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RNA stem stability in the formation of a self-cleaving hammerhead structure

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ABSTRACT

The proposed single-hammerhead structure of the self-cleaving newt RNA is unstable due to a weak stem III and therefore is unable to mediate self-cleavage. A double-hammerhead structure with greater theoretical stability has been shown to mediate the self-cleavage of this RNA (Forster *et al.*, 1988, Nature **334**, 265). We have found that the double-hammerhead mediated self-cleavage reaction of a 40 base RNA containing the newt sequence (termed nCG) can be converted to a single-hammerhead reaction by increasing the size of stem III and/or of its loop, thereby enabling a single-hammerhead structure to form. In addition, the 5'-self-cleavage fragment of the nCG RNA can act in *trans* to mediate the self-cleavage of a full-length RNA by the formation of a partial double-hammerhead structure.

INTRODUCTION

Site specific self-cleavage of certain low molecular-weight plant pathogenic RNAs occurs *in vitro* in the presence of Mg^{2+} but in the absence of proteins, yielding 5'-hydroxyl and 2',3'-cyclic phosphodiester termini. This reaction is believed to be important *in vivo* in the replication of these RNAs by a rolling circle mechanism, in which multimeric RNAs undergo site specific cleavage to generate monomer units (1-6).

Hammerhead-shaped secondary structures have been proposed for the sequences around the sites of *in vitro* self-cleavage of plus and minus dimeric RNAs of avocado sunblotch viroid (ASBV; ref. 3), the plus RNA of the satellite of tobacco ringspot virus (sTRSV; refs. 4,5), the plus and minus RNAs of the virusoid of lucerne transient streak virus (vLTSV; ref. 6), the plus RNA of the virusoid of subterranean clover mottle virus (vSCMoV; refs. 7,8) and an RNA transcript containing a dimeric copy of the tandemly repeated, 330-base-pair satellite 2 sequence of the newt (*Notophthalmus viridescens*) genome (9). The hammerhead structures consist of three base-paired stems enclosing inner single-stranded regions and include 13 conserved bases (10,11).

Whereas the hammerhead stuctures of the plus RNA of sTRSV and the virusoids appear stable (Fig. 1a), the hammerhead structures of plus and minus ASBV and newt RNAs appear unstable due to the presence of weak stem IIIs with sterically constraining loops (3,10; Fig. 1b). Forster *et al.* (11) proposed more stable secondary structures for these RNAs which involve the interaction of two hammerhead sequences to form a double-hammerhead structure (Fig. 1c) that

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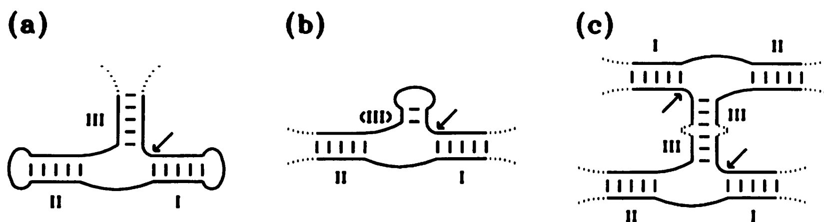


Fig. 1. Schematic diagrams of the three types of hammerhead structures. (a) General hammerhead structure for the virusoid RNAs and the plus RNA of sTRSV showing the stable open-ended stem III with a minimum of 5 base-pairs (6,7). (b) Single-hammerhead structure of the newt and plus and minus ASBV RNAs showing weak two or three base-pair stem III with the constraining two or three base loop (11). (c) Double-hammerhead structure (11) of the newt and plus and minus ASBV RNAs showing the more stable stem III generated by the juxtaposing of two hammerhead sequences. The newt stem III consists of six consecutive base-pairs, the plus ASBV stem III contains six base-pairs with an interior loop of two bases, and the minus ASBV stem III contains eight base-pairs with an interior loop of two bases (11). Stems are numbered I to III (after ref. 6), sites of cleavage, base-pairs, and the interior loops present in plus and minus ASBV are indicated by arrows, dashes and dotted bulges, respectively. The fact that the stem III of the structure in (b) may not form is indicated by parentheses around the stem number.

has a more stable stem III and maintains the other features of the hammerhead model (13 conserved residues and three stems; ref. 11).

Forster *et al.* (11) provided evidence for the double-hammerhead model by demonstrating that the kinetics for the self-cleavage reaction of a 40 base RNA containing the approximate sequence of the newt hammerhead (the nCG RNA, previously termed the newt-like RNA; ref. 11; Fig. 2a) approximated those expected for a bimolecular reaction, as predicted by the double-hammerhead model.

