# HAMMERHEAD MEDIATED SELF-CLEAVAGE OF PLANT PATHOGENIC RNAs

A thesis submitted to the University of Adelaide
for the degree of Doctor of Philosophy

by

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CONTENTS

## CHAPTER 6 ALTERNATIVE HAMMERHEAD STRUCTURES IN THE *IN VITRO* SELF-CLEAVAGE OF ASBV RNAs

**STATEMENT**

This thesis contains no material which has previously been submitted for an academic record at this or any other University, and is the original work of the author except where due reference is made in the text. The citations of my collaborative papers refer to work of the other authors and not my own. I consent to this thesis being made available for photocopying and loan.

Candice C. Sheldon

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## ABBREVIATIONS

| | |
|---|---|
| ASBV | avocado sunblotch viroid |
| BCIG | 5-brom-4-chlor-3-indolyl-β-D-galactopyranosid |
| BSA | bovine serum albumin |
| cDNA | complementary DNA |
| DTT | dithiothreitol |
| *E. coli* | *Escherichia coli* |
| EDTA | ethylenediaminetetraacetate |
| HDV | hepatitis delta virus |
| IPTG | isopropyl β-D-thiogalactopyranoside |
| LTSV-N | lucerne transient streak virus - New Zealand isolate |
| PCR | polymerase chain reaction |
| PEG | polyethylene glycol |
| NMP | nucleoside monophosphate |
| NTP | nucleoside-5'-triphosphate |
| RNase | ribonuclease |
| SDS | sodium dodecyl sulphate |
| sArMV | satellite RNA of arabis mosaic virus |
| sBYDV | satellite RNA of barley yellow dwarf virus |
| sCYMV-S1 | satellite RNA (1) of chicory mottle virus |
| sTRSV | satellite RNA of tobacco ringspot virus |
| Tris | tris(hydroxymethyl)aminomethane |
| vLTSV-A | virusoid of lucerne transient streak virus - Australian isolate |
| vLTSV-N | virusoid of lucerne transient streak virus - New Zealand isolate |
| vLTSV-Ca | virusoid of lucerne transient streak virus - Canadian isolate |
| vSCMoV | virusoid of subterraneum clover mottle virus |
| vVTMoV | virusoid of velvet tobacco mottle virus |

## SUMMARY

Viroids, virusoids and satellite RNAs are believed to replicate by rolling circle mechanisms, involving the production of multimeric plus and minus RNA species, which are processed to monomeric forms. RNA-mediated self-cleavage reaction, observed *in vitro* for many plant pathogenic RNAs, are believed to be responsible for this *in vivo* processing event. Hammerhead-shaped RNA secondary structures (containing three base-paired stems and 13 conserved nucleotides) have been proposed for the sequence around the site of *in vitro* self-cleavage for the many plant pathogenic RNAs, and also a self-cleaving newt RNA.

The work presented in this thesis has investigated the sequence and structural requirements for this *in vitro* hammerhead-mediated self-cleavage reaction.

1) Mutagenesis of an RNA containing the plus vLTSV hammerhead sequence revealed flexibility in the sequence requirements for self-cleavage *in vitro*, however, alterations of the conserved sequence or predicted secondary structure generally reduced the efficiency of self-cleavage.

2) The plus RNA of ASBV and an RNA containing the newt hammerhead sequence were demonstrated to undergo *in vitro* self-cleavage by a variation of the hammerhead structure: the double-hammerhead structure. It was demonstrated that these two RNAs were unable to form (single-) hammerhead structures due to the low stability of one stem (stem III) in the single-hammerhead structure. The newt RNA could self-cleave by a single-hammerhead structure, if its stem III was made more stable, by increasing the size of stem III and/or its loop.

The possible importance of the hammerhead self-cleavage reaction *in vivo* in the replication of the vLTSV was investigated.

3) Mutations were introduced into the full-length vLTSV sequence that abolished self-cleavage of the minus RNA *in vitro*. Surprisingly, when these mutated sequences were inoculated onto host plants with a helper virus, monomeric minus RNAs were produced *in vivo*. The introduced mutations had reverted in a

small proportion of the progeny RNA, suggesting that there was selection for the wild-type sequence. Possibly, the mutations lowered the efficiency of the *in vivo* self-cleavage reaction, rather than abolishing it; the self-cleavage of the mutated RNAs may have been enhanced by the intracellular environment. Interestingly, the mutated virusoids accumulated base changes at other sites in the virusoid molecule, possibly reflecting an adaptive response of the virusoid molecule to the introduced mutations.

# CHAPTER 1

# GENERAL INTRODUCTION

## 1-1 *In vitro* RNA-mediated self-cleavage reactions

RNA-mediated site-specific self-cleavage reactions occur *in vitro* in a variety of low molecular weight single-stranded RNAs. These *in vitro* RNA self-cleavage reactions occur in the absence of proteins and require only the presence of a divalent cation, such as $Mg^{2+}$, and around neutral pH conditions to yield 5'-hydroxyl and 2',3' cyclic phosphodiester termini. In at least two cases the *in vitro* self-cleavage reactions are reversible (Buzayan *et al.*, 1986a,c, Saville and Collins, 1991).

Table 1.1 lists all naturally occurring RNAs which undergo, or are predicted to undergo, *in vitro* self-cleavage. These RNA species are described briefly below. There appear to be four types of RNA structures which mediate the self-cleavage reactions, and these are also described below. The self-cleavage reaction is believed to be important *in vivo* in the replication of the pathogenic RNAs by rolling circle mechanisms (Branch and Robertson, 1984, Hutchins *et al.*, 1985), in which multimeric RNAs undergo site specific cleavage to generate monomer units.

## 1-2 RNAs in which *in vitro* self-cleavage occurs

### 1-2-1 Viroids

Viroids are the smallest known infectious agents of plants (reviewed by Diener, 1983, Keese and Symons, 1987a), they are composed solely of RNA and are not encapsidated in a protein coat. Their RNA genomes are single-stranded, circular and are highly base-paired into rod-like secondary structures, which are believed to possess no major tertiary structural interactions. The different viroids range in size from approximately 240 to 390 nucleotides.

Viroids replicate autonomously, that is, unlike the structurally similar virusoids (see below), they do not require a helper virus for their replication. The lack of conserved open reading frames between viroid species suggests that viroids have no mRNA activity (Diener, 1983), therefore, the enzymes responsible for their replication, presumably, are derived

**Table 1.1** RNAs which self-cleave *in vitro*.

| RNA and self-cleavage structure | Size (nucleotides) | Reference (reporting or predicting self-cleavage and/or ligation) |
|---|---|---|
| **1. Cleavage by hammerhead structure, reaction not reversible** | | |
| Avocado sunblotch viroid (plus and minus) | 246 | Hutchins *et al.* (1986) |
| Encapsidated linear satellite RNAs of : | | |
| Barley yellow dwarf virus (sBYDV) (plus and minus) | 322 | Miller *et al.* (1990) |
| Tobacco ringspot virus (sTRSV) (plus) | 359 | Prody *et al.* (1986) |
| *Arabis mosaic virus (sArMV) (plus) | 300 | Kaper *et al.* (1988) |
| *Chicory yellow mottle virus (sCYMV-S1) (plus) | 457 | Rubino *et al.* (1990) |
| Encapsidated circular satellite RNAs (virusoids) of : | | |
| Lucerne transient streak virus (vLTSV) (plus and minus) | 324 | Forster and Symons (1987) |
| Velvet tobacco mottle virus (vVTMoV) (plus only) | 366 | S. McNamara (unpublished) |
| Subterranean clover mottle virus (vSCMoV) (plus only) | 332 & 388 | Davies *et al.* (1990) |
| RNA transcript of newt satellite II DNA | 330 | Epstein and Gall (1987) |
| **2. Cleavage by hairpin structure, reaction reversible** | | |
| Encapsidated linear satellite RNAs of : | | |
| Tobacco ringspot virus (sTRSV) (minus) | 359 | Buzayan *et al.* (1986a,c) |
| *Arabis mosaic virus (sArMV) (minus) | 300 | Kaper *et al.* (1988) |
| *Chicory yellow mottle virus (sCYMV-S1) (minus) | 457 | Rubino *et al.* (1990) |
| **3. Cleavage by axehead/psuedoknot structure, reaction not reversible(?)** | | Sharmeen *et al.* (1988) |
| Hepatitis delta virus RNA (HDV RNA) (genomic and antigenomic) | 1700 | Kuo *et al.* (1988) Wu *et al.* (1989) |
| **4. Undefined cleavage structure, reaction reversible** | | |
| *Neurospora* mitochondrial VS RNA | 881 | Saville and Collins (1990, 1991) |

