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The Role of Ribonuclease H in Multicopy Single-Stranded DNA Synthesis in Retron-Ec73 and Retron-Ec107 of *Escherichia coli*

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Bacterial reverse transcriptase is responsible for the synthesis of multicopy single-stranded DNA (msDNA). Reverse transcriptases from retron-Ec73 and retron-Ec107 do not contain an RNase H domain. Cellular RNase H is therefore considered to be required to make the mature form of msDNA. We found that RNase HI, but not RNase HII, is required for the production of the mature form of both msDNAs.

Multicopy single-stranded DNA (msDNA) is a multicopy satellite DNA in which a single-stranded DNA is branched out from a single-stranded RNA by a 2′,5′-phosphodiester linkage (see references 4 through 6 for reviews). msDNAs have been found in a number of prokaryotes, including myxobacteria; *Rhizobium* spp. (12); and enteric bacteria, such as *Escherichia coli* (see references 4 through 6 for reviews), *Salmonella* spp. (12), *Klebsiella* spp. (12), and *Proteus* spp. (12). The genetic element responsible for the synthesis of msDNA is termed a retron and is a retroelement which includes the gene for reverse transcriptase (RT).

Figure 1 shows the biosynthetic pathway of msDNA (see references 4 through 6 for reviews). The primary transcript, which includes two sets of inverted repeats, a1-a2 and b1-b2, is made from the single promoter of the retron. The a1 and a2 sequences form a stem structure, placing a G residue at the end of the stem (Fig. 1, step 1). cDNA synthesis is thought to be primed from the 2′-OH group of the G residue by RT (Fig. 1, step 2). After the primary reaction, DNA synthesis proceeds by RT using the *msd* region of the primary transcript as a template. As the cDNA chain is elongated, the template RNA is thought to be concomitantly processed by RNase H (Fig. 1, step 2). The cDNA synthesis terminates at a specific site for each msDNA, leaving a DNA-RNA duplex at their 3′ ends (Fig. 1, step 3).

Unlike eukaryotic RTs, the bacterial retron RTs so far studied do not have the RNase H domain, with the exception of RT-Ec67. Cellular RNase H is therefore considered to play an important role in msDNA synthesis. To date, two distinct RNase H genes have been found in *E. coli*. The major RNase H is called RNase HI, and it is encoded by the *rnhA* gene (9). The second RNase H gene (*rnhB*), which encodes RNase HII, has been isolated to complement an *rnhA* mutation (7).

In the present study, we investigated the role of RNase H in the biosynthesis of two different msDNAs, msDNA-Ec73 (15) and msDNA-Ec107 (3). For this purpose, three distinct *E. coli* RNase H mutants, carrying mutations *rnhA*, *rnhB*, and *rnhA-rnhB*, as well as their parental strain, were used. We found that RNase HI is required for the production of the mature forms of the msDNAs.

To examine the exact role of RNase H in the synthesis of msDNA-Ec73, three *E. coli* RNase H mutants [MIC73 (*rnhA*::*cat*), MIC2075 (*rnhB*::*kan*), and MIC2067 (*rnhA*::*cat rnhB*::*kan*)] and their parental strain (W3110) were transformed with p23S3.5 containing the entire retron-Ec73 (14). The experimental procedure used to detect msDNA is shown in Fig. 2A. The total RNA fraction (1) isolated from each transformant was treated with RNase A with or without heat denaturation and then analyzed by polyacrylamide gel electrophoresis. Band b was detected in both the wild-type strain and the *rnhB* strain (Fig. 2B, lanes 1 and 3, respectively). This band corresponds to msDNA-Ec73 linked to a triribonucleotide. This is the expected size, since RNase A cuts the 3′ ends of pyrimidine nucleotides to leave the 5′-A-G-C-3′, including the branching G residue attached to the msDNA (see the band b product in Fig. 2A). However, band b was not observed for either the *rnhA* strain or the *rnhA-rnhB* strain (Fig. 2B, lanes 2 and 4, respectively). On the other hand, a distinct higher-molecular-weight band (band a) was detected in all strains (Fig. 2B, lanes 1 through 4). This band corresponds to the RNA-DNA heteroduplex shown as the band a product in Fig. 2A. Even in the wild-type strain, a small amount of band a was detected (Fig. 2B, lane 1). For the wild-type strain, however, this band was not observed when retron-Ec73 was harbored in a low-copy-number plasmid (data not shown).