In the work described here, the self-cleavage reaction of the nCG RNA was changed from a double-hammerhead to a single-hammerhead reaction by stabilising the stem III of the single-hammerhead structure; this was achieved by increasing the size of stem III and/or its loop. Also, the 5'-self-cleavage fragment of the nCG RNA was able to catalyse in *trans* the cleavage of the full-length RNA via the formation of a partial double-hammerhead structure.

MATERIALS AND METHODS

Preparation of RNAs

RNAs were produced by oligodeoxynucleotide directed transcription using T7 RNA polymerase (10,12). Oligodeoxynucleotide templates were synthesized on an Applied Biosystems Model 380B DNA synthesizer (Bresatec, Adelaide) and annealed to an 18-mer primer at an equimolar concentration of 0.2 pM, by heating at 65°C for 3 minutes in 10 mM Tris-HCl, pH 7.0, and snap-cooling on ice. Non-radioactive transcription reactions contained 40 mM Tris-HCl, pH 7.5, 12 mM $MgCl_2$, 1 mM spermidine trihydrochloride, 10 mM DTT, 2 mM of

each NTP, 0.01% Triton X-100, 0.05 µg/µl BSA and 10 U/µl T7 RNA polymerase (Bresatec, Adelaide). Radioactive transcriptions differed by having 6 mM $MgCl_2$, 0.5 mM ATP, CTP, GTP and 0.025 mM UTP (to generate a low concentration of RNA), 1.25 µCi/µl α-^{32}P-UTP (Bresatec, Adelaide) and 1 U/µl T7 RNA polymerase. Incubations were at 37°C for 1.5 h. The transcription products were separated by denaturing polyacrylamide gel electrophoresis and, where appropriate, the full-length RNAs were excised, eluted and ethanol precipitated. RNA concentrations were estimated by U.V. spectroscopy or liquid scintillation counting. The sequences of the full-length RNAs were checked by enzymic RNA sequencing (13).

Self-cleavage reactions

0.05 ng/µl of ^{32}P-labelled RNA was used in all reactions and non-radioactive RNA added to achieve the required concentrations. Prior to incubation, RNAs in 1 mM sodium EDTA, pH 6.0, were heated at 80°C for 1 min and snap-cooled on ice. The self-cleavage reactions were incubated under two conditions, either at 37°C in 50 mM $MgCl_2$, 0.5 mM sodium EDTA, 50 mM Tris-HCl, pH 9.0, (Buffer A) or at 55°C in 10 mM $MgCl_2$, 0.5 mM sodium EDTA, 50 mM Tris-HCl, pH 8.0, (Buffer B) for various times (5 µl final volume). The higher pH and $MgCl_2$ was used at 37°C to give efficient self-cleavage; however, at 55°C this buffer resulted in a high level of non-specific degradation of the RNA. Therefore, a lower pH and a lower $MgCl_2$ concentration was used in Buffer B. Reaction mixes incubated at 55°C were covered by liquid paraffin to prevent evaporation. Reactions were terminated by the addition of an excess of EDTA over $MgCl_2$ and an equal volume of formamide (6). Products were resolved on 7 M urea, 10% polyacrylamide gels run in 90 mM Tris-borate, pH 8.3, 2 mM sodium EDTA. To decrease the migration of the small RNA fragments, an ionic-strength gradient in the gel was generated by the addition of 3 M sodium acetate, to a final concentration of 0.5 M, to the bottom buffer tank. The bands were excised and liquid scintillation counting used to determine the extent of self-cleavage.

RESULTS

The approach used for the conversion of self-cleavage of the nCG RNA (Fig. 2) from a double- to a single-hammerhead reaction involved site-directed mutagenesis of the nCG RNA to enlarge the size of the two base-pair stem III and/or its loop. Cleavage by a double-hammerhead structure is a bimolecular reaction (Fig. 3a), and therefore, the efficiency of cleavage is dependent on the concentration of the RNA. The efficiency of single-hammerhead cleavage, however, as it is a unimolecular reaction, is independent of RNA concentration. Transcription reactions to generate radioactively-labelled RNA were carried out under conditions of low UTP concentration (0.025 mM UTP), yielding a low concentration of RNA. Virtually complete cleavage of an RNA during the transcription reaction was taken to indicate single-hammerhead cleavage. This was further verified by the efficient cleavage of low concentrations (0.05 ng/µl) of the isolated full-length RNA when incubated with $MgCl_2$.