* RNAs which are predicted to self-cleave *in vitro*

from the host plant. Replication of viroids is believed to occur by a rolling-circle mechanism (1-4-1) involving complementary minus species.

About 15 different viroid species have been sequenced so far. Avocado sunblotch viroid is distinct from the other viroids due to its lack of sequence homology with the other viroids, and its high A,U content (Koltunow and Rezaian, 1989). It is also the only viroid so far that has been shown to self-cleave *in vitro* (Table 1.1).

### 1-2-2 Virusoids (circular satellite RNAs)

Virusoids are structurally similar to viroids. They are composed of circular single-stranded RNA of approximately 320 to 390 nucleotides, and adopt a highly base-paired rod-like structure. Like viroids, it is believed they have no mRNA activity, and are replicated by a rolling circle mechanism, involving a minus RNA species (reviewed by Francki, 1985).

Unlike viroids, however, virusoids are satellite RNAs, as they are dependent on a helper virus for their replication, but are not required for the replication of the helper viral RNA (Jones *et al.*, 1983, Francki *et al.*, 1986, Keese, 1986). As is typical of satellite RNAs, they have no sequence homology with the helper viral RNA, and are encapsidated by the helper viral coat protein.

Virusoids have been found encapsidated in four plant sobemoviruses (Hull, 1988); lucerne transient streak virus (LTSV; Tien-Po *et al.*, 1981), velvet tobacco mottle virus (VTMoV; Randles *et al.*, 1981), solanum nodiflorum mottle virus (SNMV; Gould and Hatta, 1981) and subterranean clover mottle virus (SCMoV; Francki *et al.*, 1983b). Each virusoid was named after the sobemovirus in which it was first found (e.g. the virusoid of lucerne transient streak virus; vLTSV). There are, however, only three different virusoids: those encapsidated by VTMoV and SNMV are sequence variants of the same virusoid (vVTMoV).

The helper viral - virusoid relationship has little specificity; vLTSV can also be supported by SNMV (Jones and Mayo, 1983) and two sobemoviruses which do not naturally support virusoids, southern bean mosaic virus (Paliwal, 1984) and sowbane mosaic virus (Francki *et al.*, 1983a). In addition, glasshouse tests have indicated that LTSV can support vVTMoV (Jones and Mayo, 1983) and vSCMoV (Keese *et al.*, 1983), indeed, a

virusoid with sequence similarity to vSCMoV has been found recently in a natural isolate of LTSV (Dall *et al.*, 1990).

### 1-2-3 Linear satellite RNAs

Three nepoviruses (Harrison and Murant, 1977) and one luteovirus (Martin *et al.*, 1990) encapsidate single-stranded linear satellite RNAs of approximately 300 to 460 nt, which undergo, or are predicted to undergo, *in vitro* self-cleavage (Table 1.1). These are the satellite RNAs of tobacco ringspot virus (sTRSV; Buzayan *et al.*, 1986b), arabis mosaic virus (sArMV; Kaper *et al.*, 1988), chicory yellow mottle virus (sCYMV-S1; Rubino *et al.*, 1990) and barley yellow dwarf virus (sBYDV; Miller *et al.*, 1990).

Although only the linear forms of the satellite RNAs are encapsidated in virion particles, circular forms are present in the plant (Linthorst and Kaper, 1984, Kaper *et al.*, 1988, Rubino *et al.*, 1990, Miller *et al.*, 1990). The circular forms are believed to be rolling circle replicative intermediates (see below).

### 1-2-4 RNA transcripts of the newt DNA satellite 2 sequence

Satellite 2 of the newt, *Notophthalmus viridescens*, is an abundant 330 base-pair tandemly repeated DNA sequence dispersed uniformly throughout the genome. In a variety of tissues, cytoplasmic RNA transcripts homologous with a subset of satellite 2 repeats are found. These transcripts correspond in size to the repeat unit or to integral multiples of the unit length (Epstein *et al.*, 1986).

Dimeric satellite 2 RNAs transcribed *in vitro* from cDNA clones were found to self-cleave *in vitro* (Epstein and Gall, 1987). The termini generated by *in vitro* self-cleavage correspond to the termini of monomeric sequences isolated from somatic tissue, but were different from those of monomeric RNAs isolated from ovarian tissue (Epstein and Pabon-Pena, 1991). The newt RNA is the only apparantly non-pathogenic RNA that has been shown to undergo *in vitro* self-cleavage.

### 1-2-5 Hepatitis delta virus

Hepatitis delta virus (HDV, also known as hepatitis delta viral agent) appears to be a satellite RNA of human hepatitis B virus, that has some interesting similarities to the plant

pathogenic RNAs described above. HDV is composed of a single-stranded circular RNA of about 1700 nucleotides and has no significant homology with its helper virus (Wang *et al.*, 1986, Kos *et al.*, 1986). A viroid-like rod-like secondary structure can be drawn for HDV (Wang *et al.*, 1986), and it appears likely that replication occurs by a rolling circle mechanism involving complementary RNA (Negro *et al.*, 1989). Like the plant viral satellite RNAs, HDV is encapsidated in the coat protein of its helper virus, hepatitis B virus, however, unlike the plant satellite RNAs, HDV encodes a protein, known as the delta antigen. Both genomic and antigenomic (complementary to genomic) RNAs undergo *in vitro* self-cleavage.

### 1-2-6 *Neurospora* mitochondrial VS RNA

The *Neurospora* mitochondrial VS RNA is an 881 nucleotide single-stranded circular RNA molecule complementary to one strand of a low copy, double-stranded circular DNA plasmid, which is organised as a population of head-to-tail multimers (Saville and Collins, 1990). The VS RNA undergoes both an RNA-mediated *in vitro* self-cleavage reaction (Saville and Collins, 1990), and a reverse ligation reaction *in vitro* (Saville and Collins, 1991). Self-cleavage may have a role in processing multimeric VS RNA *in vivo* (Saville and Collins, 1990).

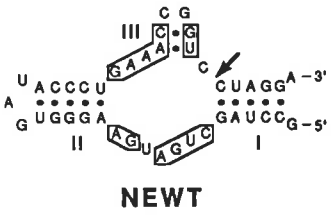
## 1-3 Structures mediating self-cleavage

There appear to be four different RNA structures which mediate *in vitro* self-cleavage (Table 1.1).

