip1: 36-55

5' GAG CTC TCC ATG CAT TTG GT 3'

Ip2: 16549-16569

5' CTT AAA TAA GAC ATC ACG ATG 3'

Ip3: 16528-16547

5' CTA AAT AGC CCA CAC GTT CC 3'

**External primers**

Ep1: 16067-16086

5' CTC ACC CAT CAA CAA CCG CT 3'
Ep2: 16034-16053
5' GGG AAG CAG ATT TGG GTA CC 3'

EP3: 15481-15500
5' CTC ACC AGA CCT CCT AGG CG 3'

EP4: 14906-14925
5' CAC TAC TCA CCA GAC GCC TC 3'

Three µg of total DNA and 1 pmole of each primer (representing a 200- to 300-fold molar excess over the template) were used. Analysis of the fixed and dried DNA gel was carried out either on a Phosphor-Imager, using, for quantification of the labeling of the extended primers, the program Image-Quant, version 1.2 (Molecular Dynamics), or by autoradiography. For the identification of the 5'-end(s) of the nascent chains originating at or close to position 57, an undried gel was exposed for autoradiography, the band of interest was excised, and the DNA, eluted and subjected to circularization by intramolecular ligation of the 5' and 3' ends of each extended primer with T4 RNA ligase (New England Biolabs). The circularized molecules were subjected to PCR amplification, and the products were separated in a native polyacrylamide gel and cloned in *E. coli* by using a TA vector, and 20-50 plasmids derived from each PCR product were then sequenced to identify the 5'-3'-end junctions (fig. S2). For more detailed information see ref. 3 and the corresponding supporting information on the *PNAS* Web Site http://www.pnas.org/cgi/content/full/242719399/DC1.

For detection of nascent H-strand chains by S1 nuclease protection, 3 µg of DNA from a HeLa cell mitochondrial fraction were subjected to asymmetric PCR, using 400 pmoles of [5'-32P]-labeled L-strand primer Ip3 and 10 pmoles of unlabeled H-strand primer (between positions 271 and 253). This procedure generated a labeled mtDNA L-strand fragment between positions 16,528 and 271. The single-strand DNA product of the PCR was separated from the double-
strand DNA by electrophoresis through a 2% agarose gel. After excision, the fragment was purified using the Perfectprep® Gel Cleanup Kit (Eppendorf). The labeled probe was tested for purity on an 8 M urea/6% polyacrylamide minigel, and then used for hybridization with 1.5 µg of DNA from the mitochondrial fraction of HeLa cells. After digestion with the S1 enzyme (Roche, 1000 U for 30 min at 41°C), the S1-resistant products were run through a 7 M urea/10% polyacrylamide gel. M1, M2, M3 are DNA markers, in particular, respectively, pBR322 DNA-MspI digest, 100 bp DNA Ladder and 1 kb DNA Ladder (New England Biolabs).

Evidence that the Psex band derived mainly from primer extension on the parental H-strand was provided by an experiment in which the extended primer obtained with the Ep2 primer on 143B.TK− cell mtDNA was digested with the restriction enzyme ScrFI or Fnu4HI or AluI. In fact, fragments of the expected sizes were observed, together with the disappearance of the Psex band.

It was found that, in mtDNA from the three main cell lines analyzed, the average amounts of nascent chains originating at position 57 and detected with the internal primer Ip3 are nearly identical (within ~12 to ~24%) to the amounts of the O57 chains detected with the external primer Ep2. By contrast, the cumulative amounts of nascent chains initiating at the 191, 167, 146-151 and 110 origins and detected with the internal primer are very significantly (~3- to ~5-fold) higher than those detected with the external primer (fig. S3A).