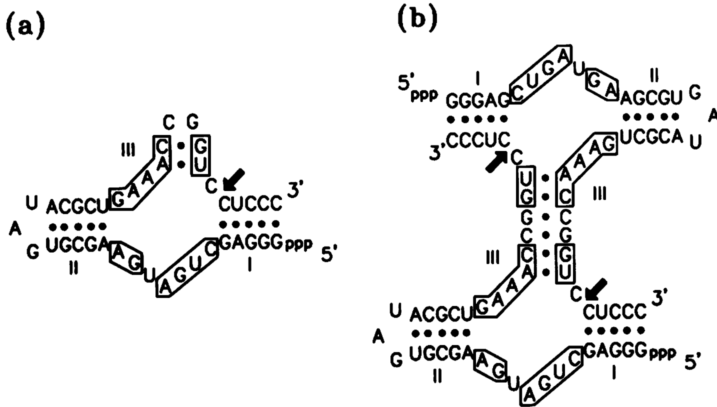


Fig. 2. Sequence of the 40 base nCG RNA (11) generated by T7 RNA polymerase transcription of a synthetic oligodeoxynucleotide template. (a) Drawn as a single-hammerhead structure. (b) Drawn as a double-hammerhead structure. Certain residues have been altered compared with the hammerhead sequence from the RNA transcript of the newt satellite 2 sequence to conform to the T7 promoter requirements and to reduce the possibility of alternative secondary structures. Stems and sites of cleavage are indicated as in Fig. 1, and bases conserved between the virusoid, ASBV, plus sTRSV and newt RNAs are boxed.

Little, or no, cleavage during the transcription reaction does not preclude the possibility of single-hammerhead cleavage, as the RNA may be folded into inactive structures as the nascent RNA emerges from the RNA polymerase during the transcription reaction. Therefore, to investigate whether an RNA that did not cleave during the transcription reaction was capable of self-cleavage by either a single- or a double-hammerhead structure, full-length RNA transcripts were isolated and incubated at various concentrations under two conditions (Buffer A at 37°C and Buffer B at 55°C; see Materials and Methods), which preliminary experiments with several RNAs had indicated usually gave efficient cleavage. Single- and double-hammerhead cleavage could be distinguished on the basis of whether the efficiency of cleavage during the reaction was dependent or independent of the RNA concentration.

Cleavage of the nCG RNA can be catalysed by the 5'-self-cleavage fragment

Figure 2 shows the 40-mer nCG RNA (11) drawn as (a) single- and (b) double-hammerhead structures. The RNA did not cleave during the transcription reaction (11). Due to the imprecise termination of the T7 RNA polymerase (12), a doublet of bands is generated (Fig. 8a, lane 1). Enzymic RNA sequence analysis (13) determined that the lower band was the full-length 40 base RNA, and this RNA was used for the experiments described below.

The graph of self-cleavage efficiency versus time for this RNA incubated at 55°C in Buffer B (Fig. 3a; taken from ref. 11) shows that the extent of self-cleavage increased with increasing concentration of RNA. The cleavage of the 40 base RNA generated a 35 base 5'-fragment and a 5 base 3'-fragment (Fig. 2). Cleavage of the lowest concentration of this RNA

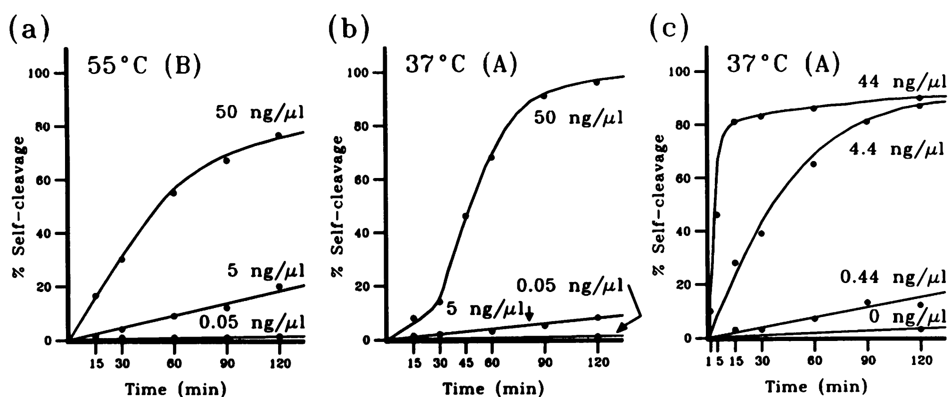


Fig. 3. Effect of RNA concentration on the extent (%) of self-cleavage of the 40 base nCG RNA as a function of time. (a) The 40 base nCG RNA at concentrations of 0.05, 5.0, 50 ng/μl, incubated at 55°C in Buffer B, taken from ref. 11. (b) As for (a), except that the RNAs were incubated at 37°C in Buffer A. (c) The 35 base 5'-self-cleavage fragment of the nCG RNA at a concentrations of 0, 0.44, 4.4, and 44 ng/μl catalysing the cleavage of 0.05 ng/μl of full-length (40 base) RNA. Reactions were carried out at 37°C in Buffer A. (44 ng/μl of 35 base 5'-fragment corresponds to the same molarity as 50 ng/μl of 40 base full-length RNA.)