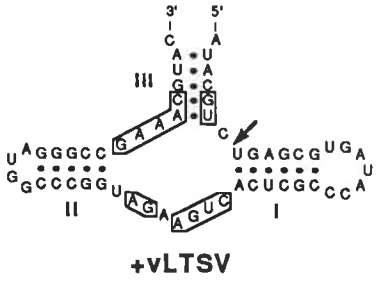
### 1-3-1 Hammerhead model

The best characterised type of *in vitro* self-cleavage is that mediated by the hammerhead structure (Forster and Symons, 1987a). This type of self-cleavage occurs, or is predicted to occur, in 12 unique species of naturally occurring RNAs, listed in Table 1.1, and the hammerhead structures of these RNAs are shown in Figure 1.1. The hammerhead structures consist of three base-paired stems enclosing inner single-stranded regions. By comparison of naturally occurring hammerhead structures, a consensus hammerhead structure can be determined containing 13 bases which are usually present (Figure 1.1; Forster and Symons, 1987a,b). Deletion of flanking sequences has confirmed that this

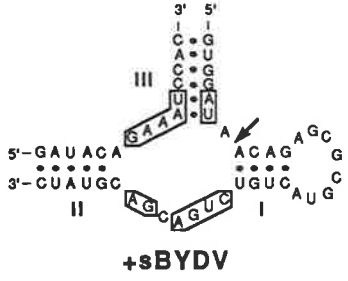
**Figure 1.1** (Single-) hammerhead structures for the RNAs listed in Table 1.1 (only one sequence variant is shown for each RNA), and the consensus hammerhead structure derived from them. Bases that are conserved between most naturally occurring hammerhead structures are boxed, sites of self-cleavage are indicated by the arrows, and base-paired stems are numbered according to Forster and Symons (1987a). In the consensus hammerhead structure, non-conserved bases are represented by N and N' (complementary to N). The base X at the self-cleavage site is either A or C.



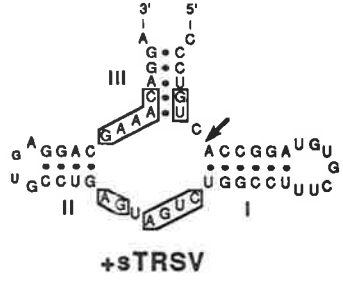
NEWT



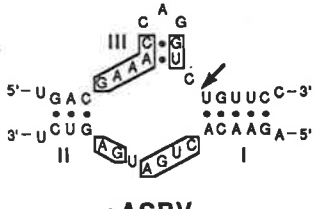
+vLTSV



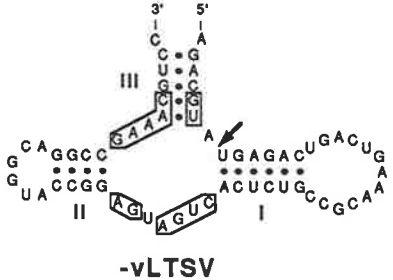
+sBYDV



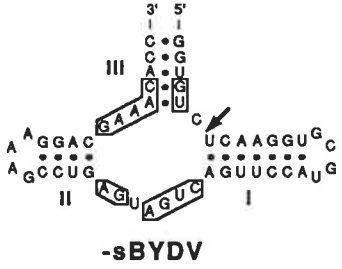
+sTRSV



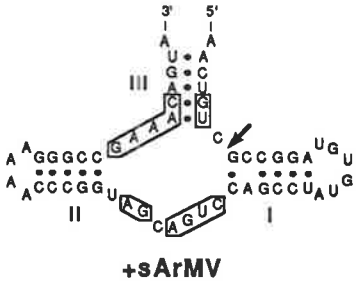
+ASBV



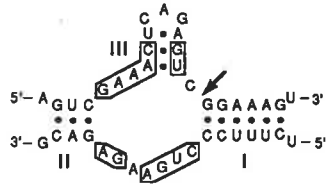
-vLTSV



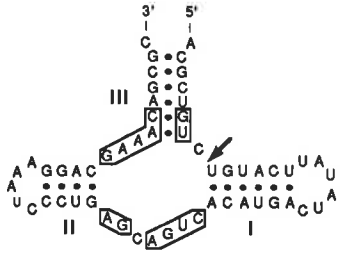
-sBYDV



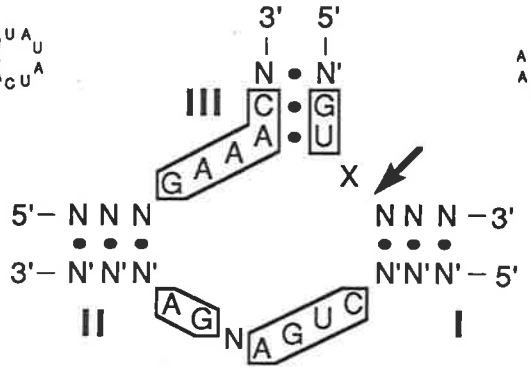
+sArMV



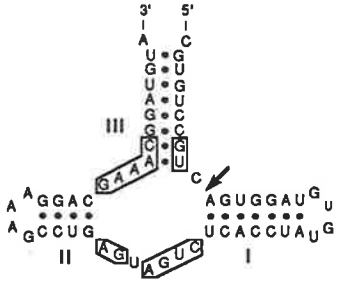
-ASBV



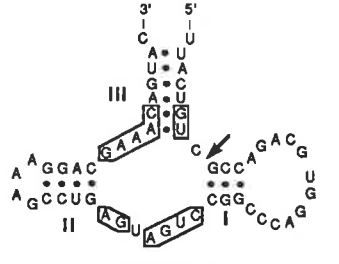
+vSCMoV



CONSENSUS



+vVTMoV



+sCYMV-S1

hammerhead sequence is all that is required for self-cleavage (Forster and Symons, 1987b). NMR studies on various non-cleavable hammerhead sequences, have provided results consistent with the three base-paired stems of the hammerhead structure (Odai *et al.*, 1990b, Pease and Wemmer, 1990, Heus and Pardi, 1991). However, no NMR data on the active tertiary structure are available. Computer modelling predictions (Mei *et al.*, 1989) have provided an interesting insight into the possible tertiary structure and the mechanism mediating the reaction.

Double-hammerhead

The hammerhead structures of most of the RNAs appear theoretically stable (Figure 1.1). However, the hammerhead structures of plus and minus ASBV and newt RNAs appear theoretically unstable due to the presence of weak stem IIIs with sterically constraining loops (Figure 1.1; Hutchins *et al.*, 1986, Forster and Symons, 1987b). Shortly before commencement of my Ph.D., more stable secondary structures were proposed for these RNAs, which involve the interaction of two hammerhead sequences to form double-hammerhead structures. These structures have theoretically more stable stem IIIs while maintaining the other features of the single-hammerhead (see Figures 4.1, 6.1).

### 1-3-2 Other structures mediating self-cleavage

Self-cleavage of the minus RNA of sTRSV (Buzayan *et al.*, 1986a) and the predicted self-cleavage of the minus RNAs of sArMV (Kaper *et al.*, 1988) and sCYMV-S1 (Rubino *et al.*, 1990) appear to be mediated by a different class of structure, involving two separate sequences, known as the hairpin structure. Mutagenesis and deletion of the minus sTRSV sequence has allowed definition of a catalytic sequence of about 50 nucleotides and a substrate sequence of about 10 nucleotides (Haseloff and Gerlach, 1989, Feldstein *et al.*, 1989, Hampel and Tritz, 1989). The *in vitro* self-cleavage reaction mediated by the hairpin structure is reversible (Buzayan *et al.*, 1986a,c).

