Cell-free Synthesis of the Branched RNA-linked msDNA from Retron-Ec67 of *Escherichia coli*

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msDNA-Ec67 is produced in a clinical strain of *Escherichia coli* and composed of a 67-base single-stranded DNA, which is linked to the 2'-OH group of the 15th rG residue of a 58-base RNA molecule by a 2',5'-phosphodiester linkage (Lampson, B. C., Sun, J., Hsu, M.-Y., Vallejo-Ramirez, J., Inouye, S., and Inouye, M. (1989) *Science* 243, 1033-1038). The production of msDNA-Ec67 is dependent upon retron-Ec67, which consists of the *msr-msd* region and the gene for reverse transcriptase (RT). These two elements were separately cloned into plasmids; p67-BH0.6 contained the *msr-msd* region and pRT-67 contained the RT gene under the *lpp-lac* promoter-operator. msDNA-Ec67 was produced only when cells were transformed with both plasmids. In addition, msDNA-Ec67 was synthesized in a cell-free system using total RNA prepared from cells harboring plasmid p67-BH0.6 and purified Ec67-RT. Using this cell-free system, the priming reaction, during initiation of DNA synthesis, was demonstrated to be a specific template-directed event; only dTTP was incorporated into a 132-base precursor RNA yielding a 133-base compound. This specific dT addition could be altered to dA or dC by simply substituting the 118th A residue of the putative *msr-msd* transcript with a T or G residue.

The priming reaction was blocked when G was substituted for G at the 15th residue of the precursor RNA transcript, which corresponds to the branched rG residue in msDNA. DNA chain elongation could be terminated by adding ddNTP in the cell-free system, forming a sequence ladder. The DNA sequence determined from this ladder completely agreed with the msDNA sequence. The RT extension reaction was completely blocked when the RNA preparation was treated with RNase A but not when the preparation was treated with DNase. This clearly demonstrates that RNA but not DNA is responsible for the msDNA production. A part of the fully extended cell-free product contained a 13-base RNA strand resistant to RNase A, which is consistent with the previously proposed model. In this model, the 5'-end sequence of the *msr-msd* transcript (a2; bases 1-13) forms a duplex with the 3'-end sequence (a1) of the same transcript, thus serving as a primer, as well as a template for msDNA synthesis by RT. Our results are inconsistent with a model recently proposed by Lease and Yee (Lease, R. A., and Yee, T. (1991) *J. Biol. Chem.* 266, 14497-14503).

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Three different retrons have been identified in *E. coli*, retron-Ec67 (Lampson et al., 1989a), retron-Ec86 (Lim and Maas, 1989), and retron-Ec73 (Inouye et al., 1991; Sun et al., 1991). MsDNAs produced from these retrons are extensively diverged, sharing no sequence homologies in either DNA or RNA structures. MsDNA-Ec67 produced by retron-Ec67 consists of a 67-base single-stranded DNA, which is linked to the 2'-OH group of a rG at the 15th residue of a 58-base msdRNA. The DNA and RNA molecules form a 7-base pair DNA-RNA hybrid at their 3'-ends. The RT gene encodes a polypeptide of 586 amino acid residues consisting of both RT and ribonuclease H (RNase H) domains (Lampson et al., 1989b). RT from retron-Ec67 (RT-67) has been partially purified, and its enzymatic activity has been characterized (Lampson et al., 1990).

In this report, we established a cell-free system for the synthesis of msDNA-Ec67 using partially purified RT-Ec67. MsDNA-Ec67 was synthesized de novo when RT-Ec67 and a total RNA fraction containing the primary transcript from the *msr-msd* region of retron-Ec67 were mixed and incubated in the presence of 4 dNTPs. Using this cell-free system, the priming as well as extension reactions were characterized, and the results thus obtained completely agreed with the proposed model described above (Lampson et al., 1989a), demonstrating that the de novo synthesis of msDNA-Ec67 is carried out on a precursor RNA molecule in a manner specific to the template sequence and dependent upon RT. The present results disagree with a model recently proposed by Lease and Yee (1991), in which a single-stranded DNA identical with the DNA molecule of msDNA is first synthesized in a conventional manner independent from RT and the single-stranded DNA is then later linked to msdRNA by a 2',5'-phosphodiester linkage.