(0.05 ng/ul) was very low, indicating that cleavage due to a single-hammerhead, if it occurred at all, was a very minor contributor to the total self-cleavage.

The graph of the cleavage reaction carried out at 37°C in Buffer A is given in Figure 3b. It differs from the 55°C graph (Fig. 3a) in that the efficiency of cleavage at 5 ng/μl is lower while the 50 ng/μl line demonstrates a sigmoidal shape, which was reproducible with different preparations of RNA.

The reason for the sigmoidal-shaped curve for the cleavage reaction of 50 ng/μl nCG RNA at 37°C in Buffer A was investigated by carrying out self-cleavage reactions in which a small amount of full-length nCG 40 base RNA (0.05 ng/μl, radioactively labelled) was incubated with various concentrations of non-radioactive 35 base 5'-self-cleavage fragment RNA. Figure 3c shows the graph of the results for these reactions carried out at 37°C in Buffer A. Clearly the 5'-fragment is capable of catalysing the cleavage of a fixed, low concentration of the full-length RNA, presumably by interacting with it to form a partial double-hammerhead structure (Fig. 4a).

On the basis of these results, the sigmoidal curve obtained at 37°C (Fig. 3b) can be interpreted as follows. The proposed nCG single-hammerhead structure (with three stems; Fig. 2a) does not form due to the low stability of stem III (11) and consequently no single-hammerhead self-cleavage occurs. Inactive structures containing stems I and II, but not stem III (Fig. 4b) (and also other inactive structures) are probably reasonably stable at 37°C, so that their denaturation would occur relatively slowly. Such structures would, therefore, reduce the ability of the double-hammerhead structure to form as this requires the denaturation of two of these stable molecules and their subsequent interaction. The 5'-fragment lacks bases 36 to 40 (part of

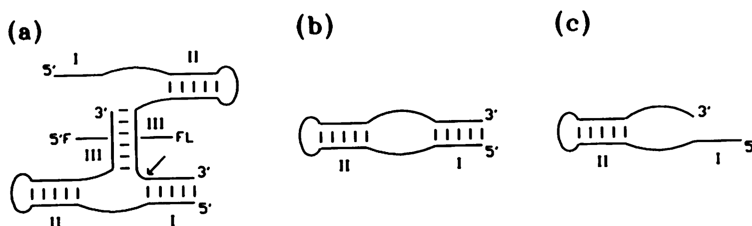


Fig. 4. Schematic diagrams of nCG full-length (FL) and 5'-fragment (5'F) secondary structures. (a) Proposed partial double-hammerhead structure formed by interaction of one full-length RNA with one 5'-fragment, capable of catalysing the cleavage of the full-length RNA. (b) Proposed structure stable at 37°C in buffer A but inactive in self-cleavage activity. The structure is similar to a single-hammerhead except that it does not have a stem III. (c) Proposed secondary structure of 5'-fragment demonstrating that this 35 base RNA can only form stem II. Stems, site of cleavage and base-pairs are as indicated in Figure 1.

stem I) and so would not be capable of forming such stable structures (Fig. 4c). As a consequence, the formation of a partial double-hammerhead structure by the interaction of a full-length RNA and a 5'-fragment (Fig. 4a), resulting in the cleavage of the full-length RNA, would occur more readily as it is easier to form than the standard double-hammerhead. Slow initial cleavage due to the standard double-hammerhead, followed by cleavage catalysed by the 5'-fragment would then occur. Hence, the rate of self-cleavage would increase as more 5'-fragment was generated and would eventually plateau as full-length RNA became exhausted, giving a sigmoidal shape when % cleavage is plotted against time (Fig. 3b).

Incubation of 0.05 ng/µl of full-length RNA with excess 5'-fragment would be expected to result in pseudo-first order reaction kinetics. However, the reaction showed roughly second order kinetics, with the increase in the initial reaction rate being approximately 100 fold for the 10 fold increase in the 5'-fragment concentration from 0.44 to 4.4 ng/µl and from 4.4 to 44 ng/µl, This indicates that only a fraction of the 5'-fragment molecules were participating in the reaction, presumably the remainder were base-paired into complexes that did not allow their interaction with the full-length RNA.

Increasing the size of the nCG single-hammerhead stem III loop from two to four bases can convert self-cleavage from a double- to single-hammerhead reaction

The 2 base single-hammerhead stem III loop of the nCG RNA was increased in size by one or two bases, and the effect on self-cleavage determined.