When these same msDNA fractions were heat denatured and treated with RNase A before electrophoresis, a major band migrated at the same position in all cases, as shown in Fig. 2B (lanes 5 through 8). These results can be explained by the pathway shown in Fig. 2A; upon heat denaturation, the RNA strand is dissociated from the DNA-RNA heteroduplex, which can then be digested with RNase A.

To compare the sizes of products in the wild-type (*rnhA*$^+$) and *rnhA* mutant strains, the band b products in lanes 5 and 6 (Fig. 2B) were isolated and labeled at the 3′ end of the DNA with terminal deoxynucleotidyltransferase and [α-^{32}P] dideoxy ATP as described previously (14). The labeled molecules were analyzed by urea-polyacrylamide gel electrophoresis, and the result is shown in Fig. 2C. msDNAs synthesized in both strains were found to consist of the same three distinct bands (Fig. 2C, bands 1 to 3). These bands were isolated from the gel, and their

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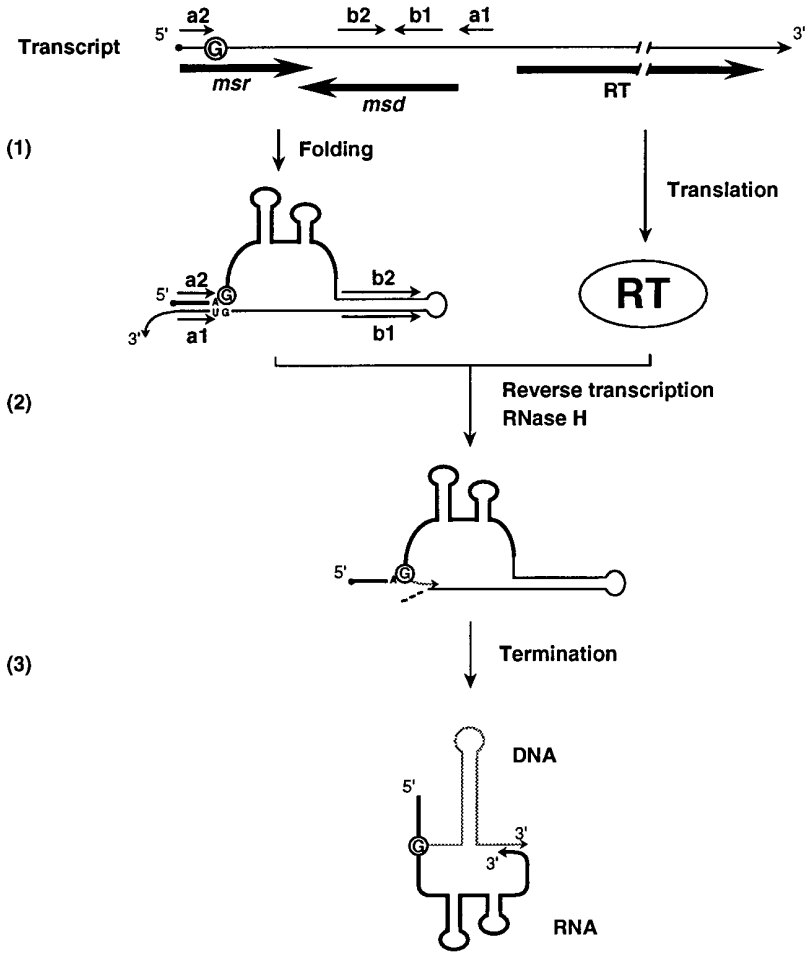

FIG. 1. Biosynthetic pathway of msDNA synthesis. Long thin arrow, RNA transcript. Short thin arrows, inverted repeats (a1-a2 and b1-b2). Thick arrows, genes. The branching G residue is circled. The thick-line region in the folded RNA structure remains as msd RNA in the mature form of msDNA. Wavy lines, cDNA synthesized by RT. The three nucleotides (A, U, and G) near the branching G residue are conserved among all msDNAs characterized so far.