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The abbreviation used is: RT, reverse transcriptase.
**RESULTS**

**Specificity of the First Base Addition in a Cell-free System**

In the proposed model for msDNA synthesis (Dhundale et al., 1987; Lampson et al., 1989a), the primary transcript from the msr-msd region functions as a precursor by forming a stable secondary structure as shown in Fig. 1C. Notably, there is a set of inverted repeat sequences a1 and a2, where a1 is immediately upstream of msd and a2 immediately upstream of the branched G residue within msr (Lampson et al., 1989b). The branched G residue (circled in Fig. 1C) is located at the end of the a1-a2 stem structure, and msDNA synthesis is primed from the 2'-OH residue of the G residue using the bottom RNA strand as a template. The first base or the 5'-end of msDNA is determined by the base at position 118 in Fig. 1C. Thus, the synthesis of msDNA-Ec67 starts from a dT residue, complementary to the A residue at position 118.

In order to investigate the validity of the proposed model, we first established a cell-free system for the synthesis of msDNA-Ec67 using purified RT-Ec67 and the RNA fraction prepared from cells harboring p67-BH0.6. This plasmid contained the msr-msd region and a truncated RT gene from retron-Ec67 as described under "Experimental Procedures." The protein pattern of the partially purified RT analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis is shown in Fig. 2. As shown in Fig. 3, [α-32P]dTTTP (lane 3) was specifically incorporated into a product migrating at the position of 123 nucleotides in size. Neither [α-32P]dGTP (lane 1) nor [α-32P]dTTP (lane 2) was incorporated into the cell-free system. In the case of [α-32P]dTTP (lane 4), two minor bands appeared at positions shorter by 4–5 bases than the major product labeled with [α-32P]dTTP (lane 3). As discussed later, these products were labeled even in the absence of the branched G residue (Fig. 3, lane 16), indicating that these were not associated with msDNA synthesis. When RT was omitted from the reaction mixture, no labeled bands were detected with [α-32P]dTTP (data not shown).

The size of the product labeled with dTTTP agrees well with that of the structure proposed in Fig. 1C; the folded RNA precursor consists of 132 bases, and the addition of a dT residue to the RNA molecule yields an oligonucleotide consisting of 133 bases (see also Fig. 4, Structure II). In order to further test the proposed model for msDNA synthesis, two mutations were constructed; in the first mutation, the A residue at position 118 of the precursor RNA molecule (Fig. 1C) was substituted with a U residue (mut-1) and in the second mutation with a G residue (mut-2). When the mut-1 RNA preparation was used for the priming reaction, [α-32P]dTTP was specifically incorporated into the major product (Fig. 3, lane 6) migrating at the same position as the product labeled with [α-32P]dTTP using the wild-type RNA fraction (lane 3). Similarly [α-32P]dTTP was specifically incorporated with the mut-2 RNA preparation (lane 12). It should be noted that the A to G substitution in mut-2 resulted in a band migrating at the same position as the product labeled with [α-32P]dTTP using the wild-type RNA fraction (lane 3).

**Biosynthesis of msDNA**

[Further content continues with describing the experimental procedures, including bacterial strains, media, and specific techniques for RNA preparation and analysis.]
FIG. 1. Restriction map of pCI-1EP5, the proposed secondary structure of msDNA-Ec67, and a putative secondary structure of the precursor RNA molecule. A, the restriction map of pCI-1EP5 (Lampson et al., 1989b). The BssHII site changed to a BamHI site and the XhoI site created by site-specific mutagenesis are shown by arrowheads. Locations and orientation of msr and msd and the RT gene are shown by arrows. The regions cloned into p67-BH0.6 and p67-RT are indicated by open boxes. B, the structure of msDNA-Ec67 (Lampson et al., 1989b). The branched G is circled and RNA is boxed. Both RNA and DNA are numbered from their 5' ends. C, a putative secondary structure of the precursor RNA molecule. The 5'-end of the RNA transcript was determined by primer extension (Hsu et al., unpublished results). The 3'-end of the RNA molecule is considered to form a stem structure using the inverted repeat sequence, a1 (nucleotides 120-132), and a2 (nucleotides 2-14) (arrows) in the primary RNA transcript (Lampson et al., 1989a, 1989b). The branched G is circled. Bases changed by mutations are indicated by arrows with individual designations.

