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R-Loop in the Replication Origin of Human Mitochondrial DNA Is Resolved by RecG, a Holliday Junction-Specific Helicase

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Stable RNA-DNA hybrids (R-loops) prime the initiation of replication in *Escherichia coli* cells. The R-loops are resolved by *Escherichia coli* RecG protein, a Holliday junction specific helicase. A stable RNA-DNA hybrid formation in the mitochondrial D-loop region is also implicated in priming the replication of mitochondrial DNA. Consistent with this hypothesis, the 3′ ends of the mitochondrial R-loop formed by in vitro transcription are located close to the initiation sites of the mitochondrial DNA replication. This mitochondrial R-loop is resolved by RecG in a dose-dependent manner. Since the resolution by RecG requires ATP, the resolution is dependent on the helicase activity of RecG. A linear RNA-DNA heteroduplex is not resolved by RecG, suggesting that RecG specifically recognizes the higher structure of the mitochondrial R-loop. This is the first example that R-loops of an eukaryotic origin is sensitive to a junction-specific helicase. The resolution of the mitochondrial R-loop by RecG suggests that the replication-priming R-loops have a common structural feature recognized by RecG.

The replication of vertebrate mitochondrial DNA (mtDNA) initiates from two origins, O_H and O_L for the heavy and light strands, respectively [1]. The replication of the heavy strand proceeds displacing the parental heavy strand. When the replication of the heavy strand completes approximately two thirds, the O_L on the parental heavy strand is exposed as a single stranded region. The replication of the light strand starts only after the O_L is exposed as a single stranded region. Thus the replication from the O_H determines the overall replication rate of mtDNA. The mammalian O_H includes the light strand promoter (LSP) and three downstream conserved sequence blocks called CSBIII, CSBII, and CSBI. The replication of the heavy strand is proposed to be primed with the transcriptional machinery from the following observations. Firstly, RNAs covalently linked to DNA are detected and their 5′ ends are identical to the major transcriptional initiation sites of mouse mitochondrial DNA [2]. Secondly, in human mitochondria, there are free RNAs whose 5′ ends are located at the major transcription start sites and 3′ ends map in the region where 5′ ends of the nascent heavy DNA strands are located [2,3]. Consistent with this, stable RNA-DNA hybrids (R-loops) were formed at the O_H during in vitro transcription of mouse [4] and human [5] mtDNA. RNase MRP, a putative enzyme responsible for making replication primer for mtDNA, cleaves the R-loop near the initiation site of replication [6].

In yeast there are several sequences, called *rep* or *ori,* comprising approximately 300 bp segment and having a very similar structure to that of mammalian O_H region. Therefore these sequences are implicated in the replication of yeast mtDNA. Indeed DNA with covalently attached-RNA maps in one of the *ori* sequences, *ori*-5 [7]. The in vitro R-loop formation is reported using the *ori*-5 as a template [8]. Thus, the R-loop formation is implicated in the replication of mtDNA.

DNA replication can be initiated in a manner that is dependent on the homologous recombination function in *Escherichia coli* (*E. coli*) cells [9,10]. RecG protein of *E. coli* by itself recognizes DNA junctions including Holliday junctions and has a weak 3′ to 5′ helicase activity. The complex of RuvA and RuvB proteins, in

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which the former is a Holliday junction specific binding protein and the latter has a 5′ to 3′ helicase activity, plays an overlapping role in homologous recombination with RecG. Mutations in the *ruv* or *recG* gene stimulate inducible stable DNA replication (iSDR), in which the involvement of D-loops is supposed. In contrast, *recG* mutants but not *ruv* mutants stimulates constitutive stable DNA replication (cSDR) which was originally found in RNase HI defective mutants. Since R-loops are thought to be required for the initiation of cSDR, RecG but not RuvAB may participate in the removal of R-loops as does RNase HI.

The R-loop formation at a replication origin is known in ColE1 [11,12], bacteriophage T7 [13], and bacteriophage T4 [14]. Among them the R-loop structure at the ColE1 replication origin is best characterized. The proper secondary structure of the transcript called RNA II is essential to the formation of R-loop. Recently RecG was shown to resolve R-loops at the ColE1 replication origin, thereby inhibiting the replication of a plasmid containing a ColE1-type replication origin [15,16]. R-loops at the bacteriophage T4 replication origin is also dissociated by bacteriophage T4 UvsW protein which is a helicase involved in phage recombination and a functional analog of *E. coli* RecG [17]. Thus, R-loops relevant to the priming of replication may have a common structural feature recognized by Holliday junction-specific helicases.