An RNA, termed nUCG, was constructed with a U residue inserted into the stem III loop to give a loop sequence of UCG. It is shown as a single-hammerhead structure, with a two base-pair stem and a three base loop, and as a double-hammerhead structure in Figure 5a. The nUCG RNA did not self-cleave during the transcription reaction and cleaved poorly even at high concentrations of RNA at both 37°C in Buffer A and at 55°C in Buffer B (5% cleavage after 2 h incubation at 50 ng/µl; results not shown). Presumably the RNA is folded into inactive structures

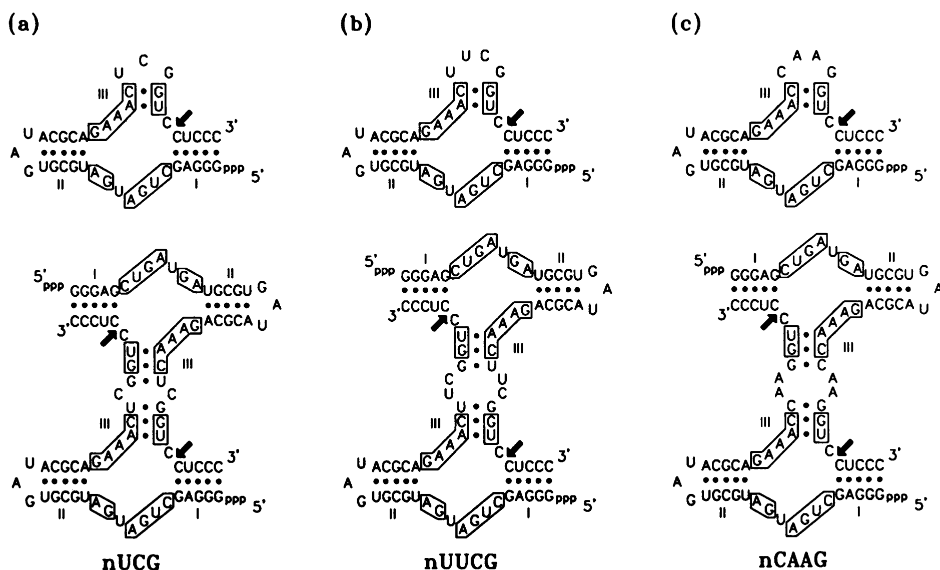


Fig. 5. Sequence of RNAs generated by T7 RNA polymerase transcription of synthetic DNA templates drawn as both single- and double-hammerhead structures. (a) nUCG RNA, (b) nUUCG RNA, (c) nCAAG RNA. In addition to the bases added to enlarge the stem III loop, one base-pair in stem II has been inverted relative to the nCG RNA to reduce the possibility of alternative secondary structures. Stems, sites of cleavage and conserved nucleotides are indicated as in Figure 2.

in preference to the hammerhead structures even though examination of the nUCG sequence (confirmed by enzymic RNA sequencing) did not reveal any major potential alternative secondary structure. These results therefore reflect the low stability of the nUCG hammerhead structures relative to the inactive structures.

An RNA with a stem III loop sequence of UUCG (two U residues inserted into the stem III loop) termed nUUCG is shown as a single-hammerhead structure, with a two base-pair stem III and a four base loop, and as a double-hammerhead structure in Figure 5b. The nUUCG RNA did not self-cleave during the transcription reaction. However, when isolated full-length nUUCG RNA at three concentrations (0.05, 0.5, 5.0 ng/µl) was incubated at 37°C in Buffer A and at 55°C in Buffer B, it self-cleaved and generated a 37 base 5'-fragment and a 5 base 3'-fragment. At 37°C (Fig. 6a) nUUCG RNA self-cleaved in a concentration dependent manner; i.e., the extent of self-cleavage was greater at the higher concentrations of RNA, with about 80% self-cleavage after 2 h at 5 ng/µl. At 55°C, however, cleavage was independent of the concentration of RNA, with about 20% cleavage after 2 h at all three concentrations (Fig. 6b).

The lack of concentration dependent cleavage at 55°C can be explained by considering that the nUUCG double-hammerhead structure is not able to form due to the presence of the

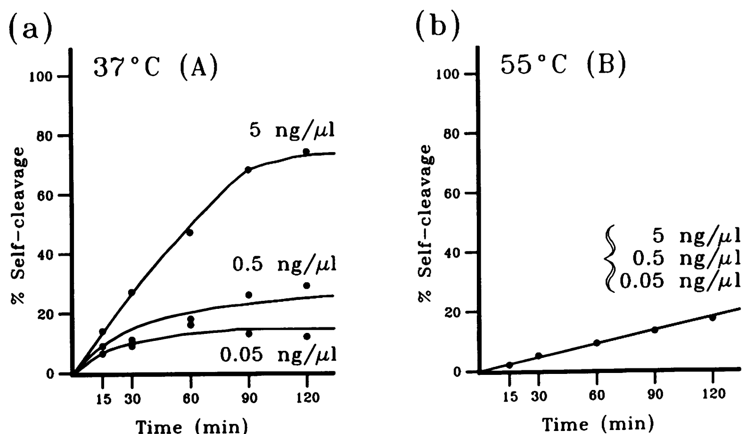


Fig. 6. Effect of nUUCG concentration on the extent (%) of self-cleavage as a function of time. The 42 base nUUCG RNA (Fig. 5b) was incubated at concentrations of 0.05, 0.5 and 5.0 ng/μl (a) at 37°C in Buffer A, and (b) at 55°C in Buffer B.

