JOURNAL OF BACTERIOLOGY, Oct. 1997, p. 6518–6521 Vol. 179, No. 20
0021-9193/97/$04.00+0

A Mutational Study of the Site-Specific Cleavage of EC83, a Multicopy Single-Stranded DNA (msDNA): Nucleotides at the msDNA Stem Are Important for Its Cleavage

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Received 5 May 1997/Accepted 7 August 1997

Multicopy single-stranded DNA (msDNA) molecules consist of single-stranded DNA covalently linked to RNA. Such molecules are encoded by genetic elements called retrons. Unlike other retrons, retron EC83 isolated from *Escherichia coli* 161 produces RNA-free msDNA by site-specific cleavage of msDNA at 5′-TTGA/A-3′, where the slash indicates the cleavage site. In order to investigate factors responsible for the msDNA cleavage, retron EC83 was treated with hydroxylamine and colonies were screened for cleavage-negative mutants. We isolated three mutants which were defective in msDNA cleavage and produced RNA-linked msDNA. They were all affected in *msd*, a gene for msDNA, with a base substitution at the bottom part of the msDNA stem. In contrast, base substitution at and around the cleavage site has no marked effect on msDNA synthesis or its cleavage. From these results, we concluded that the nucleotides at the bottom of the msDNA stem, but not the nucleotides at the cleavage site, play a major role in the recognition and cleavage of msDNA EC83.

Multicopy single-stranded DNA (msDNA) is a short molecule covalently linked to RNA by a 2′-5′ phosphodiester bond (see Fig. 1) (for reviews, see references 4, 11, and 13). msDNA was found in some gram-negative bacteria, including many isolates of *Escherichia coli* and myxobacteria (2, 6, 8–10, 12, 19, 25). msDNA is produced by a bacterial retroelement called a retron (24). Retrons consist of three genes: *msd* for msDNA, *msr* for msRNA, and *ret* for reverse transcriptase (RT). These genes are on the chromosome in the order *msr*, *msd*, *ret*. They form a single transcription unit called the msDNA operon. The primary transcript contains a pair of inverted repeats at the 5′ end of *msr* and at the upstream end of *msd*. The RNA transcript is able to form a stable secondary structure in which the branched guanine residue of msRNA is positioned by the stem formed by the inverted repeats. RT, the translation product of *ret*, recognizes the structure of the *msr-msd* region of the precursor RNA transcript and initiates reverse transcription (7, 12). The 2′ OH of the branched guanine residue functions as a primer, and the *msd* region of the primary transcript serves as a template (3, 20–22). As reverse transcription progresses, the RNA at the RNA-DNA hybrid region is digested by host RNase H (5, 14, 23). The reaction stops when reverse transcription reaches about the middle of the template. For termination of msDNA synthesis, digestion of template RNA by RNase H is important (14, 23). Since the 2′ OH of the template serves as a primer and the reverse transcription stops at the middle of the template, the product is a single-stranded DNA covalently linked to RNA.

Several years ago one of us showed that, unlike other msDNAs, the msDNA produced by retron EC83 of clinical *E. coli* isolate 161 was not linked to RNA; it was a 79-nucleotide-long single-stranded DNA with a 5′ monophosphate (msDNA EC83) (9). The genomic organization of this retron resembled that of the other retrons with *msr*, *msd*, and *ret*. These three genes were necessary and sufficient for the production of RNA-less msDNA in *E. coli* K-12. It was shown that the msDNA without RNA was derived from an msDNA linked to RNA by nucleolytic cleavage between the fourth and the fifth residues of the DNA component of the RNA-DNA copolymer (9). Nothing is known about the enzyme responsible for such a site-specific cleavage of msDNA. In this study, we investigated the msDNA structure responsible for such a site-specific DNA cleavage.

It was reasonable to assume that the hypothetical enzyme carrying out site-specific cleavage of msDNA EC83 recognizes the bases at the cleavage site, since many sequence-specific endonucleases recognize the base sequence at their cleavage sites and the sequences at the cleavage sites of the two known RNA-less msDNAs, EC83 (9) and EC78 (15), both contain the sequence 5′-TTGA/-3′, where the slash indicates the cleavage site (Fig. 1A). Therefore, we thought the conserved tetranucleotides (5′-TTGA-3′) would play an important role in the recognition and cleavage of msDNA. We tested this idea by performing site-directed mutagenesis of the conserved tetranucleotides into various sequences. The msDNAs produced by these mutants were analyzed, and the results are shown in Fig. 1B.