In this study, we show that the 3′ ends of the mitochondrial R-loop are located close to the initiation sites of the heavy strand replication and the human mitochondrial R-loop is resolved by RecG in an ATP-dependent manner, further supporting an idea that the mitochondrial R-loop formed during the transcription primes the replication of the leading strand. The replication-priming R-loops may have a common structure recognized by RecG from *E. coli* to mammals.

MATERIALS AND METHODS

Materials. RNase H, SP6 RNA polymerase, poly (A) polymerase, and RNA PCR kit (AMV) were purchased from Takara (Seta, Japan). RNase A and ReverScript I were from Boehringer-Mannheim (Mannheim, Germany). TA cloning vectors pCR 2.1 and pCR 3 were from Invitrogen (SanDiego, USA), and pGEM-T Easy was from Promega (Madison, USA). Ethylenediamine-N,N,N′,N-tetraacetic acid (EDTA) was from Dojin (Kumamoto, Japan). Other reagents were of analytical grade.

Construction of plasmids containing the D-loop region of human mtDNA. A fragment of human mtDNA from nucleotide position (np) 16036 to 394 containing CSB III, II, I, and termination-associated sequence was amplified by polymerase chain reaction (PCR). The PCR products were cloned into pGEM-T Easy. One clone which contains a fragment with the correct direction and DNA sequence was selected and named pGEMhmD (Fig. 1A, top). The DNA sequence of the insert was determined by the dye terminator method with an ABI 370 sequencer. The insert of pGEMhmD was cut out with EcoRI and ligated to pCR 3 cleaved with EcoRI. A plasmid containing the insert with the same direction as pGEMhmD was cloned and named pCR3hmD (Fig. 1A, bottom).

Formation and resolution of in vitro R-loop. Recombinant *Escherichia coli* RecG protein was purified as previously described [16].

The R-loop reaction mixture containing 5 nM pGEMhmD (or pCR3hmD), 50 mM KCl, 20 mM Tris-HCl, pH 8.0, 10 mM $MgCl_2$, 1 mM dithiothreitol (DTT), 0.1 mM NTPs, and 0.2 u/μl SP6 RNA polymerase was incubated at 37°C for 30 min essentially according to the method of Lee and Clayton [4]. To remove NTPs, the reaction mixture was applied on to a gel filtration spin column, CENTRI-SEP (Princeton Separations Inc., Adelphia, USA). The R-loops in the elution was ethanol precipitated, dried, and resolubilzed in water.

The R-loop resolution reaction was performed in 20 μl of the resolution buffer containing 0.1 pmol of the R-loop, 20 mM Tris-HCl, pH 8.0, 50 mM KCl, 4 mM $MgCl_2$, 1 mM DTT, 2 mM ATP, and 0.05% bovine serum albumin in the presence of the indicated concentration of RecG at 37°C for 10 min. For the resolution by RNase H (6 units) and RNase A (10 ng), ATP was omitted. The reaction was stopped by the addition of 1 μg/ml proteinase K and 0.5% sodium dodecylsulfate (SDS) and incubated for another 10 min. The R-loop was analyzed by 0.7% agarose gel electrophoresis in the buffer consisting of 89 mM Tris base, 89 mM boric acid, and 2 mM EDTA.

Linear RNA–DNA heteroduplex. pCR3hmD (Fig. 1A, bottom) was linearized the BamHI site which is located in the multicloning site downstream of the insert. The linearized pCR3hmD was used as a template for synthesizing an RNA complementary to the light strand of human mitochondrial D-loop region with SP6 RNA polymerase. The synthesized RNA was gel eluted and quantified by measuring A_{260}. Using this RNA (0.1 pmol) as a template, the linear RNA-DNA heteroduplex was produced by a reverse transcriptase that is devoid of RNase H activity, ReverScript I. The linear RNA-DNA heteroduplex was treated with RecG/ATP or RNase H as described above.