FIG. 2. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the partially purified RT preparation. RT was purified as described under "Experimental Procedures." Lane 1, molecular weight standards: a, bovine serum albumin (M, 66,000); b, ovalbumin (M, 45,000); c, carbonic anhydrase (M, 31,000). Lane 2, 15 μl of the partially purified RT was analyzed. 10% polyacrylamide was used, and the gel was stained by Coomassie Brilliant Blue.

Type product (lane 3) and the mut-1 product (lane 6).

Previously, we have demonstrated that the branched G residue is essential since the substitution of the G residue with an A residue completely blocked the synthesis of msDNA-Mxl62 in vivo (Hsu et al., 1990). Similarly, in the present cell-free system, the G to A substitution (mut-3 at position 15 in Fig. 1C) completely abolished the specific [α-32P]dTP incorporation into the precursor RNA (compare lane 15 with lane 3 in Fig. 3). Note that the doublet bands are still produced with [α-32P]dCTP even with the mut-3 RNA preparation (lane 16), indicating that these bands are not associated with msDNA synthesis.

In all known msDNAs from both myxobacteria and E. coli (see Inouye and Inouye (1991b) for a review), the base directly opposite to the branched G residue in the folded RNA precursor is always an G residue without exception (Fig. 1C, residue 119). When this G residue at position 119 was changed to A (mut-4), the specific dT incorporation was still observed, but the incorporation was substantially reduced (approximately to 5% of the wild-type incorporation) (data not shown). This indicates that this G residue on the template strand plays an important role in the priming reaction. When the products from the priming reaction in Fig. 3 were digested with RNase A, all yielded products of small molecular weights migrating almost at the front of gel electrophoresis (data not shown).

Elongation of the DNA Strand in the Cell-free System—The procedures of the proposed msDNA synthesis are summarized in Fig. 4. In the experiments described above, it was clearly demonstrated that the first base was added to the precursor RNA molecule in a specific manner such that the first base is complementary to the base position at the A
The reaction for the first base addition was carried out as described under "Experimental Procedures"; the reaction mixture contains an RNA fraction from a 1-ml culture and the mixture was incubated at 37°C for 30 min. The reaction was carried out with the RNA fraction from SB221 cells harboring p67-BH0.6 (wild-type) (lanes 1–4), from cells harboring p67-mut-1 for the A to T mutation at position 118 in Fig. 1C (lanes 5–8), from cells harboring p67-mut-2 for the A to G mutation at position 15 in Fig. 1C (lanes 9–12), and from cells harboring p67-mut-3 for the G to A mutation at position 118 in Fig. 1C (mutation 2) (lanes 9–12), and from cells harboring p67-mut-3 for the G to A mutation at position 15 in Fig. 1C (lanes 13–16). [α-32P]dNTP used for each lane is indicated at the top of each lane. The MspI digest of pBR322 labeled with the Klenow fragment and [α-32P]dCTP was applied to the far left lane as molecular weight markers. Numbers indicate sizes of fragment in bases. An arrowhead indicates the position of the precursor RNA molecule specifically labeled with dNTP for each RNA preparation.

residue in Structure II in Fig. 4. Furthermore, the addition of the first base has been demonstrated to be dependent upon the RT preparation added in the reaction and also upon the rG residue (circled in Fig. 4) at the end of the α1-α2 stem. The model further proposes that the T residue linked to the branched rG residue then serves as a primer to further extend the DNA chain along the RNA template. As the DNA strand is extended, the RNA template is concomitantly removed as shown in Structure III so that the total number of bases of Structure III is almost identical with that of Structure II.

In order to confirm this model, the chain elongation reaction was carried out using the same cell-free system as used for the first base addition in Fig. 3; in addition to [α-32P]dTTP, three other dNTPs, as well as dideoxynucleotides (ddNTPs) were added for separate chain termination reactions (Sanger et al., 1977). After the chain elongation reaction, the products were treated with RNase A to remove single-stranded RNA attached to them. As can be seen in Fig. 5, a ladder is formed, clearly indicating that the DNA chain was elongated using a specific template sequence. The sequence determined from the ladder is identical with the DNA sequence from base 24 to base 54 of msDNA-Ec67 (Fig. 1B). Although some termination of msDNA synthesis occurred at around positions 42–44, most of the reaction terminated at around position 69, forming a strong band in all lanes at position a. This product is most likely the fully extended msDNA-Ec67 (67-base single-stranded DNA) that is linked to a 4-base RNA, AGAU, resulting from RNase treatment (Fig. 4, Structure IVa). The DNA strand is considered to be branched out from the 2'-OH group of the G residue of the tetranucleotide. Note that every band in the sequencing ladder
migrated at a position longer by 2 bases than what was expected from the size of the DNA strand. This was probably caused by the extra 4-base RNA attached at the 5'-end of the DNA strand. The 2-base discrepancy in the mobility in the gel is likely to be due to the branched RNA structure.