The sequences around the *in vitro* site of self-cleavage of the genomic and anti-genomic RNAs of HDV have a high degree of sequence similarity, however, they have no sequence similarities to either the hammerhead or the hairpin structures. Several similar secondary structures (termed either the axehead or psuedoknot structures) have been proposed for the self-cleavage sequences of both genomic and anti-genomic HDV RNAs

**Table 1.2** Single-stranded RNA pathogens, the type of rolling circle mechanism by which they are proposed to replicate, and whether *in vitro* self-cleavage has been detected.

| Class of Rolling Circle Mechanism | RNA pathogen | *In Vitro* Self-cleavage Detected (1) |
|---|---|---|
| ASYMMETRIC (predominantly high molecular weight minus RNAs present) | several non-ASBV viroids | plus NO<br>minus NO |
| | vVTMoV (2)<br>vSCMoV | plus YES<br>minus NO |
| SYMMETRIC (monomeric minus RNAs present) | ASBV<br>vLTSV<br>sTRSV<br>HDV<br>sBYDV | plus YES<br>minus YES |

(1) Refer to Table 1.1

(2) *In vitro* self-cleavage ability of the minus RNA of vVTMoV has not been investigated

(Branch and Robertson, 1991, Belinsky and Dinter-Gottlieb, 1991, Perrotta and Been, 1991).

The *Neurospora* mitochondrial RNA, although having some limited sequence similarity around its site of *in vitro* (reversible) self-cleavage to the HDV RNAs (Saville and Collins, 1990), appears to self-cleave by another, as yet, undefined structure (Saville and Collins, 1991).

## 1-4 Possible role of self-cleavage *in vivo*

### 1-4-1 Rolling circle mechanism in the replication of RNA pathogens

Circular monomeric plus RNAs, and higher multimeric plus RNAs have been detected by Northern hybridization for all the RNA pathogens listed in Table 1.2 (Branch *et al.*, 1981, Kiefer *et al.*, 1982, Bruening *et al.*, 1982, Branch and Robertson, 1984, Ishikawa *et al.*, 1984, Hutchins *et al.*, 1985, Negro *et al.*, 1989, Davies *et al.*, 1990, Miller *et al.*, 1991). This led to the proposal of rolling circle models for their replication, based on the model of Brown and Martin (1965). Two types of models (Figure 1.2) have been developed to account for the difference in the nature of the minus (complementary to plus) RNAs detected by Northern hybridization for the different pathogens. A symmetrical model (Hutchins *et al.*, 1985; Figure 1.2a) has been proposed for those pathogens for which monomeric (as well as higher multimeric) minus RNAs have been detected (Table 1.2). An asymmetrical model (Branch *et al.*, 1981, Branch and Robertson, 1984; Figure 1.2b) has been proposed for those pathogens for which predominantly high molecular weight minus RNAs, with little or no monomeric minus species have been detected (Table 1.2).

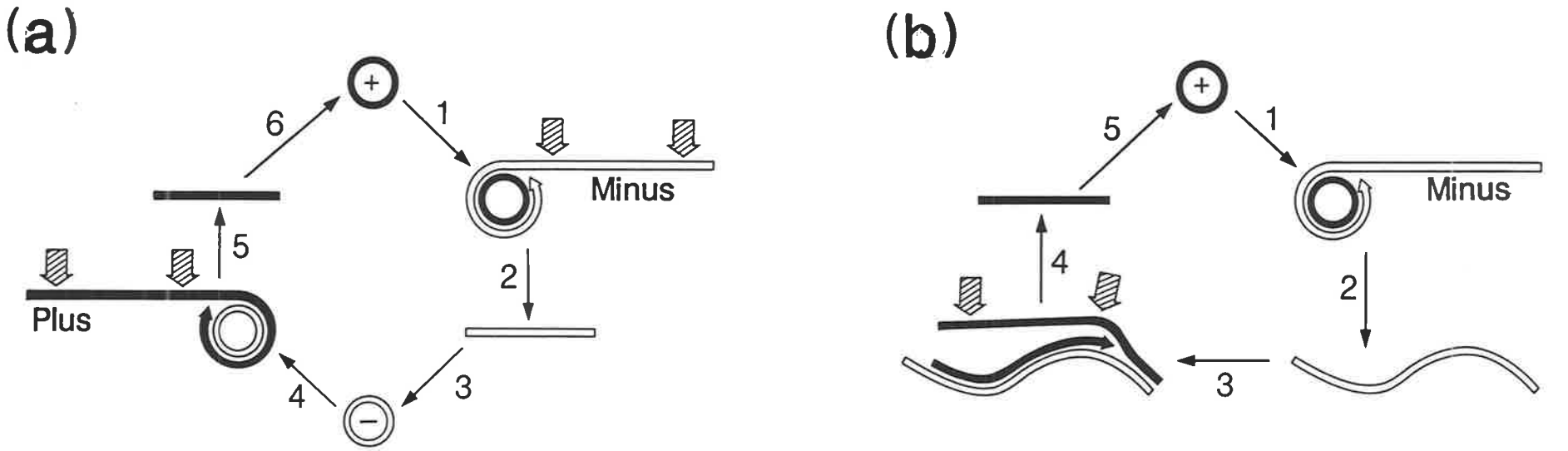
### 1-4-2 Enzymic activities involved in the rolling-circle mechanism

In both classes of rolling circle mechanisms, three types of enzymic activity are required. The RNA polymerase (Figure 1.2, steps a1, a4, b1, b3) and the RNA ligase (Figure 1.2, steps a3, a6, b5) activities are believed to be either host and/or helper virus enzymes. (In addition, it is possible that an auto-catalytic ligase activity is responsible for the ligation of minus sTRSV.) *In vivo* self-cleavage reactions (similar to the *in vitro* reaction) have been suggested to account for the processing of multimeric RNAs to linear unit-length RNAs (Figure 1.2, steps a2, a5, b4), at least for the virusoids, sTRSV, ASBV

**Figure 1.2** Rolling-circle models for the replication of viroids, virusoids and satellite RNAs.

(**a**) Symmetrical model. A circular monomeric plus RNA is copied by an unidentified RNA polymerase to give longer than unit length minus RNAs that are processed to give unit-length linear minus RNAs. Processing may occur by *in vivo* self-cleavage at specific sites, as indicated by the striped arrows. The unit-length, linear minus RNA is circularised by an RNA ligase, or possibly self-circularised in the case of sTRSV (Buzayan *et al.*, 1986a), and copied to give a longer than unit-length plus strand. This is processed, possibly by *in vivo* self-cleavage, to unit-length linear RNAs which are then circularized to form the progeny cicular RNA. Therefore, the proposed templates for plus and minus RNA synthesis are circular monomeric minus and plus RNAs, respectively. In the case of the linear satellite RNAs, the first step in the cycle is step 6, circularisation of the linear RNA, and the final step in the cycle, is step 5.

(**b**) Asymmetrical model. Similar to (a), except that the longer than unit-length minus strand is not processed, but rather is copied to give longer than unit-length plus strands. Therefore the proposed templates for plus and minus RNA synthesis are linear multimeric minus, and circular monomer plus RNAs, respectively. It has been suggested that the formation of the multimeric series in both types of models is due to incomplete cleavage of the multimeric RNAs, and also, possibly in part, to ligation of monomeric units together.


(a)
6
1
Minus
5
2
Plus
4
3
(b)
5
1
Minus
4
2
3

and HDV. It is unknown whether an, as yet, unidentified self-cleavage reaction is involved in the processing of the non-ASBV viroid RNAs (presumably only the plus RNA, Table 1.2), or whether a host enzyme is responsible.