Determination of the 3′ ends of R-loop. After the R-loop formation, plasmids with R-loops were purified by gel elution. The free RNAs were isolated from the plasmids by hydrolyzing DNA with DNase I and precipitating with ethanol. Then, a poly-adenine tail was added to the 3′ ends with a poly (A) polymerase. The cDNAs were prepared from the polyadenylated RNAs using the oligo dT-adaptor primer containing the sequence of M13 primer M4 (GTTTTCCCAGTCACGAC) with RNA PCR kit (AMV) according to the manufacturer's instructions. The cDNAs were PCR-amplified using the oligo dT-adaptor primer and an oligonucleotide corresponding to np 286-233 of the heavy strand as primers. The PCR products were cloned into TA vectors (pCR 2.1, Invitrogen). Independent 33 clones were sequenced and the poly A sites were determined.

RESULTS AND DISCUSSION

R-loop formation by in vitro transcription. We used SP6 RNA polymerase instead of mitochondrial RNA polymerase for the mitochondrial R-loop formation. This was justified based on the reports by Clayton et al.; (1) SP6 RNA polymerase forms the mitochondrial sequence-dependent R-loop that is comparable to that generated by human mitochondrial RNA polymerase [4] and (2) the upstream elements of CSBIII are not critical for the R-loop formation [5,8]. The recombinant plasmid pGEMhmD contains the D-loop control region of human mitochondrial mtDNA (np 394-16036) downstream of an SP6 promoter (Fig. 1A, top). SP6 RNA polymerase efficiently formed R-loop on a supercoiled pGEMhmD plasmid (Fig. 1B). The R-loop formation was observed as an RNA-DNA complex with altered electrophoretic migration as reported by Lee and Clayton (Fig. 1B, lane 2). The R-loop formation was not observed when the D-loop region was inserted into the

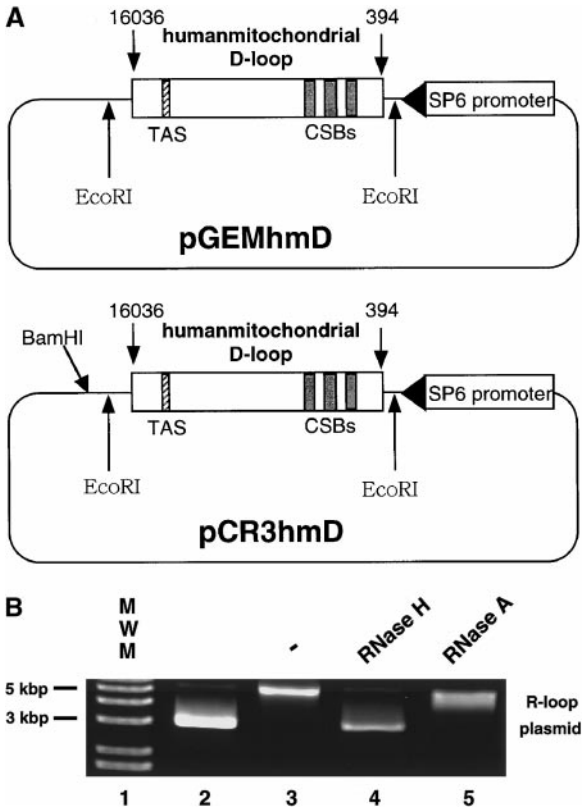

FIG. 1. R-loop formation. (*A*) The diagrams of pGEMhmD (top) and pCR3hmD (bottom). CSBs, conserved sequence blocks; TAS, termination associated sequence. (*B*) The R-loop (lane 2) was formed from the closed circular plasmid pGEMhmD (lane 1) as a template. The mitochondrial R-loop was treated with 6 units of RNase H (lane 3) or 10 ng of RNase A (lane 4).