When the RNA preparation was treated with RNase-free deoxyribonuclease I (DNase I; Boehringer Mannheim), it was still fully capable of producing msDNA, as can be seen in Fig. 6A, lane 3; three major bands obtained by RT extension reaction in the control experiment (lane 2) were also observed. Note that multiple bands are formed in the present cell-free system (see Fig. 7). In contrast, when treated with RNase A, the RNA preparation completely lost the ability to produce msDNA (lane 4). It should be noted in Fig. 6B, lane 3, that the RNA preparation treated with DNase I completely lost the plasmid DNA, which can be observed in the untreated RNA preparation (lane 2, band c). Note that the RNA band indicated by arrow b was not affected by the DNase I treatment. In contrast, when the RNA preparation was treated with RNase A, the RNA band completely disappeared, whereas the DNA band was unchanged (Fig. 6B, lane 4). These results clearly demonstrate that RNA but not DNA is responsible for the production of msDNA.

**RNA Structure at the 5'-End**—The structure of msDNA-Ec67 produced in vivo has been determined as shown in Fig. 1B (Lampson et al., 1989b), which corresponds to Fig. 4, Structure IVb. On the basis of the proposed model shown in Fig. 4, Structure IVb may also be produced, in which the 5'-end arm of the msdRNA (upstream of the branched rG residue and the sequence from base 1 to 14 in Fig. 1B) forms a double-stranded RNA (14 base pairs), which represents the remaining a1-a2 stem structure from the folded precursor RNA template. In Fig. 5, band b migrated at around 82 bases, which is longer by 13 bases than band a. Since the double-stranded RNA is resistant to RNase A treatment, the 5' end of msdRNA (the a2 region) remained intact as shown in Fig. 4 (IVb). Upon heating before gel electrophoresis the 14-base RNA (a1) was dissociated from the 5' end of msdRNA (a2), resulting in the entire 5' end arm of msdRNA remaining with the DNA strand (see Fig. 4). Thus, band a and b products consist of 71 and 84 bases, respectively, which migrated at 69- and 82-base positions, respectively, in Fig. 5.

To unambiguously prove the existence of Structure Vb, the band b product was extracted from the gel and retreated
with RNase A. As shown in Fig. 7, the purified band b product (lane 3) changed its mobility to the band a position in a sequencing gel when it was treated a second time with RNase A (lane 5). No change in the mobility was observed before and after RNase treatment of band a (lanes 4 and 6, respectively).

Interestingly, the size difference between bands d and c in Fig. 5 is also approximately 13 bases; the size difference between bands d and b or between bands c and a is approximately 35 bases. On the basis of these sizes, the band c product is likely a result of further extension of the single-stranded DNA all the way to the branched G residue using the msdRNA as a template (see Fig. 4). This extension elongates the msDNA by another 35 bases at its 3' end, which agrees well with the size of band c. Such DNA elongation from the 3' end of msDNA has been demonstrated for msDNA-Ec67 with a partially purified RT-Ec67 (Lampson et al., 1990). Thus the band d product is considered to consist of the fully extended msDNA strand (102 bases) plus the 17-base RNA similar to the RNA structure of the band b product (Fig. 4, Structure VIIb).

**DISCUSSION**

All bacterial retroons so far characterized consist of the msr-msd region and the RT gene under a single promoter upstream of the msr-msd region (see Inouye and Inouye, 1991a, 1991b for reviews). However, these two genetic elements function in trans and complement each other to produce msDNA. In the present study, the msr-msd region from retroon-Ec67 was cloned in p67-BH0.6 separately from the RT gene. In addition, the RT gene was cloned in a separate plasmid (pRT-67) under the lpp5::lac promoter. Cells harboring either plasmid could not produce msDNA, whereas cells doubly transformed with both plasmids were able to produce msDNA-Ec67 (Lampson et al., 1989b). This indicates that the msr-msd region cloned in p67-BH0.6 produces an RNA transcript, which functions as a precursor template for the synthesis of msDNA-Ec67.