### 1-4-3 Evidence for self-cleavage *in vivo*

Monomeric forms are detected by Northern analysis for the plus and minus RNAs of ASBV, vLTSV, sTRSV and the plus RNAs of vSCMoV and vVTMoV, but not for the minus RNA of vSCMoV(see Table 1.2). The correlation between the presence or absence of monomeric forms *in vivo* for these RNAs, and the ability or lack of ability, respectively, to self-cleave *in vitro* (Tables 1.1, 1.2), suggests that the self-cleavage observed *in vitro* may be involved in the processing of the multimeric RNAs to monomeric linear forms *in vivo*.

Further, in a number of cases, the site of self-cleavage *in vitro* is implicated as the *in vivo* site of processing to form monomeric RNAs. The *in vivo* termini of linear plus satellite RNAs (sTRSV, sArMV, sCYMV-S1, and sBYDV) correspond to those obtained (or predicted) by self-cleavage of the plus RNA *in vitro*, and the encapsidated RNAs have cyclic 2',3'-phosphodiester and 5'-hydroxyl terminal groups (Buzayan *et al.*, 1986b, Kaper *et al.*, 1988, Piazzola *et al.*, 1989, Miller *et al.*, 1990). 2'-phosphate moieties on encapsidated circular vVTMoV RNAs have been detected (Kibertstis *et al.*, 1985) on the base corresponding to the *in vitro* self-cleavage site of vVTMoV (S. McNamara, unpublished). These moieties may have been produced during the *in vivo* ligation of linear monomeric RNA with 5'-hydroxyl, and 2',3'-cyclic phosphodiester termini, and therefore suggest the site of *in vivo* cleavage.

In addition, the termini of the RNA transcripts of the newt DNA satellite 2 found in non-ovarian tissue correspond to those obtained by *in vitro* self-cleavage. Transcripts isolated from ovarian tissue, however, have termini about 50 nucleotides upstream from the *in vitro* site. This suggests that either different mechanisms are involved in the processing of RNAs from the different tissues, or dimeric transcripts isolated from non-ovarian tissue underwent *in vitro* self-cleavage during the extraction procedure.

Although it appears that the *in vitro* self-cleavage site is the site of *in vivo* cleavage for at least a number of the RNAs, there is no evidence that the mechanism of *in vivo*

cleavage is the same as *in vitro* self-cleavage, or that *in vivo* cleavage occurs by a protein independent reaction.

## 1-5 Aims

The aims of this work were:

(1) To investigate further the sequence requirements for the hammerhead self-cleavage reaction *in vitro*, by mutagenesis of the plus vLTSV hammerhead self-cleavage structure.

(2) To investigate the possible involvement of the double-hammerhead structure in the *in vitro* self-cleavage of the newt RNA, and plus and minus ASBV RNAs, and thereby confirm the requirement for the stability of stem III for self-cleavage. Further, it was hoped to determine the minimum stem III requirement for *in vitro* self-cleavage by a single-hammerhead self-cleavage.

(3) To investigate the possible involvement of self-cleavage *in vivo* in the replication of vLTSV.

# CHAPTER 2

# MATERIALS AND METHODS

## Materials

### 2-1 Reagents

General laboratory reagents were of analytical grade.

Acrylamide : Sigma Chemical Co.

Agarose : Sigma Chemical Co. and SeaKem GTG (FMC)

Amberlite MB-1 ion exchange resin : Sigma Chemical Co.

Ampicillin (sodium salt) : Sigma Chemical Co. Stock solutions (100 mg/ml in water) stored at -20°C.

Ammonium persulphate : May and Baker.

Bacto-tryptone, Bacto-agar and yeast extract : Difco Labs., U.S.A.

BCIG : United States Biochemical Corporation. Stored at 24 mg/ml in redistilled dimethyl formamide at -20°C.

Bromophenol blue : B.D.H. Laboratories Aust.

Bovine Serum Albumin : Bresatec (Adelaide).

3'-CMP : Sigma Chemical Co.

dNTPs and NTPs : Sigma Chemical Co. Stock solutions were prepared in water, and stored at -20°C.

Dithiothreitol : Sigma Chemical Co. Stored as 0.1 M solution in water at -20°C.

*E. coli* tRNA : Sigma Chemical Co.

EDTA (disodium salt) : Sigma Chemical Co.

Ethidium bromide : Sigma Chemical Co.

Formaldehyde : B.D.H. Laboratories Aust.

Formamide : B.D.H. Laboratories Aust. Deionised and stored at -20°C in the dark.

IPTG : Sigma Chemical Co. Stored as 0.1 M solution in water at -20°C.

N,N'-methylene-bis-acrylamide : Bio-rad Laboratories.

Nonidet P40 : B.D.H. Laboratories Aust.

Phenol : B.D.H. Laboratories Aust. Redistilled and stored in the dark at -20°C.

PEG 8000 : Sigma Chemical Co.

SDS : Sigma Chemical Co.

Spermidine trihydrochloride : Sigma Chemical Co.

Tetramethylammonium chloride : Aldrich Chemical Co.

Tris : Sigma Chemical Co.

Toluidine blue : Aldrich Chemical Co.

Urea : Sigma Chemical Co.

Xylene cyanol-FF : Ajax Chemicals Aust.

Nitrocellulose membrane (BA85 0.45μm) : Schleicher and Schuell.

DNA sequencing kits using $\alpha$-$^{32}$P-dATP and the Klenow fragment of DNA polymerase I were purchased from Bresatec.

## 2-2 Enzymes

Klenow fragment of DNA polymerase I, T4 DNA Ligase, T7 RNA polymerase, SP6 RNA polymerase and T4 polynucleotide kinase were from Bresatec (Adelaide). Calf intestinal phosphatase was from Boehringer Mannheim. RNase A, RNase $P_1$ and RNase $T_1$ were from Sigma Chemical Co. Restriction Endonucleases were from Pharmacia or Boehringer Mannheim. Vent DNA polymerase was from New England Biolabs. M-MLV RNase H$^-$ reverse transcriptase (superscript) was from Gibco-BRL. RNase $U_2$ was from Calbiochem. RNase PhyM was prepared from *Physarium polycephalum* as described by Donis-Keller (1980) by J. Cassidy. T4 RNA ligase was from Pharmacia.

## 2-3 Radioisotopes

$\alpha$-$^{32}$P-dATP, $\alpha$-$^{32}$P-dCTP, $\alpha$-$^{32}$P-UTP,and $\gamma$-$^{32}$P-ATP (3000 Ci/mmole) were from Bresatec (Adelaide).

## 2-4 Synthetic oligodeoxynucleotides

Synthetic oligodeoxynucleotides were synthesized by Bresatec (Adelaide) on an Applied Biosystems DNA Synthesizer and supplied in a crude form.

The oligodeoxynucleotides used as templates for the synthesis of RNAs in Chapters 3, 4 and 5 are not detailed here. They are complementary to the RNA transcribed from

them, and in addition possess the T7 promoter on their 3'-end (3'- ATT ATG CTG AGT GAT ATC CCT C...-5'), the five 5'-nucleotides of the promoter sequence are transcribed, and appear on the 5'-end of the RNA transcript. The 18-mer primer that is annealed to the template oligodeoxynucleotides is : 5'- TAA TAC GAC TCA CTA TAG -3'.