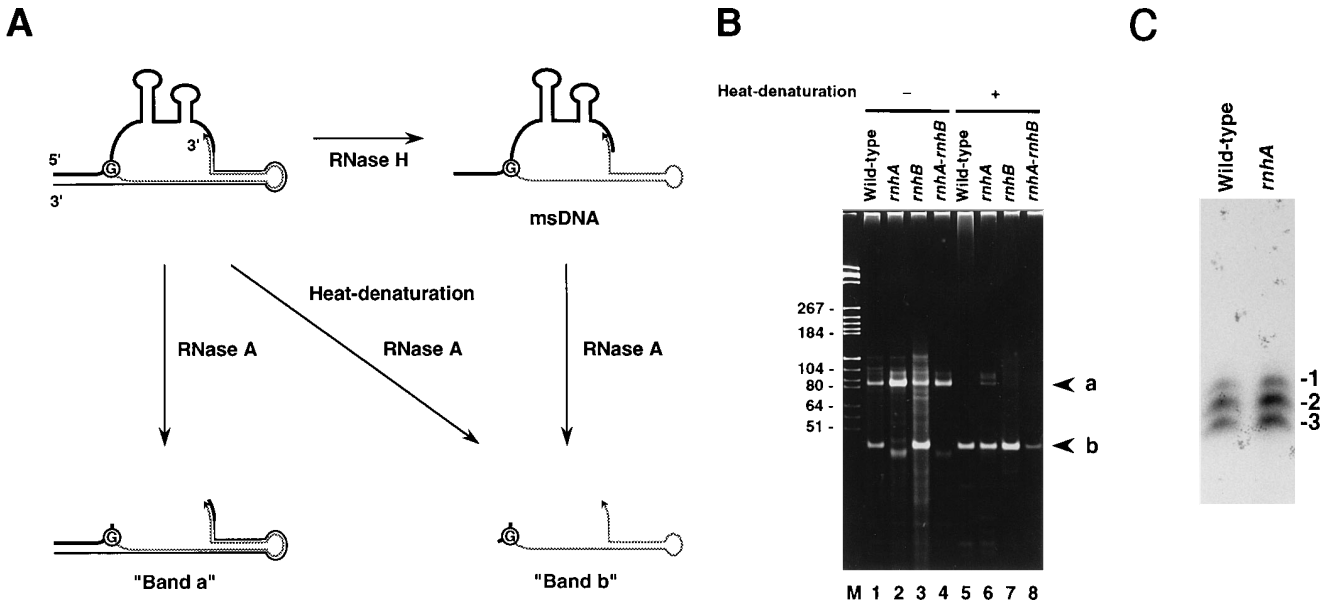

FIG. 2. Effect of RNase H on the synthesis of msDNA-Ec73. (A) Schematic representation of digestion of *msr-msd* RNA from retron-Ec73 with RNase H or RNase A. Thin lines, *msr-msd* from retron-Ec73; thick lines, *msr* RNA attached to msDNA. Wavy lines, msDNA synthesized by RT-Ec73. The branching G residue is circled. (B) msDNA-Ec73 synthesis in RNase H mutants. Lanes 1 and 5, msDNA fraction isolated from W3110/p23S3.5; lanes 2 and 6, MIC73 (*rnhA*)/p23S3.5; lanes 3 and 7, MIC2075 (*rnhB*)/p23S3.5; lanes 4 and 8, MIC2067 (*rnhA-rnhB*)/p23S3.5. Total RNA fractions were treated with RNase A before (lanes 1 to 4) and after (lanes 5 to 8) boiling and quick cooling. msDNAs were separated on a 10% polyacrylamide gel and visualized by staining with ethidium bromide. Bands a and b correspond to bands a and b, respectively, in panel A. The numbers on the left are double-stranded DNA markers (in base pairs). (C) Urea-polyacrylamide gel electrophoresis analysis of msDNA isolated from lanes 5 and 6 in panel B. Products were labeled at the 3′ end as described in the text. msDNA isolated from strain W3110/p23S3.5 (wild-type) and msDNA from MIC73 (*rnhA*)/p23S3.5 (*rnhA*) are shown.