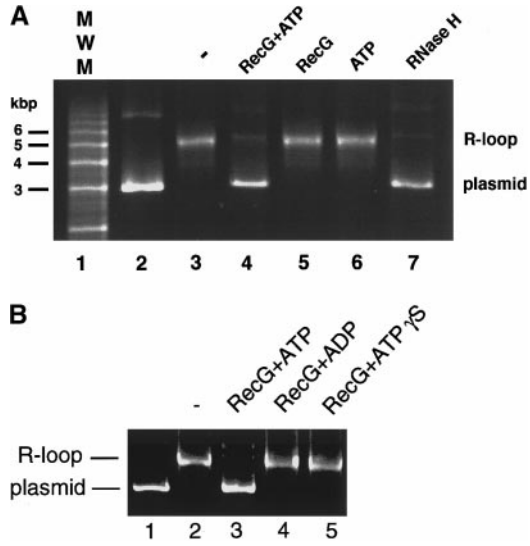

FIG. 2. Resolution of R-loop by RecG. (*A*) The template plasmid pGEMhmD is shown in lane 2. The R-loop (lane 3) was treated with 200 nM RecG and 2 mM ATP (lane 4), 200 nM RecG alone (lane 5), ATP alone (lane 6), or 6 units of RNase H (lane 7). (*B*) The R-loop (lane 2) was treated with 200 nM RecG and 2 mM ATP (lane 3), 200 nM RecG and 2 mM ADP (lane 4), or 200 nM RecG and 2 mM ATPγS (lane 5).

plasmid in the opposite direction (results not shown). Digestion with RNase H, an endonuclease specific for the RNA strand of RNA-DNA hybrids, reversed the shifted mobility (Fig. 1B, lane 3). In consistence with a RNA-DNA hybrid formation, RNase A, an endonuclease specific for single-stranded RNA, did not reversed the shifted mobility although the band of RNA-DNA complex became broad (Fig. 1B, lane 4). Ten-fold amount of free RNA was completely degraded with the same amount of RNase A (results not shown). The broad band might reflect the existence of single stranded region in the R-loop as proposed for the mouse mitochondrial R-loop [4]. The same R-loop formation was observed with pCR3hmD (results not shown).

The resolution of R-loop with RecG. The pre-formed R-loop was treated with RecG, a Holliday junction specific helicase. RecG completely reversed the shifted mobility in the presence of ATP (Fig. 2A, lane 4), indicating that the R-loop is resolved. RecG or ATP alone had no effect (Fig. 2A, lanes 5 and 6). ADP and ATPγS, a nonhydrolyzable ATP analog, did not support the resolution by RecG (Fig. 2B, lanes 4 and 5), indicating that the resolution by RecG is dependent on the hydrolysis of ATP. Taken together, these results demonstrate that the R-loop may be resolved by the helicase activity of RecG. RecG resolved the R-loop in a dose-dependent manner. The half maximal effect was observed at approximately 100 nM (Fig. 3).

Using RNA complementary to the heavy strand as a template (Fig. 4, lane 2), a linear RNA-DNA hybrid was produced (Fig. 4, lane 3). The increase in the staining intensity may be due to the formation of double strand. RNase H decreased the intensity of staining, which may reflects the degradation of RNA resulting in the formation of single strand DNA (Fig. 4, lane 5). RecG did not affect the linear RNA-DNA hybrids (Fig. 4, lane 4), indicating that the R-loop resolution activity of RecG is not due to any contaminating RNase H-like enzymes and that RecG does not simply recognize the primary sequence of the hybrid but specifically recognizes the higher structure of the R-loop.

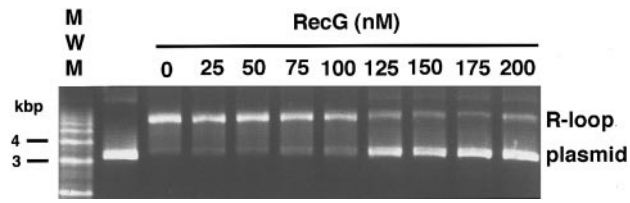

FIG. 3. Dose-dependent effect of RecG on the resolution of R-loop. The R-loop was treated with indicated concentrations of RecG.

3′ ends of R-loop. We precisely determined the 3′ end of RNA moiety of the R-loop at one base resolution. Fig. 5 shows that many 3′ ends of the R-loop are located in the area where the replication origins of the heavy strand are located [3].