To our surprise, most base substitution mutations do not affect synthesis of msDNA or its cleavage (Fig. 1B). For example, substitutions of three of four bases hardly affected the synthesis and cleavage of msDNA (Fig. 1B, lane 8). Interestingly, however, when the G at the third position is changed to C, the efficiency of cleavage is moderately reduced (Fig. 1B, lanes 4, 6, and 7). At present the role of this nucleotide in msDNA synthesis is not clear. Next, we asked whether the fifth nucleotide (5′-TTGA/A-3′) plays any role in msDNA cleavage. This was evaluated by mutation of nucleotide A in the sequence 5′-TTGA/A-3′ to C, G, or T. As shown in Fig. 1C, no marked effect on msDNA cleavage was observed. However, substitution of A by either pyrimidine reduces overall msDNA accumulation (Fig. 1C, lanes 2 and 4).

Since the above results showed that the sequence at the cleavage site does not play a major role in msDNA cleavage, we decided to search for mutants defective in msDNA cleavage (cleavage-negative mutants) after random hydroxylamine mu-

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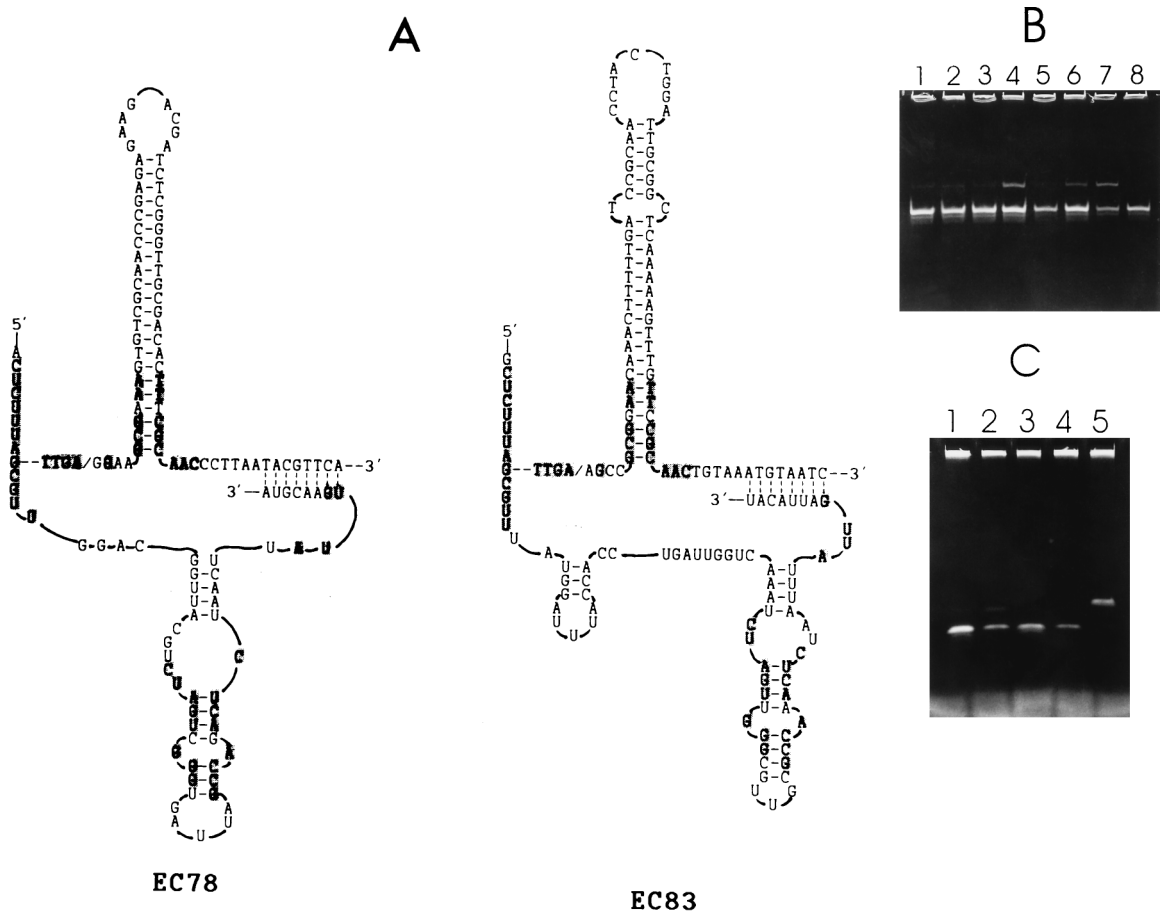