destabilising interior loop in stem III (Fig. 5b). The concentration independent cleavage that occurs indicates that the single-hammerhead structure is stable. At 37°C it appears that both single- and double-hammerhead structures are stable and therefore there is a proportion of single-hammerhead cleavage with an additional amount of double-hammerhead cleavage which is greater at the higher RNA concentrations. Very similar results (not shown) were also obtained for an RNA with a stem III loop sequence of CAAG (termed nCAAG, Fig. 5c).

Overall, these results indicate that the double-hammerhead cleavage reaction of the nCG RNA can be converted to a single-hammerhead cleavage reaction under appropriate conditions by increasing the stem III loop size from two to four bases with a stem III of two base-pairs.

RNAs with a three base-pair stem III and three or four base loop can self-cleave by a single-hammerhead structure

Recently Tuerk *et al.*(14) reported that RNA base-paired stems closed by a C·G base pair and with loops of sequence UUCG were unusually stable. Switching the top base pair from a C·G to a G·C reduced the stability of the stem markedly, as did substituting the C in the loop for a U (14). RNAs with sequence based on the nCG RNA with three base-pair stem IIIs and four base loops (termed nUUCG(CG), nUUCG(GC) and nUUUG(CG) to indicate the loop sequence and the orientation of the closing base-pair) were made using these data (Fig. 7a-c).

Both the nUUCG(CG) and nUUUG(CG) RNAs self-cleaved by a single-hammerhead structure, as indicated by their nearly complete cleavage during the transcription reaction (Fig. 8a, lanes 2,3) and the efficient self-cleavage of the isolated RNA when incubated at low concentration (0.05 ng/μl) in Buffer A at 37°C and in Buffer B at 55°C for 10 min (results not shown).

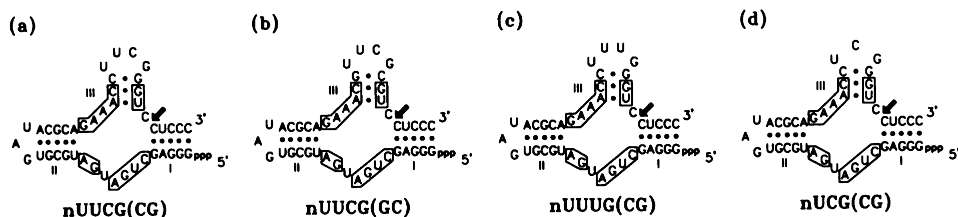


Fig. 7. Single-hammerhead structures of RNAs with stem IIIs of three base-pairs and increased loop size relative to the nCG RNA, generated by T7 RNA polymerase transcription of synthetic DNA templates. The RNAs are based on the nCG sequence (Fig. 2a) but in addition to the bases inserted into the stem III and loop, one base-pair in stem II was inverted to reduce the possibility of alternative secondary structures. (a) nUUCG(CG) RNA. (b) nUUCG(GC) RNA. (c) nUUUG(CG) RNA. (d) nUCG(CG) RNA. Stems, sites of cleavage and conserved nucleotides are indicated as in Fig. 2.

nUUCG(GC) RNA, in contrast, cleaved poorly during the transcription reaction (Fig. 8a, lane 4). Isolated full-length nUUCG(GC) RNA was therefore incubated under self-cleavage conditions at concentrations of 0.05, 0.5, 5.0 ng/µl. Self-cleavage occurred and was independent of RNA concentration when carried out at 55°C in Buffer B (Fig. 8b), indicating that the RNA was cleaving as a single-hammerhead structure. At 37°C in Buffer A, the initial reaction rate was independent of the RNA concentration although the total % cleavage was greater at the higher concentrations of RNA (Fig. 8c). This indicates that single-hammerhead cleavage occurred at all concentrations of RNA and in addition to this, double-hammerhead cleavage occurred at the higher concentrations of RNA. It appears that at the start of the self-cleavage reaction, after the heating and snap-cooling step, the RNA was initially a mixture of active and inactive structures. The high initial rate of cleavage reflects the rapid cleavage of the active structures. The inactive structures presumably underwent slow transformation to active structures, resulting in the plateau in the graph of cleavage efficiency versus time (Fig. 8c). In contrast to the rapid initial rate of cleavage at 37°C, at 55°C in Buffer B there was a more gradual transition from inactive to active structures, resulting in a slower initial rate of self-cleavage.