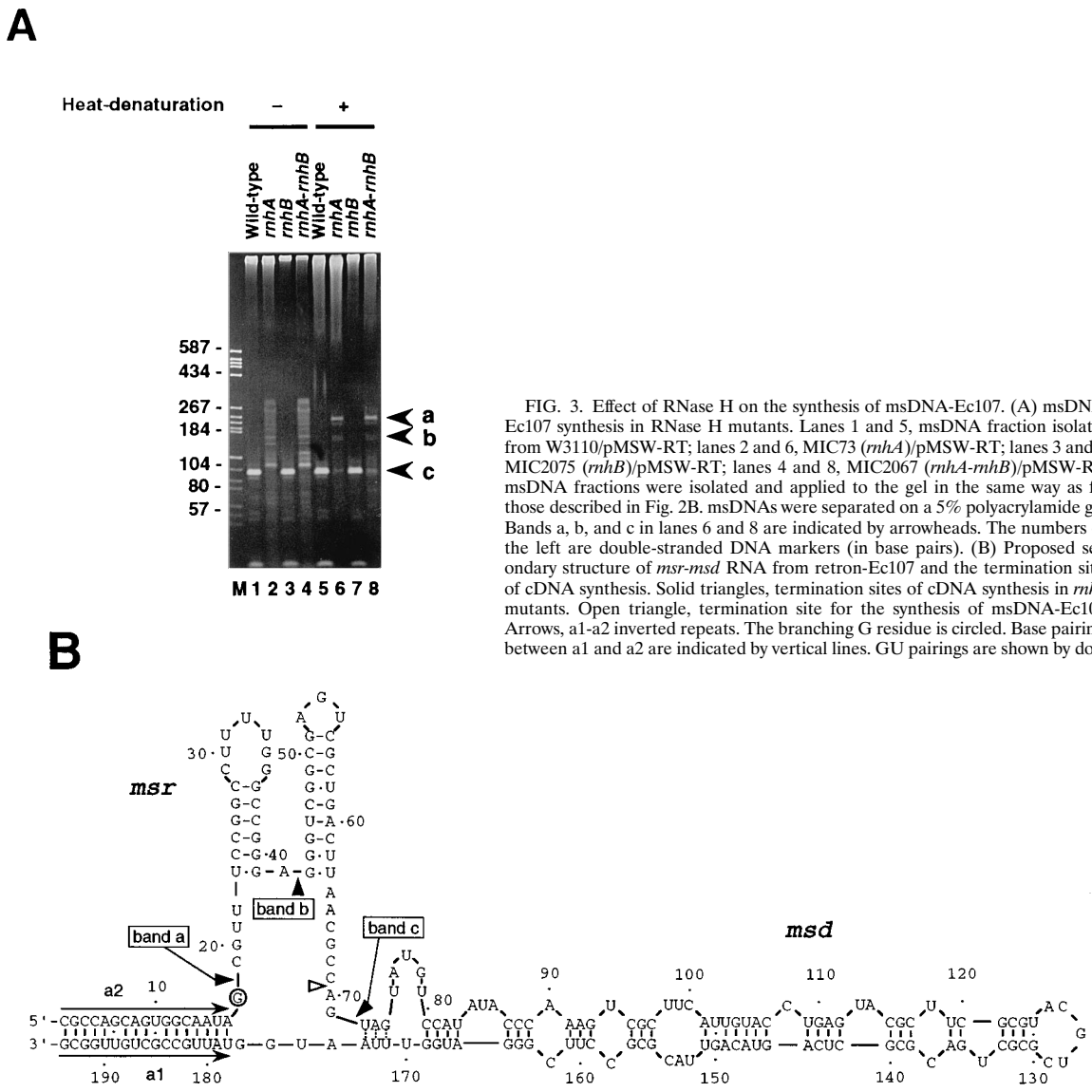

FIG. 3. Effect of RNase H on the synthesis of msDNA-Ec107. (A) msDNA-Ec107 synthesis in RNase H mutants. Lanes 1 and 5, msDNA fraction isolated from W3110/pMSW-RT; lanes 2 and 6, MIC73 (*rnhA*)/pMSW-RT; lanes 3 and 7, MIC2075 (*rnhB*)/pMSW-RT; lanes 4 and 8, MIC2067 (*rnhA-rnhB*)/pMSW-RT. msDNA fractions were isolated and applied to the gel in the same way as for those described in Fig. 2B. msDNAs were separated on a 5% polyacrylamide gel. Bands a, b, and c in lanes 6 and 8 are indicated by arrowheads. The numbers on the left are double-stranded DNA markers (in base pairs). (B) Proposed secondary structure of *msr-msd* RNA from retron-Ec107 and the termination sites of cDNA synthesis. Solid triangles, termination sites of cDNA synthesis in *rnhA* mutants. Open triangle, termination site for the synthesis of msDNA-Ec107. Arrows, a1-a2 inverted repeats. The branching G residue is circled. Base pairings between a1 and a2 are indicated by vertical lines. GU pairings are shown by dots.