FIG. 1. TTGA/A mutants. (A) Structures of msDNA EC78 (15) and msDNA EC83 (9). The bases conserved in the two msDNAs are shaded. The slash indicates the cleavage site, and the final product is 74 (EC78) or 79 (EC83) nucleotides long (single-stranded DNA). (B) msDNAs produced by TTGA/A mutants. A DNA fragment from the 5′ end to the *Sac*II site of msDNA EC83 (from positions 1 to 10) was replaced with synthetic oligonucleotide [5′-GGCT(T/A)(C/G)(A/T)(A/T)GTAAAGAGCCTG-3′ and 5′-GATCCAGGCTCTTTAC(T/A)(T/A)(G/C)(A/T)AGCCGC-3′], and appropriate mutants were screened by sequencing. msDNAs were extracted from pT161, a positive control (lane 1); the TTGA/A wild type (lane 2); the TTGA/A→TTGT/A mutant (lane 3); the TTGA/A→TTCA/A mutant (lane 4); the TTGA/A→TAGA/A mutant (lane 5); the TTGA/A→TACT/A mutant (lane 6); the TTGA/A→ATCT/A mutant (lane 7); and the TTGA/A→AAGT/A mutant (lane 8). (C) The fifth nucleotide in the sequence TTGA/A was changed into various nucleotides by using synthetic oligonucleotides (5′-GGNTCAAGTAAAGAGC CTG-3′ and 5′-GATCCAGGCTCTTTACTTGANGCCGC-3′), and msDNAs produced by mutants were analyzed. Lanes: 1, wild type as a control; 2, TTGA/A→TG A/C; 3, TTGA/A→TTGA/G; 4, TTGA/A→TTGA/T; 5, RNA-linked msDNA produced by cleavage-negative mutant 31 (see Fig. 2) as a control.

tagenesis of the whole retron. For the screening of a large number of mutants, we took advantage of the mutagenic effect of msDNA (16). It was shown that msDNA with mismatched base pairs is highly mutagenic as a consequence of titration of the MutS protein (17). We assumed that retrons defective in msDNA synthesis would not show such mutagenic effect. Plasmid pT161 contains *msd*, *msr*, and *ret* and confers a strong mutator phenotype (16). *E. coli* CC107 is a Lac^- strain with a frameshift mutation in *lacZ* (1). When this strain is transformed with plasmid pT161 and transformants are spread on a MacConkey-lactose agar plate, it forms a colony with many red papillae because of the high frequency of Lac^+ reversion induced by msDNA (16, 17). Plasmid pT161 was treated with hydroxylamine and transformed into *E. coli* CC107. We looked for colonies producing significantly fewer papillae than the strain with the wild-type plasmid. From about 20,000 transformants examined, 820 colonies with reduced numbers of papillae were picked and their msDNAs were examined by gel electrophoresis. Most of them (715 colonies) produced very little or no msDNA, showing a correlation between msDNA production and mutator function. A total of 105 colonies produced a reduced but moderate amount of msDNA whose size was the same as that of wild-type msDNA. However, we found three colonies which produced msDNA slightly longer than that of the wild type (Fig. 2A, lanes 4, 6, and 8). We thought these might be cleavage-negative mutants since the msDNA obtained from branched RNA-linked msDNA (msDNA EC83) by RNase A treatment is 7 nucleotides longer than the wild-type RNA-free msDNA (9). This is because the three ribonucleotide residues attached to the 5′ end of msDNA are resistant to RNase A digestion (9). To confirm that the msDNAs slightly longer than wild-type msDNA are RNA-linked msDNA, we carried out an RT extension analysis (12). In the RT extension reaction, msDNA serves as a primer, associated RNA serves as a template, and msDNA is extended to the branched residue of RNA by RT. It was shown that RNA-linked msDNA, but not RNA-free msDNA, is extended by RT (9). As shown in Fig. 2A, the msDNAs isolated from the three mutants are extended efficiently (lanes 5, 7, and 9) but the wild-type msDNA, as previously shown, is not (lane 3). Therefore, it is concluded that the mutants we isolated produce