These results indicate that all three RNAs (nUUCG(CG), nUUUG(CG) and nUUCG(GC)) are capable of single-hammerhead cleavage. The reported destabilization of stems by switching the top base-pair or altering the sequence of the loop (14) does not appear to have been sufficient to weaken the nUUCG(GC) and nUUUG(CG) single-hammerhead structures, compared with the nUUCG(CG) structure, to the extent of abolishing single-hammerhead cleavage.

An RNA with a 3 base-pair stem III and a 3 base loop of sequence UCG (termed nUCG(CG), Fig. 7d) did not cleave during the transcription reaction (results not shown), indicating that the nascent RNA adopted an inactive conformation. The isolated full-length RNA gave a virtually identical cleavage pattern to nUUCG(GC); i.e., it showed non-concentration

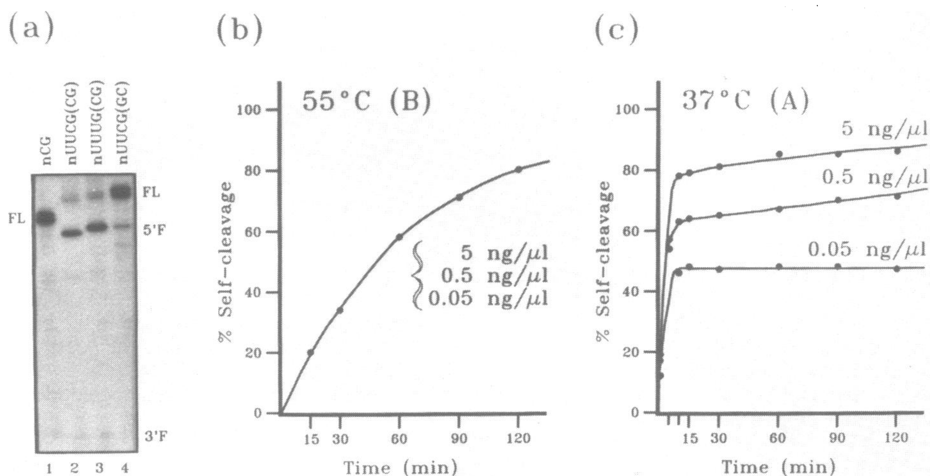
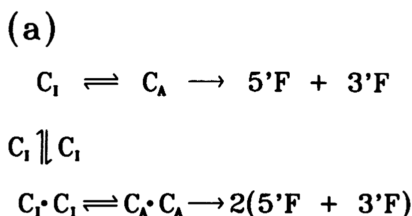


Fig. 8. Self-cleavage of RNAs with enlarged stem IIIs and loops. (a) T7 RNA polymerase transcriptions of oligonucleotide templates. Detection of transcription products was by autoradiography after denaturing polyacrylamide gel electrophoresis. Lane 1, transcription of newt-like template, yielding the 40 base full-length RNA, and a 41 base RNA resulting from imprecise termination of the polymerase. Lane 2, transcription of nUUCG(CG) template. Lane 3, transcription of nUUUG(CG) template. Lane 4, transcription of nUUCG(GC) template. FL; full-length RNA. 5'F; 5'-self-cleavage fragment. 3'F; 3'-self-cleavage fragment. Labels on the left refer to the nCG track and the labels on the right refer to the other tracks. (b) Plot of % self-cleavage of nUUCG(GC) RNA at concentrations of 0.05, 0.5 and 5 ng/µl versus time. Reactions undertaken at 55°C in Buffer B. (c) As for (b), except reactions undertaken at 37°C in Buffer A.



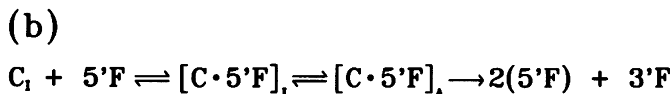


Fig. 9. Proposed interaction between inactive and active conformations of the various RNAs and the pathways leading to their self-cleavage. (a) Self-cleavage by single-hammerhead (C_A) and double-hammerhead (C_AC_A) structures. Self-cleavage generates a 5'-fragment (5'F) and a 3'-fragment (3'F), A and I indicate active and one or more inactive conformations, respectively. (b) Self-cleavage of the nCG RNA catalysed by the 5'-fragment (5'F). $[C.5'F]_A$ and $[C.5'F]_I$ represent active and one or more inactive conformations, respectively, of the full-length RNA:5'-fragment complex.

dependent cleavage at 55°C in Buffer B, but demonstrated both double- and single-hammerhead cleavage at 37°C in Buffer A (results not shown).

