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Mutagenesis analysis of a self-cleaving RNA

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ABSTRACT

The hammerhead structural model proposed for sequences that mediate self-cleavage of certain RNAs contains base-paired three stems and 13 conserved bases. Insertion, deletion and base substitution mutations were carried out on a 58 base RNA containing the sequence of the single-hammerhead structure of the plus RNA of the virusoid of lucerne transient streak virus, and the effects on self-cleavage assessed. Results showed that there is flexibility in the sequence requirements for self-cleavage *in vitro*, but alterations of the conserved sequence or predicted secondary structure generally reduced the efficiency of self-cleavage.

INTRODUCTION

RNA-mediated site-specific self-cleavage of the phosphodiester backbone of certain plant pathogenic RNAs (1-6) and of an RNA transcript of a repetitive DNA sequence in the newt genome (7) occurs *in vitro* in the presence of Mg^{2+} to generate 5'-hydroxyl and 2',3'-cyclic phosphodiester termini.

Primary and secondary structural homology exists around the site of self-cleavage for nine self-cleaving RNAs, and consists of three base-paired stems and 13 conserved nucleotides which form a hammerhead-shaped secondary structure (4,8). Deletion of flanking sequences has confirmed that this hammerhead structure is all that is required for self-cleavage (9).

The sequence requirements for the hammerhead structure were investigated by introducing substitutions, deletions and insertions into a short RNA containing the hammerhead sequence of the plus RNA of the virusoid of lucerne transient streak virus (vLTSV; ref. 9). We found that the hammerhead structure can tolerate insertions and deletions in some regions better than others and that both biologically conserved and non-conserved bases can be altered and self-cleavage activity retained.

MATERIALS AND METHODS

All methods were essentially the same as in ref. 10. RNAs were produced by oligodeoxynucleotide directed transcription using T7 RNA polymerase; the method differed from that published (10) in that radioactive transcriptions were done with 0.5 mM UTP. If required, the full-length RNAs were isolated and, after heating at 80°C for one minute in 1 mM

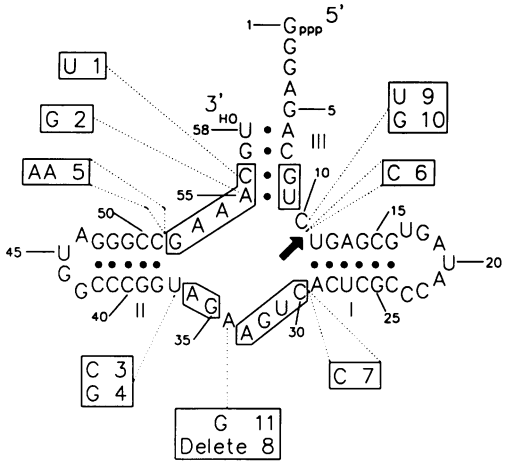

Fig. 1. Mutants of the hammerhead structure of the 58 base plus vLTSV RNA produced by transcription of an oligodeoxynucleotide template (9). The sequence is derived from nucleotides 164 to 216 of plus vLTSV (4), except for the five 5'-terminal nucleotides which were derived from the T7 RNA polymerase promoter. The bases changed in the variant RNAs are indicated together with the number assigned to each mutant for reference to Table 1. Stems are numbered I to III (after ref. 4), the site of cleavage is indicated by an arrow, and bases conserved between all virusoids (4), avocado sunblotch viroid (1), the plus satellite RNA of tobacco ringspot virus (4) and the newt RNA (7) are boxed.

EDTA, pH 6, and snap-cooling on ice, were incubated under two conditions (a) 50 mM $MgCl_2$, 0.5 mM sodium EDTA, 50 mM Tris-HCl, pH 9.0 (Buffer A) at 37°C for 1 h; or (b) 10 mM $MgCl_2$, 0.5 mM sodium EDTA, 50 mM Tris-HCl, pH 8.0 (Buffer B) at 55°C for 1 h. In addition, end nucleotide analysis using nuclease P1 was done essentially as by Uhlenbeck (11).

RESULTS AND DISCUSSION

The RNA used as the wild-type sequence for this mutagenesis study was the 58 base RNA used by Forster and Symons (9). This RNA was produced by transcription from a synthetic DNA template, and contained the sequence of the plus vLTSV hammerhead structure plus five extra 5' terminal nucleotides dictated by the T7 RNA polymerase promoter (Fig. 1). As this structure has a stable stem III, it cleaves by a single-hammerhead structure (9). During the transcription reaction this wild-type 58-mer self-cleaved to about 95%, generating a 48 base 3'-fragment and a 10 base 5'-fragment (Fig. 2, lane 1).