Primers for site directed mutagenesis of the vLTSV-A M13mp18 cDNA clone (Chapter 7) :

M1 : (DS839) 5'- CAA TTG AGC GGA CGT TCG GCC TGC CAT GGC -3'

M2 : (DS841) 5'- TTT CAG TCA GTC TCA CTA CGT CTG AGC GTG A -3'

M3 : (DS840) 5'- TTT CAG TCA GTC TCA ACG TCT GAG CGT GAT -3'

Primers for first strand cDNA synthesis and PCR amplification (Chapter 7) :

Primer 1 : (RS1369) 5'- ACT ATC CAC CTC CAG GGG TCA T -3'

Primer 2 : (RS1370) 5'- AAC GAC ATC CCG AGA TCT GAG C -3'

## 2-5 Vector DNA

Bacteriophage M13mp18 and M13mp19 RF DNAs (Yanish-Peron *et al.*, 1985), and the plasmid DNAs pSP64 and pSP65 (Krieg and Melton, 1987) were from Bresatec (Adelaide). The plasmid DNA pGem2 was from Promega Biotech.

## 2-6 Bacterial strains

*E. coli* JM101 : Δ(lac, pro) supE44 thi F' traD36 proAB laci$^q$ ZΔM15.

*E. coli* MC1061 : araD139 Δ(ara,leu)7697 ΔlacX74 gal U$^-$gal K$^-$ hsr$^-$ hsm$^+$strA.

## 2-7 Media and Solutions

L(Luria) broth : 1 % (w/v) bacto-tryptone, 0.5 % (w/v) yeast extract, 1 % (w/v) NaCl, pH 7.0.

2 x YT broth : 1.6 % (w/v) bacto-tryptone, 1 % (w/v) yeast extract, 0.5 % (w/v) NaCl, pH 7.0.

M13 minimal medium : 1.05 % (w/v) $K_2HPO_4$, 0.45 % (w/v) $KH_2PO_4$, 0.1 % (w/v) $(NH_4)_2SO_4$, 0.05 % (w/v) $Na_3citrate.2H_2O$, made to a volume of one litre. This solution was autoclaved, cooled to 45°C and the following added from separately made solutions: 10 ml of 20 % glucose, 0.8 ml of 1 M $MgSO_4$, 0.5 ml of 1 % (w/v) thiamine-HCl.

100 x Denhart's solution : 2 % (w/v) Ficoll 400, 2 % BSA, 2 % (w/v) polyvinyl pyrrolidone.

Acrylamide gel solution : 50 % (w/v) acrylamide, 2 % (w/v) N,N'-methylene-bis-acrylamide. The solution was deionized with amberlite MB-1 ion exchange resin (2 g/100 ml) for at least one hour, with gentle stirring, followed by filtration through Whatmann 541 paper, and stored at room temperature in the dark.

2 x Formamide loading solution : 95 % (v/v) deionised formamide, 10 mM EDTA, 0.02 % (w/v) xylene cyanol FF, 0.02 % (w/v) bromophenol blue.

3 x Urea loading solution : 2 M urea, 30 % (w/v) sucrose, 5 mM EDTA, 0.1 % (w/v) bromophenol blue, 0.1 % (w/v) xylene cyanol FF.

5 x glycerol loading solution : 0.5 % (w/v) SDS, 25 % (v/v) glycerol, 25 mM EDTA, 0.025 % (w/v) bromophenol blue.

## 2-8 Buffers

1 x TAE : 40 mM Tris-acetate, 20 M Na-acetate, 1 mM EDTA, pH 8.2.

1 x TBE : 89 mM Tris-HCl, 89 mM boric acid, 2 mM EDTA, pH 8.3.

20 x SSC : 3 M NaCl, 0.3 M $Na_3$citrate, pH 7.4.

1 x TE : 10 mM Tris-HCl, pH 8.0, 0.1 mM EDTA.

## 2-9 DNA molecular weight markers

Phage SPP1 DNA digested with EcoRI. Obtained from Bresatec (Adelaide) at 0.5 µg/µl in water.

Fragment sizes in base-pairs : 7840, 6960, 5860, 4690, 3370, 2680, 1890, 1800, 1450, 1330, 1090, 880, 660, 480 and 380.

Plasmid pUC19 DNA digested with HpaII. Obtained from Bresatec (Adelaide) at 0.5 µg/µl in water.

Fragment sizes in base-pairs : 501, 489, 404, 331, 242, 190, 147, 111, 110, 67, 2 x 34 and 26.

## Methods

### 2-10 *In vitro* synthesis of RNAs from synthetic DNA templates

RNAs were produced by oligodeoxynucleotide directed transcription using T7 RNA polymerase (Milligan *et al.*, 1987, Forster and Symons, 1987b). Oligodeoxynucleotide templates were 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. 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) or 0.5 mM UTP, 1.25 mCi/ml $\alpha$-$^{32}$P-UTP and 1 U/µl T7 RNA polymerase. Incubations were at 37°C for 1.5 h.

### 2-11 *In vitro* synthesis of RNAs from plasmid templates

Plasmid clones were digested with the appropriate restriction enzyme, according to the manufacturers' specifications, and transcribed with SP6 or T7 RNA polymerase. Transcription reactions contained 0.1 µg/µl DNA template, 0.5 U/µl SP6 or T7 RNA polymerase, 40 mM Tris-HCl, pH 7.5, 6 mM $MgCl_2$, 0.1 µg/µl bovine serum albumin, 10 mM DTT, 0.5 mM ATP, 0.5 mM CTP, 0.5 mM GTP, 0.025 mM UTP and 2 µCi/µl $\alpha$-$^{32}$P-UTP, and were incubated at 37°C for 1-1.5 h.

### 2-12 Purification of synthetic oligodeoxynucleotides and RNAs by polyacrylamide gel electrophoresis

Samples were added to an equal volume of formamide loading solution (2-7) and heated at 80°C for 1 min and snap cooled on ice before loading on 35 x 20 x 0.05 cm, polyacrylamide, 7 M urea gels run in TBE buffer (2-8). RNAs or synthetic oligodeoxynucleotides were detected by either autoradiography, or by staining with 0.05 % toluidine blue, 1 mM EDTA, and were eluted from gel slices in 0.1 % SDS, 1 mM $Na_2EDTA$, 10 mM Tris-HCl, pH 7.5, overnight at 37°C. Nucleic acid was recovered by the addition of sodium acetate to 0.3 M and 2 - 2.5 volumes of ice-cold ethanol, followed by storage at -20°C or -80°C for at least 30 min and centrifugation in an eppendorf centrifuge for

30 - 60 min. Samples were washed in 70 % ethanol, and resuspended in 1 mM EDTA (RNA) or $H_2O$ (DNA). RNA concentrations were estimated by U.V. spectroscopy or liquid scintillation counting.

### 2-13 *In vitro* self-cleavage reactions of purified RNAs

Purified RNAs, after heating at 80°C for one minute in 1 mM EDTA, pH 6, and snap-cooling on ice, were incubated in self-cleavage buffer (5 µl reaction volume) as described in each Chapter. Reaction mixes incubated at 55°C were covered with liquid parafin, and 37°C reactions were conducted in a 37°C oven, to prevent evaporation. Reactions were terminated by the addition of an excess of EDTA over $MgCl_2$ and an equal volume of formamide loading solution (2-7). Products were resolved on 7 M urea, polyacrylamide gels run in TBE buffer (2-8). If necessary, 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. Products were identified by autoradiography, and if required, the bands were excised and liquid scintillation counting used to determine the extent of self-cleavage.

### 2-14 5'-$^{32}$P-labelling of RNAs

RNAs in 1 mM EDTA were heated at 80°C for 1 min, and snap-cooled on ice, prior to incubation in 50 mM Tris-HCl, pH 9, 10 mM $MgCl_2$, 10 mM DTT, with 3 U T4 polynucleotide kinase and 10 µCi $\gamma$-$^{32}$P-ATP (10 µl reaction volume).