The surprising finding of a new major origin of H-strand replication in the D-loop which had been missed in numerous investigations extending over a period of ~25 years demanded a reevaluation of the previous studies. A careful analysis of the protocols followed in the earlier
work and further analysis of the nascent H-strand chains originating at position 57 led to the conclusion that the new origin could not have been detected in the previous investigations. This was mainly due to the PAGE conditions utilized to map the origins. For instance, Chang and Clayton (4), in 1985, ran the product of an S1 protection assay on a denaturing 6% polyacrylamide gel (Fig. 2 of their paper); that gel was clearly run too long in order to see any product shorter than 100 bp. Kang et al. (5), in 1997, utilized ligation-mediated PCR, and the final product was run again through a 7 M urea/5% polyacrylamide gel. Also in this case, any band shorter than 100 bp would have run off the gel and could have not been detected.

The labeling pattern of the extended primers synthesized on nascent chains initiated at different previously known origins in every cell line was reproducibly and characteristically different in the three main cell lines analyzed here, independently of the primer used for their detection. However, a comparison of the data for each species of extended primers did not reveal any similarity to the pattern observed for the extended primers synthesized on the O57 chains, as concerns the relative abundance of the "true" replicating chains and overall nascent chains.
REFERENCES


**fig. S1.** Primer extension products obtained on "true" replicating nascent H-strand chains from the indicated cell lines, using the [5'-32P]-labeled L-strand external primer Ep3. M2: DNA marker (N.E. Biolabs). See Materials and Methods for marker identification and PAGE conditions.
**fig. S2.** Approach followed for detection of the extended primers produced on mtDNA nascent H-strand chains using L-strand primers, and for identification of the 5'-ends (origins) of the nascent chains. The example shown used the Ip3 primer. $O_H$: origin of nascent H-strand chains.
fig. S3. Contribution of O$_{57}$ and of the multiple previously known origins to mtDNA replication in HeLa, A549 and 143B.TK$^{-}$ cells. (A) The relative amounts of nascent chains, estimated in multiple experiments, are expressed relative to the cumulative amount of nascent chains initiated at the 191 to 110 origins in each experiment. Bars: averages with standard error of the mean (SE) for HeLa cells (n=5, from three DNA preparations), A549 cells (n=3, from one DNA preparation) and 143B.TK$^{-}$ cells (n=3, from two DNA preparations). (B) Ratios of amounts of "true" replicating O$_{57}$ nascent chains to total nascent chains, as determined from the data in panel (A). Bars: average ±SE.
**fig. S4.** Growth behavior of HeLa cells (A) and 143B.TK- cells (B) during exposure to 50 ng/ml of ethidium bromide (EtBr) for 3 days and subsequent recovery after drug withdrawal.
**fig. S5.** (A) Relative amounts of total mtDNA per cell in 143B.TK- cells during exposure to 50 ng/ml of EtBr for 3 days, or in cells exposed to the drug for 3 days and then transferred to drug-free medium. (B) Quantification of *all* mtDNA nascent H-strand chains (open symbols), as detected with the internal Ip3 primer, and of "true" replicating chains (filled symbols), as detected with the external Ep1 primer, which were initiated at one or the other of the previously known 191 to 110 origins (blue symbols), or initiated at the novel 57 origin (red symbols) during exposure of the cells to EtBr for 3 days and after their transfer to drug-free medium for 7 days. The data are expressed relative to the cumulative amount of nascent chains initiated at the 191 to 110 origins, as detected with the Ip3 primer before exposure of the cells to EtBr.
**fig. S6.** Primer extension products obtained on *all* nascent H-strand chains [using internal primer Ip3 (A)], or on "true" replicating nascent H-strand chains [using external primer Ep2 (B)] from HeLa cells before or after exposure to 50 ng/ml of EtBr for 3 days and after drug withdrawal.
**fig. S7.** Primer extension products obtained on all nascent H-strand chains [using internal primer Ip3 (A)], or on "true" replicating nascent H-strand chains [using external primer Ep2 (B)] from HeLa cells before or during exposure to 20 µM 2',3'-dideoxycytidine for 3 days and after drug withdrawal.