Variants of the plus vLTSV hammerhead sequence were created by transcription of the appropriate template DNAs and their capacity for cleavage during the transcription reaction assessed by polyacrylamide gel electrophoresis and autoradiography. The results are summarized in Table 1 (refer to Fig. 1 for the numbering of the mutants). The full-length RNAs of those

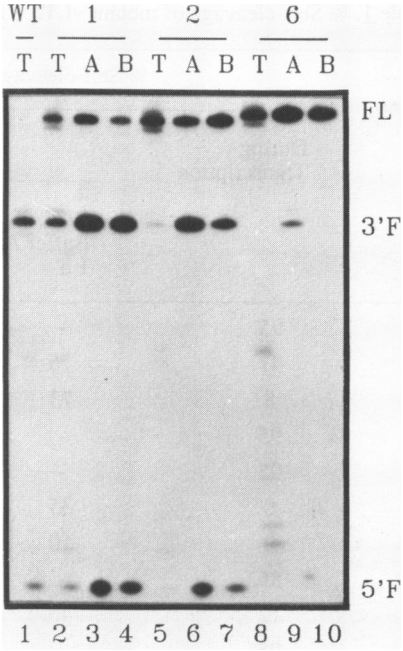

Fig. 2. Self-cleavage of mutant plus vLTSV RNAs analysed by polyacrylamide gel electrophoresis. Lane 1, T7 RNA polymerase transcription of wild-type plus vLTSV hammerhead template DNA. Lane 2, T7 RNA polymerase transcriptions of Mutant 1 DNA template. Lane 3, self-cleavage of Mutant 1 RNA in Buffer A at 37°C for 1 h. Lane 4, as for lane 3, except that incubations were done in Buffer B at 55°C for 1 h. Lanes 5-7, as for lanes 2-4, except that the RNA was Mutant 2. Lanes 8-10, as for lanes 2-4 except that the RNA was Mutant 6. Detection of transcription products was by autoradiography after denaturing polyacrylamide gel electrophoresis. FL; full-length RNA. 5'F; 5'-self-cleavage fragment. 3'F; 3'-self-cleavage fragment.

mutants that cleaved less than 50% during transcription were isolated and incubated for 1h at 37°C in Buffer A and at 55°C in Buffer B after heating and snap-cooling. These conditions were known to result in the efficient cleavage of other RNAs (10).

Self-cleavage occurs in hammerhead structures with the conserved bases altered in stem III

The two mutants in the stem III region (Fig. 1, mutants 1 and 2) demonstrate that the conserved bases can be altered and self-cleavage activity retained, although at a lower level than wild-type. Mutant 1 cleaved to 47% during the transcription reaction (Fig. 2, lane 2; Table 1) and cleaved to high levels when incubated under the two Buffer conditions (Fig. 2, lanes 3,4; Table 1). Mutant 2 cleaved less efficiently (8%) during the transcription reaction (Fig. 2, lane 5; Table 1) than at 37°C in Buffer A (73%; Fig. 2, lane 6; Table 1) or at 55°C in Buffer B (39%; Fig. 2, lane 7; Table 1). Base-pairing within the stem III was possible in these mutants through G·U

Table 1. % Self-cleavage of mutant vLTSV RNAs

RNA	% Self-cleavage		
Wild-type or Mutant No.*	During Transcription	Isolated RNA+	
		37°C Buffer A 1 h	55°C Buffer B 1 h
Wild-type	95	-	-
1	47	76	82
2	8	73	39
3	95	-	-
4	95	-	-
5	0	35	5
6	0	10	0
7	85	-	-
8	12	40	15
9	95	-	-
10	30	67	30
11	95	-	-

* Sequence variants as in Fig. 1.
\+ Only the purified full-length transcripts of sequence variants which self-cleaved less than 50% during the transcription reaction were incubated under the two conditions.

base-pairs, which have approximately the same stability as A·U base-pairs (12). The lowering of self-cleavage efficiency by the substitutions may reflect a lowering of the stability of stem III, and/or a modification of the tertiary interaction within the hammerhead structure. The results indicate that the conserved bases can be varied as long as the stability of stem III is maintained. The importance of the stability of stem III in the formation of the hammerhead structure has been demonstrated (10), and others have found that mutations that abolished the potential base-pairing in stem III also abolished self-cleavage (13-15).

Analysis of the naturally occurring hammerhead RNAs indicates that there is no sequence or size conservation of stem I and II or of their loops (4-8), in fact the loops are not even required for self-cleavage (11,14,16,17). Stem III therefore is unique in having conserved residues (albeit these have been shown not to be crucial) and therefore may be more intimately associated with the self-cleavage site than the other stems.

Is U37 hydrogen-bonded to G52?