DNA sequences were determined by the method of Maxam and Gilbert (11) (data not shown). It was found that msDNA in these strains was extended by a few bases at the 3′ end: band 1 had four extra A's, band 2 had three extra A's, and band 3 had two extra A's in comparison with the msDNA-Ec-73-produced strain C1-23 (15).

These results demonstrate that RNase HI plays a major role in the degradation of a template RNA and that the length of the DNA molecule of msDNA-Ec73 produced in the absence of RNase H is identical to that for the wild type.

The role of RNase H in the synthesis of msDNA-Ec107 was also examined, in the same manner as described above. Three RNase H mutants and the wild-type strain were transformed with pMSW-RT containing the entire retron-Ec107 (*msr-msd* and the RT gene). pMSW-RT was constructed as follows. A 1-kb *Xba*I-*Bam*HI fragment containing the RT-Ec107 gene from pRT-107 (13) was inserted into the *Xba*I and *Bam*HI sites of pUC19, and the resulting plasmid was designated pUC107-RT. The promoter and the *msr* and *msd* regions were amplified by PCR with oligonucleotides 2851 and 2852 (13) as primers. The amplified DNA fragment was digested with *Hin*dIII and *Xba*I and ligated with pUC107RT. The sequence of the PCR-amplified region was confirmed, and the resulting plasmid was designated pMSW-RT. The msDNA fraction, which was isolated in the same way as described for msDNA-Ec73, was analyzed by polyacrylamide gel electrophoresis. As shown in Fig. 3A, the msDNA fractions from the wild-type and the *rnhB* strains containing msDNA-Ec107 were found to be the same size (band c, lanes 1 and 3, respectively). The *rnhA* and *rnhA-rnhB* strains did not produce the major band (band c) seen for the wild-type strain (Fig. 3A, lanes 2 and 4, respectively). However, when the same fractions were heat denatured and treated with RNase A, three distinct bands became apparent (Fig. 3A, bands a, b, and c in lanes 6 and 8).

To determine the structures of these molecules, each band was isolated from the gel and then labeled as described earlier, and their nucleotide sequences were determined by the method of Maxam and Gilbert (11) (data not shown). The major band (Fig. 3A, band a) was found to consist of 158 nucleotides of cDNA, which encompasses the DNA molecule

of msDNA-Ec107 plus the sequence complementary to the *msr* region (Fig. 3B). The middle band (Fig. 3A, band b in lanes 6 and 8) consists of 134 nucleotides of cDNA, indicating that cDNA synthesis terminated between the two stem-loop structures in the *msr* region (Fig. 3B). The band c molecule was found to be shorter by only 2 nucleotides at its 3′ end than the DNA molecule of the wild-type msDNA-Ec107 (Fig. 3B). When the *rnhA* strains were cotransformed with a plasmid, pGB-RNHI, containing the *rnhA* gene, the transformants produced the same msDNA as that in the wild-type strain. pGB-RNHI was constructed as follows: the 0.75-kb *Eco*RI-*Pst*I fragment, which was carrying the *rnhA* gene from pSK760 (9), was ligated at the *Eco*RI and *Pst*I sites of pGB2 (2).

These results indicate that, like RT-Ec73, RT-Ec107 can synthesize msDNA in the absence of RNase H. However, unlike RT-Ec73, RT-Ec107 requires RNase HI for the correct termination of msDNA synthesis. In the absence of RNase HI, most of the cDNA synthesis passes beyond the normal termination site.

Bacterial RTs are evolutionarily related to eukaryotic RTs (16). According to the three-dimensional structure of the human immunodeficiency virus type 1 RT, the thumb domain of the small subunit (p51) contacts with the RNase H domain of the large subunit (p66) (8, 10). The thumb domain of the bacterial RT may also be responsible for the interaction with *E. coli* RNase H. However, in light of the fact that the amino acid sequences of the bacterial thumb domains are highly diverse, the stability of the RT-RNase H interaction may be significantly different from retron to retron. Such a difference may explain the difference in punctuation observed for msDNA-Ec73 and msDNA-Ec107 in the absence of RNase HI. Namely, RT-Ec73 may normally terminate msDNA synthesis with or without RNase H, while RT-Ec107 may functionally require RNase H more stringently for normal termination. Alternatively, the structures of msDNAs themselves may have effects on termination in the absence of RNase H. Further experiments are needed to answer these questions.

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