### 2-15 3'-$^{32}$P-labelling of RNAs

If the RNA to be labelled possessed a 2',3'-cyclic phosphodiester group, this was removed, by incubating the RNA in 10 µl of 10 mM HCl at 25°C for two hours (to decyclise the cyclic phosphodiester, Forster and Symons, 1987a), followed by incubation with calf intestinal phosphatase as described by Maniatis *et al.* (1982), to remove the phosphates.

RNAs with 3'-hydroxyl groups were 3' terminally labelled with 5'-$^{32}$P-pCp using T4 RNA ligase (England *et al.*, 1980). 5'-$^{32}$P-pCp was prepared by incubation of 100 µCi $\gamma$-$^{32}$P-ATP, 25 mM Tris-HCl, pH 9.0, 5 mM $MgCl_2$, 3 mM DTT, 0.05 µg/µl BSA, 5 mM 3'-CMP and T4 polynucleotide kinase at 37°C for 1 h (20 µl reaction volume). The enzyme

was inactivated by heating at 65°C for 10 min. To label RNA, 2 µl of the 5'-$^{32}$P-pCp reaction mix was incubated for 24 h at 0°C with the RNA, 50 mM Hepes-KOH, pH 7.5, 15 mM $MgCl_2$, 3.3 mM DTT, 0.015 mM ATP, 5 % (v/v) redistilled dimethyl sulphoxide and 2 U of T4 RNA ligase (20 µl reaction volume).

## 2-16 Enzymatic sequencing of end-labelled RNAs

Partial enzymic hydrolysis methods were used to sequence purified 5'- or 3'- $^{32}$P-labelled RNAs (2-14, 2-15). Partial digestions were carried out with RNase $T_1$, RNase $U_2$ and RNase PhyM as described by Haseloff and Symons (1981).

10 µl of $^{32}$P-end-labelled RNA with 2.5 µg/µl *E. coli* tRNA (carrier RNA) was dispensed into 5 x 2 µl aliquots and dried *in vacuo*. Tubes 1 ($T_1$), 3 (PhyM), 5 (No enzyme) were resuspended in 9 µl of 20 mM sodium citrate, pH 5.0, 1 mM EDTA, 7 M urea; tube 2 ($U_2$) in 9 µl of 20 mM sodium citrate, pH 3.5, 1 mM EDTA, 7 M urea; and tube 4 (Ladder) in 10 µl 1 mM $MgCl_2$ in formamide. Tubes 1, 2, 3 and 5 were heated at 80°C and snap-cooled on ice, prior to the addition of ribonucleases, as follows : tube 1, 10 U RNase $T_1$; tube 2, 5 U RNase $U_2$; tube 3, 1 µl RNase PhyM extract; tube 5, no enzyme. The tubes were incubated at 50°C for 20 min. Tube 4 was heated at 100°C for 90 seconds to generate a ladder of fragments. 10 µl formamide loading solution (2-7) was added to each tube, and samples were heated at 80°C and snap-cooled on ice prior to electrophoresis on a 20 % polyacrylamide, 7 M urea gel in TBE buffer (2-8).

## 2-17 Terminal nucleotide analysis of end-labelled RNAs

5'-$^{32}$P-labelled RNA (2-14), mixed with nonradioactive *E. coli* tRNA (10 µg), was incubated in 20 mM $NH_4OAc$, 5 mM NaOAc, 0.1 µg/µl nuclease $P_1$, at 37°C for 5 h.

3'-$^{32}$P-labelled RNA (2-15) was incubated in 10 mM NaOAc, pH 4.5, 0.5 U/µl RNase $T_2$, at 37°C for 16 h.

The 5' NMPs and 3' NMPs from the $P_1$ and $T_2$ digests, respectively, were fractionated (along with the appropriate marker NMPs) by thin layer chromatography on polyethyleneimine-cellulose plates in 1 M LiCl and detected by autoradiography and U.V. absorbance.

## 2-18 Preparation of plasmid DNA

Small amounts of plasmid DNA were prepared by a variation of Birnboim and Doly (1979) method. All centrifugations were conducted in Eppendorf centrifuges at room temperature. A single bacterial colony was grown for at least 8 h at 37°C in L broth (2-7) with 100 µg/ml ampicillin. 1.5 ml was centrifuged for 1 min and the pelleted cells resuspended in 100 µl 25 mM Tris-HCl, pH 8.0, 10 mM EDTA, 15 % (w/v) sucrose, and left on ice for 5 min. 200 µl of 0.2 M NaOH, 0.1 % (w/v) SDS was added, mixed and left on ice for 10 min. 125 µl 3 M sodium acetate, pH 4.6 was then added, mixed and left on ice for 10 min. Following this, the solution was centrifuged for 1 min and approximately 400 µl of the supernatent recovered. The DNA was precipitated by the addition of 800 µl ice cold ethanol and centrifugation for 1 min, and washed with 70 % ethanol, dried *in vacuo* and resuspended in 20 µl TE (2-8).

Larger scale preparations (500 ml) were a scale up of the small scale method described above. In addition, the nucleic acid was treated with RNase A overnight, extracted with phenol twice. The plasmid DNA was purified by HPLC fractionation on a Sepharose 6 (Sigma Chemical Co.) size exclusion column; elution was with 100 mM sodium acetate, 20 % (v/v) ethanol, 0.05 % (w/v) SDS, with detection at 260 nm (Skingle *et al.*, 1990).

## 2-19 Large scale preparation of M13 RF DNA

Large scale M13 RF DNA was prepared essentially as described by Yanisch-Perron *et al.* (1985), followed by HPLC purification as in 2-18.

## 2-20 Preparation of M13 single-stranded DNA

Single-stranded M13 DNA was prepared by the following protocol. Centrifugations were done in Eppendorf centrifuges. An overnight culture of E. coli JM101, grown in minimal medium at 37°C, was inoculated (1 : 100 dilution) into 2 ml of 2 x YT broth with phage toothpicked from single plaques, and incubated in 10 ml plastic tubes in a rotating verticle wheel at 37°C for 5 h. 1.5 ml of the culture was centrifuged for 15 min at 4°C, and the supernatent centrifuged for a further 15 min at 4°C. 1 ml of the supernatent was transferred to a 1.5 ml tube containing 200 µl of 20 % (w/v) PEG 8000 and 2.5 M NaCl.

After 5 min at room temperature and 15 min on ice, the tube was centrifuged for 10 min at 4°C and the phage pellet resuspended in 120 µl of 50 mM Tris-HCl, pH 7.5, 5 mM EDTA and 0.5 % (w/v) SDS. To this was added 60 µl of Tris-saturated phenol and 60 µl chloroform, and mixed gently but thoroughly three times over five min at room temperature. 100 µl of the aqueous phase was recovered after centrifuging for two min at room temperature, and to this was added 8 µl of 3 M sodium acetate, pH 5.2, and 250 µl of ice cold ethanol. After at least 30 min at -20°C, the DNA was pelleted by centrifuging for 1 h at 4°C, washed with 500 ml of 70 % ethanol, dried *in vacuo* and resuspended in 20 µl TE (2-8).

## 2-21 Site-directed M13 mutagenesis

Oligonucleotide directed mutagenesis was carried out by the method of Zoller and Smith (1983). Screening of plaques for mutants was done using the oligonucleotide hybridization procedure of Wood *et al.* (1985).