It is reported that mitochondrial RNA polymerase can form R-loop on a plasmid containing the mitochondrial D-loop region as efficiently as SP6 polymerase in vitro [4]. From the efficient formation of mitochondrial R-loop during in vitro transcription, it is likely that the mitochondrial R-loop is also formed in vivo. Given that R-loops are efficiently produced in vivo, it is unlikely that every transcription accompanies the replication, particularly in postmitotic non-dividing cells such as terminally differentiated muscles and neurons. The double mutant of *rnh1* encoding RNase H1 and *recG* is lethal in *E. coli* [18]. It suggests that unregulated replication primed by R-loop is toxic to cells. In *E. coli*, the overproduction of RecG causes the marked decrease in the copy number of ColE1 plasmid [16]. Our observations in this study suggest a possible regulatory mechanism in the mtDNA replication by a RecG-like enzyme. Recently the presence of recombination activity in mammalian mitochondria was detected [19]. There abundantly exist small polyadenylated RNAs, called 7S RNA, which begin from the major transcription initiation site and end near the replication origins for the heavy strand of human mtDNA [20]. It was supposed that the 7S RNA can be independently regulated from whole L-strand transcription leading to polycistronic transcripts [21]. The 7S RNA might be RNA moiety of the R-loop released by an RecG-like enzyme. Consistent with this, the 3′ ends of the mitochondrial R-loops formed in vitro map close to the initiation sites of the heavy strand replication (Fig. 5). The fact that the R-loop formation in the mitochondrial D-loop region spontaneously ceases very near the 5′ ends of the nascent heavy strands raises the possibility that a part of R-loops prime mitochondrial replication without RNase H-like activity of RNase MRP or Endonuclease G. The RNase H1 activity is not necessarily required for the replication from the ColE1 origin in *E. coli* [22].

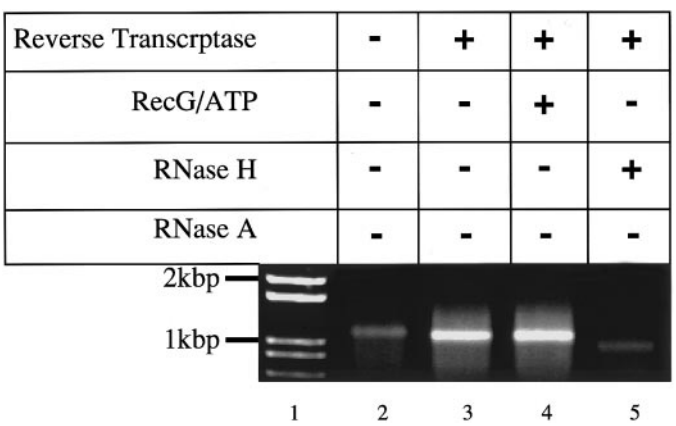

FIG. 4. Linear RNA-DNA hybrid is not sensitive to RecG. The template RNA (0.1 pmol) is shown in lane 2. A linear RNA-DNA hybrid was produced using 0.1 pmol RNA (lane 3). The linear RNA-DNA hybrid was treated with 200 nM RecG/2 mM ATP (lane 4), 6 units of RNase H (lane 5). All samples were electrophoresed on 0.8% agarose gel and stained with ethidium bromide.

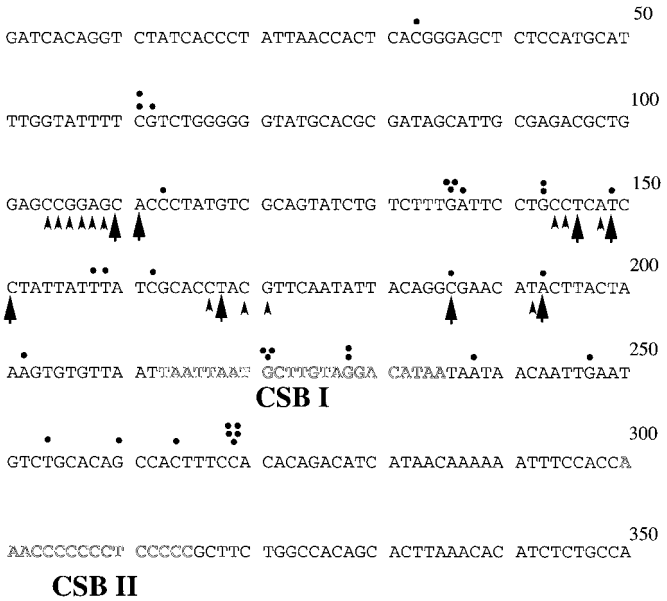

FIG. 5. The 3′ ends of RNA in the R-loop. The dots above the sequence are 3′ ends of RNA molecule in the R-loop. The big arrows and small arrows below the sequence indicate major and minor replication origins, receptively, determined in the previous study [3].

The observation that mitochondrial R-loop formed by in vitro transcription is resolved by RecG suggests that the mitochondrial R-loop shares structural characteristics with Holliday recombination intermediates and R-loops formed at the origin of ColE1 type plasmids [16].

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