The plus vLTSV hammerhead structure is unique in containing a residue (U37) between

the conserved bases of the lower single-stranded region (bases 30-36) and stem II (4). Relative to the hammerhead structures of other RNAs, this extra base represents an insertion of a U. Whether this base is hydrogen-bonded to G52 or whether it is unpaired was investigated by substituting U37 for C and G residues (Fig. 1, mutants 3 and 4, Table 1). Both of these variant RNAs self-cleaved as efficiently as the wild-type sequence, even though only C had the potential to form a Watson-Crick base-pair with G52. This indicates that bases 37 and 52 are not base-paired; presumably it is the tertiary structure of the hammerhead that prevents this from occurring.

Insertion and deletion in the hammerhead structure affects self-cleavage

An AA insertion was made between C51 and G52 (Fig. 1, mutant 5), with the rationale that one A would base-pair with U37 to extend stem II by one base-pair and thereby remove the extra U (see above) from the lower single-stranded region. The second A would then serve as an insertion to the upper left single-stranded region (bases 52-54). It is possible that the tertiary structure that prevented the base-pairing between G52 and U37 also would prevent base-pairing of the inserted A and U37. If that occurred, then there would be five unpaired bases in the upper, left-hand single-stranded region. This RNA did not cleave when transcribed from its DNA template (Table 1); however, the isolated full-length RNA self-cleaved to 35% when incubated in Buffer A (37°C), but to only 5% in Buffer B (55°C) (Table 1). Presumably the high Mg^{2+} concentration and high pH in Buffer A stabilized the active structure of mutant 5, whereas the conditions in the transcription mix (6 mM Mg^{2+}, pH 7.5) were insufficient to do so.

An RNA with a C inserted between bases C10 and U11 (adjacent to the site of cleavage in the wild-type sequence) (Fig. 1, mutant 6) did not cleave during the transcription reaction (Fig. 2, lane 8, Table 1), nor when incubated at 55°C in Buffer B (Fig. 2, lane 10, Table 1), but did self-cleave to about 10% when incubated at 37°C in Buffer A (Fig. 2, lane 9, Table 1). RNA sequencing and 5' end nucleotide analysis of the 3'-self-cleavage fragment of mutant 6 identified that the majority of self-cleavage (about 75%) occurred after the second C, with about 25% of the cleavage occurring after the first C.

An RNA with a C inserted between A29 and C30 (Fig. 1, mutant 7) cleaved to about 85% during the transcription reaction (Table 1). Presumably the active structure of this mutant is stable under the conditions present in the transcription mix, and does not require the high Mg^{2+} concentration and high pH conditions to stabilise it. An RNA with the non-conserved base A34 deleted (Fig. 1, mutant 8) self-cleaved to about 12% during the transcription reaction but self-cleaved more efficiently when the isolated RNA was incubated under the two conditions (40% in Buffer A at 37°C and 15% in Buffer B at 55°C; Table 1).

Previously, deletion of an A from the GAAAC sequence (bases 52-56, Fig. 1) in the double-hammerhead structure of avocado sunblotch viroid was observed to abolish cleavage (8). This, and the results of the insertion and deletion variants presented here indicate that the lower single stranded region of the hammerhead is more tolerant to changes in the number of bases than the upper single-stranded regions. This suggests that the lower single-stranded region is

spatially removed from the critical centre of the self-cleavage structure.

Variants of two non-conserved bases : C10 and A34

The base 5' to the cleavage site is a C in all natural hammerhead RNAs, except for minus vLTSV, in which it is an A (4). RNAs made with the other two bases at this site (Fig. 1, mutants 9 and 10) both self-cleaved. RNAs with a U at that site cleaved as efficiently as the wild type RNA; however, RNAs with a G at this site cleaved to only about 30% during the transcription reaction, 67% at 37°C in Buffer A, and 30% at 55°C in Buffer B (Table 1). Koizumi *et al.* (13), using gel purified RNAs based on the newt hammerhead sequence, also found that a U at this site cleaved efficiently; however, they obtained no cleavage with a G at this site. This difference in results may be due to some effect of the rest of the hammerhead sequence.

Hammerhead structures containing A, C and U, but not G, residues at position 34 have been found in the natural RNAs studied so far (4-7). The efficient self-cleavage of an RNA made with a G at this position (Fig. 1, mutant 11, Table 1) indicates that the lack of an RNA in nature with a G at this site is not due to the inability of such an RNA to self-cleave. It is feasible that naturally occurring self-cleaving RNAs with a G at this site will eventually be discovered.

In conclusion, it appears that there is flexibility in terms of the sequence requirements for self-cleavage *in vitro*. Whilst it appears that the identity of conserved bases and the spacing of the single-stranded regions is not crucial, it is notable that the substitutions generally reduce the efficiency of cleavage. Therefore, *in vivo*, there may be selective pressure on the natural self-cleaving RNAs to maintain the conserved sequences and hence secondary structure.

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