In this report, we were able to achieve this complementation in a cell-free system using the RNA fraction from cells harboring p67-BH0.6 and RT partially purified from cells harboring pRT-67. The cell-free synthesis of msDNA-Ec67 was initiated de novo by the bacterial RT and from the expected first base. The following features of the cell-free system listed below are consistent with our proposed model for the msDNA system depicted in Fig. 4 (Dundale et al., 1987; Lampson et al., 1989a). 1) The incorporation of the first dNTP, as well as further extension of the DNA chain is dependent upon the addition of RT and the transcript from the msr-msd region. 2) The first base linked to the precursor RNA molecule is always complementary to the base at the 118th position of the precursor RNA molecule (see Fig. 1C). 3) The 15th residue of the primary transcript is a G residue, which is essential for the priming reaction, and corresponds to the branched G residue of msDNA-Ec67 (see Fig. 1, B and C). 4) The compound to which the first dNTP is linked is sensitive to RNase A and detected as a single band in acrylamide gels. From its mobility, the compound appears to consist of 133 bases. 5) When all four dNTPs are added in the reaction mixture, the DNA chain is elongated, forming the major product of approximately 69 bases. 6) The sequence read from the ladder formed in the presence of dNTPs completely matches with the DNA sequence of msDNA-Ec67. 7) The production of msDNA on the RT extension reaction was sensitive to RNase A but not to DNase I. 8) The RNA molecule attached to the 5'-end of the extended DNA molecule is protected from RNase A digestion. This protection from RNase A is due to the formation of a double-stranded structure that represents the remaining a1-a2 stem structure from the folded precursor RNA molecule. 9) The size of RNA removed by the RNase A treatment after denaturation by boiling is 13 bases.

These results do not directly address the exact nature of the base in the precursor RNA molecule from which msDNA synthesis is primed or the exact nature of the linkage between this base and the first base linked to it. However, the properties of the cell-free product agree very well with the established structure of msDNA-Ec67, in which the first base, e dT residue, is linked to the 2'-OH group of the 15th rG residue of the msDNA RNA molecule (Fig. 1B; Lampson et al., 1989b). Attempts to synthesize msDNA-Ec67 in the cell-free system with a msr-msd RNA transcript produced in vitro by SP6 RNA polymerase have been unsuccessful. It is possible that the branched rG residue may be modified or activated in the in vitro msr-msd transcript so that the 2'-OH group of the rG residue is readily used for the priming reaction.

Recently, Lease and Yee proposed an alternate model for msDNA synthesis in which a single-stranded DNA corresponding to the DNA portion of msDNA is first synthesized in a conventional manner (by a 3' to 5' priming reaction). This DNA strand is then ligated to the 2'-OH group of the branched rG residue of msDNA at its 5'-end forming a 2',5'-phosphodiester linkage (Lease and Yee, 1991). The present results are inconsistent with this model.

Although the precise molecular mechanism of the msDNA priming reaction from the 2'-OH group of a specific rG residue remains to be determined, it appears that msDNA is synthesized directly on an RNA template by RT. The Lease and Yee model is based on kinetic (pulse-chase) experiments that were carried out with permeabilized Myxococcus xanthus cells treated with phenylethyl alcohol (Lease and Yee, 1991). Because of very high backgrounds of this system that were
eliminated by computer image processing, the biochemical characterization of the DNA fragment has not been carried out. It should also be noted that in their model, RT does not play a role in msDNA synthesis. RT requirement for msDNA synthesis has been unambiguously established not only for msDNA-Ec67 but also for other msDNAs (Lampson et al. (1989b), Lim and Maas (1989), and the present data; also see Inouye and Inouye (1991a, 1991b) for a review). In this respect, it is interesting to note that msDNA-Ec67 can be produced in yeast if retron-Ec67 is cloned under the GAL10 promoter (Miyata et al., 1992). This product is also dependent on RT-Ec67. This further supports our model.

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