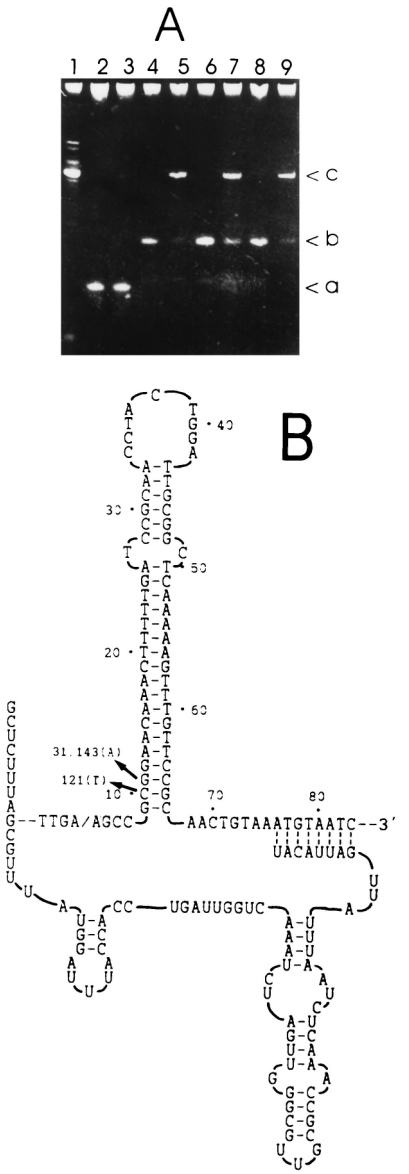

FIG. 2. Isolation and characterization of cleavage-negative mutants. Cleavage-negative mutants were isolated from pT161 by hydroxylamine treatment (18). We isolated three such mutants (mutants 31, 121, and 143) from about 20,000 colonies screened. msDNAs produced by these mutants were analyzed by the RT extension method. Altered bases were identified by sequencing, and their locations are indicated. (A) Mutant msDNAs and their RT extension products. RT extension was carried out as previously described (9, 12), and reaction products were treated with RNase A. Mutant msDNAs and their extension products were analyzed on a 10% polyacrylamide–urea gel. Lane 1, RT-extended *E. coli* B msDNA as a control; lane 2, msDNA from wild-type plasmid pT161; lane 3, wild-type msDNA treated with RT; lane 4, msDNA from mutant 31; lane 5, msDNA from mutant 31 treated with RT; lane 6, msDNA from mutant 121; lane 7, msDNA from mutant 121 treated with RT; lane 8, msDNA from mutant 143; lane 9, msDNA from mutant 143 treated with RT. Bands a, b, and c indicate RNA-free msDNA, RNA-linked msDNA, and RT-extended msDNA, respectively. Note that msDNA from the wild type was not extended, but msDNAs from mutants were efficiently extended by RT. (B) Location of mutation. The locations of mutations were determined by sequencing of the *msr-msd* region of each mutant, and the sites are indicated.

RNA-linked msDNA and thus are defective in msDNA cleavage.

Since it was known that the *msd*, *msr*, and *ret* genes were required for the synthesis of RNA-free msDNA, we asked which of these genes was altered in the cleavage-negative mutants. To that end, the DNA fragment with the *msr-msd* region of each mutant was subcloned into pACYC184 and the resulting plasmid was transformed into an *E. coli* strain producing wild-type RT protein. When msDNA was examined, it was found that the resulting strains still produce RNA-linked msDNAs (data not shown). These results showed that in all three mutants the cleavage-negative phenotype is due to a mutation(s) in *msr-msd*. We determined the nucleotide sequence of the *msr-msd* region of mutant plasmids (Fig. 2B). In one mutant (121) the C at position 10 was changed to a T, and in two other mutants (31 and 143) the G at position 11 was altered to an A. As shown in Fig. 2B, these base substitutions are located at the lower part of the stem of msDNA and they disrupt the stem structure of msDNA EC83.

In this paper, we have shown that for the cleavage of msDNA EC83, specific bases are not required at the cleavage site but the bases at the bottom of the msDNA stem play a major role in the synthesis of RNA-less msDNA. Base substitutions observed in the cleavage-negative mutants break the msDNA stem, but regeneration of the stem structure by secondary mutation on the opposite strand was not sufficient for cleavage of msDNA to occur (data not shown). These results suggest that specific bases in the lower stem are required for the synthesis and cleavage of msDNA, in agreement with previous findings that showed that proper structure of the lower stem is important for the synthesis of RNA-linked msDNA in retron EC107 (20).

This work was supported by the Genetic Engineering Research Program (1995), Ministry of Education, Republic of Korea.

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