These results indicate that self-cleavage occurs in single-hammerhead structures with a 3 base-pair stem III and a 3 or 4 base loop.

DISCUSSION

Stabilizing the single-hammerhead stem III converts a double-hammerhead reaction into a single-hammerhead reaction

The results reported in this paper demonstrate that the double-hammerhead self-cleavage reaction of the nCG RNA (Fig. 2a) can be converted into a single-hammerhead reaction by enlarging stem III and/or its loop to give a more stable single-hammerhead structure.

This work and the previous work by Forster *et al.* (11) have demonstrated that the stability of stem III is important in the formation of the active self-cleaving hammerhead structure. The stem III of the nCG single-hammerhead structure is unable to form due to its low stability and consequently the RNA is unable to adopt the correct tertiary structures required for self-cleavage. The double-hammerhead structure of the nCG RNA, on the other hand, is sufficiently stable to allow the adoption of the correct tertiary structure leading to the lowering of the activation energy of the specific phosphodiester bond breakage, resulting in self-cleavage. However, when the single-hammerhead stem III is made more stable, by increasing the size of the stem and/or the loop, then the single-hammerhead structure is able to form and mediate self-cleavage. Our results indicate that the minimum stem III requirement for single-hammerhead cleavage is a stem III of two base-pairs with a loop of four bases or a three base-pair stem with a three base loop.

The relative stabilities of inactive and active structures determine the pathway and extent of the self-cleavage reaction

On the basis of the work presented here, and from earlier results (6,15), we predict that Figure 9a describes the reaction pathways for the self-cleavage of the variant RNAs. In order for self-cleavage to occur, the inactive structures (represented by monomer C_I and dimer C_IC_I in Fig. 9a) undergo transformation to an active structure, either single-hammerhead (C_A; Fig. 9a) or double-hammerhead (C_AC_A; Fig. 9a). An additional pathway (Fig. 9b) is open to the nCG RNA as self-cleavage can be mediated in *trans* by the 5'-self-cleavage fragment (5'F; Fig. 9b)(Figs. 3c,4). In this pathway, the full-length and 5'-fragment RNAs initially undergo denaturation to allow their interaction. The resulting inactive full-length RNA:5'-fragment structure ($[C.5'F]_I$; Fig. 9b) then undergoes a conformational change to the active structure ($[C.5'F]_A$; Fig. 9b) and cleavage occurs in the full-length RNA. Which pathway the cleavage reaction of any particular RNA follows depends on the relative stabilities of the single-, double-, or partial double-hammerhead and inactive structures under the conditions used.

The rate limiting step of the self-cleavage reaction appears to be the formation of the active structures (C_A or C_AC_A, and $[C.5'F]_A$ for the nCG RNA; Fig. 9). Forster and Symons (10)

found that quantitative cleavage of a 52-mer containing the plus vLTSV hammerhead sequence occurred in less than one minute, indicating that once the active structure had formed, self-cleavage was very rapid. This is consistent with the results obtained here. At 37°C, RNAs with a three base-pair stem III (nUCG(CG) and nUUCG(GC); Fig. 7c,d) appear to form the active structure rapidly as the majority of the self-cleavage occurred in less than fifteen minutes (Fig. 8c; and results not shown). At 55°C, when the hammerhead structures are presumably less stable, and would therefore form less readily, the rate of cleavage was slower (Fig. 8b; and results not shown), indicating that the transition from inactive to active structures occurred more slowly.

The sigmoidal-shaped curve for the self-cleavage of 50 ng/µl of nCG RNA (Fig. 3b) also demonstrates that the formation of the active structure is rate-limiting. The rate of cleavage is slow until the generation of the 5'-fragment allows the formation of the partial double-hammerhead at which stage the reaction rate increases markedly, as the active structure becomes easier to form.

Inactive structures have a major impact on the nature of the reaction. During the transcription reaction, nUUCG(CG) and nUUUG(CG) self-cleaved to approximately 95%, whereas nUUCG(GC) self-cleaved to only about 5% (Fig. 8a, lane 4). The isolated full-length RNA of all three variants, however, self-cleaved to approximately the same extent (60%-80%, 0.05 ng/µl RNA, 37°C in Buffer A for 15 min; results not shown). Examination of the sequence of nUUCG(GC) reveals regions of alternative base-pairing that may form preferentially to the active structure as the nascent RNA emerges from the RNA polymerase resulting in an inactive structure. Presumably, upon isolation of the RNA from the gel, ethanol precipitation and heating and snap-cooling prior to the incubation, the inactive structures are refolded into a mixture of active and inactive structures. The fraction of RNA that does not cleave presumably is folded into stable inactive structures.

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