4 ng of kinased mutagenesis primer and 4 ng of kinased universal sequencing primer were annealed to single-stranded M13 DNA (1 µl of 20 µl prep; 2-20) in a final volume of 13 µl, by the addition of 10 µl 1M Tris-HCl, pH 7.6, 200 mM $MgCl_2$, 400 mM NaCl and heating at 70°C for 5 min and then 25°C for 5 min. The volume was adjusted to 50 µl by the addition of 5 µl 10 mM ATP, 5 µl 0.5 mM dNTPs, 5 µl 10 mM DTT, 20 µl $H_2O$, 1 µl 10 U/ µl Klenow and 1 µl 2 U/ µl T4 DNA ligase. The reaction for 4 h at 25°C. 0.5 µl, 1 µl or 2 µl of this reaction was used to transform 200 µl of competant JM101. The transformed bacteria were mixed with 3 ml YT-sloppy agar, 480 µg IPTG, 400 µg BCIG, poured onto minimal media plates and incubated overnight at 37°C.

The resultant phage were lifted onto nitrocellulose and baked for 2 h at 80°C *in vacuo*. The filter was then prehybridized at 42°C overnight in the presence of 90 mM Tris-HCl, pH 7.5, 0.9 M NaCl, 6 mM EDTA, 5 x Denhart's solution, 100 µl/ml salmon sperm DNA, 0.5 % NP40 detergent.

The probe for detection of plaques containing mutated DNA was 50 ng of 5'-end-labelled mutagenesis primer, prepared by incubating 50 ng of the primer with 25 µCi $\gamma$-$^{32}$P-ATP, 50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 1 mM DTT and 3 U of T4 polynucleotide kinase in a volume of 10 µl, for 1 h at 37°C. The enzyme was subsequently inactivated by

heating at 70°C for 5 min. The probe was added to the prehybridization solution, to give a concentration of the probe of 10 ng/ml, and hybridized to the filter at 42°C for 4 h.

The filter was washed twice for 10 min at room temperature in 6 x SSC, once for 10 min at room temperature in TMAC solution (3 M tetramethylammonium chloride, 50 mM Tris-HCl, pH 8, 2 mM EDTA, 0.1 % SDS), and twice for 30 min at 69°C in TMAC solution. The dissociation temperature for a 30-mer oligonucleotide, in the presence of TMAC, is about 74°C (Wood *et al.*, 1985). The filter was autoradiographed overnight.

Single-stranded M13 DNA prepared from positive plaques were sequenced (2-22) to confirm the presence of the mutation.

## 2-22 DNA sequencing

The dideoxy chain termination sequencing technique (Sanger *et al.*, 1977, 1980) was used to determine DNA sequence. The reactions were performed using Bresatec Dideoxy Sequencing kits with $\alpha$-$^{32}$P-dATP and the Klenow fragment of DNA polymerase I, with a modification of the provided protocol.

Annealing 6 µl single-stranded M13 DNA template (2-20), 1 µl 100 mM Tris-HCl, pH 8.0, 100 mM $MgCl_2$, 1 µl (25 ng) universal sequencing primer, 1 µl $H_2O$ was heated at 75°C for 3 min and then 37°C for 15 min. Subsequently 10 µCi (1 µl) of $\alpha$-$^{32}$P-dATP and 0.1 µl 10 U/µl Klenow was added.

Sequencing Sequencing reactions were incubated in a sterile petri dish floating on a 50°C water-bath. 2 µl of each dN°/ddNTP (Bresatec) was dotted onto the petri dish and 2 µl of the annealing reaction was added to the appropriate spot. The reactions were incubated for 10 min and 1 µl of the chase solution (Bresatec) was added to each spot. After a further 10 min, the reactions were terminated by the addition of 4 µl of loading dye (Bresatec). Approximately 1 µl of the reactions were electrophoresed on a 0.25 mm thick, 5 %, 7 M urea polyacrylamide gel. The gels were fixed in a bath containing 2 litres of 10 % acetic acid, 20 % ethanol for 30 min and dried on a Biorad 583 gel dryer, and autoradiographed overnight without an intensifying screen.

## 2-23 First-strand cDNA synthesis

10 ng of primer 1 (2-4) was annealed to 1 µl of viral RNA in a volume of 12 µl, by heating at 80°C for 1 min and then room temperature for 15 min. Reverse transcription was done using M-MLV RNase H$^-$ reverse transcriptase (Superscript, Gibco BRL) with the supplied buffer, and with only slight modifications of the recommended protocol. The reaction contained annealed primer and RNA, 50 mM Tris-HCl, pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT, 0.5 mM dNTPs and 200 U of reverse transcriptase in a final volume of 20 µl. The reaction proceeded at 37°C for 1 h and the products were then phenol/chloroform extracted and ethanol precipitated.

## 2-24 PCR

Vent DNA polymerase was used to produce the second strand of the cDNA and to amplify the double-stranded cDNA, it was also used to amplify the vLTSV sequence from single-stranded M13 DNA. 'No template' controls were included in all PCRs to guard against contamination, and extreme caution was taken to eliminate the possibility of contamination. The reactions were done using the provided Vent buffer and acetylated BSA. 2 µl of first strand cDNA or 1 µl of single-stranded M13 DNA, 100 ng of phosphorylated primer 1 and primer 2 (2-4), 20 mM Tris-HCl, pH 8.8, 10 mM $(NH_4)_2SO_4$, 2 mM $Mg_2SO_4$, 0.1 % Triton X-100, 0.1 µg/µl acetylated BSA, 0.2 mM dNTPs and 1 U of Vent DNA polymerase were placed in a 0.5 ml tube and covered with 30 µl of sterile parafin oil and subjected to 30 cycles of 94°C for 1 min, 50°C for 1 min, 72°C for 2 min in a Corbett Research FTS-1 thermal cycler machine.

The reaction was electrophoresed on a 2 % low melting point agarose (TAE) gel and the band containing the PCR product, excised and extracted (2-25). The 5'-overhanging ends of the PCR cDNA were then end-filled using the Klenow fragment of DNA polymerase I as described by Maniatis *et al.* (1982).

## 2-25 Agarose gel electrophoresis and purification of DNA

DNAs were run in TAE (2-8) buffered horizontal agarose (or low melting point agarose) gels. Samples were prepared for loading by the addition of 1/3 volume of urea loading solution (2-7). Gels were stained with 10 µg/µl ethidium bromide, and destained

with water, and DNA bands visualised under ultraviolet light. DNA was extracted either by electroelution (Maniatis *et al.*, 1982) or from low melting point gels, by the following method. Gel slices were incubated in an equal volume of 20 mM Tris-HCl, pH 7.4, 0.2 M NaCl, 2 mM EDTA at 65°C for 5 min, and phenol extracted twice, and ethanol precipitated.

## 2-26 Ligation of cDNA into DNA vectors and transformation into *E. coli*

Double stranded plasmid and M13 DNAs were linearised with the appropriate restriction enzymes according to the manufacturer's specifications, and dephosphorylated using calf intestinal phosphatase essentially as described by Maniatis *et al.* (1982).

Approximately 20 ng of linearised, dephosphorylated vector DNA was incubated with the insert DNA at a molar ratio of about 1 : 3 (vector : insert) in the presence of 50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 10 mM DTT, 1 mM ATP and either 0.025 U/µl (blunt ended insert) or 0.005 U/µl (sticky ended insert) of T4 DNA ligase. Reactions were conducted at 16°C for at least 1 h for sticky ended inserts or overnight for blunt ended insert DNA.

*E. coli* (pGem2 into MC1061, M13 into JM101; 2-6) was transformed by a method based on the technique of Hanahan (1985). A 1 in 100 dilution of an overnight culture was grown in liquid media at 37°C to log phase ($A_{600}$ 0.4 - 0.6). After chilling on ice, the cells were pelleted by centrifugation, and resuspended in 0.1 M $CaCl_2$. The resuspended cells were left on ice for at least 1 h prior to use.

Recombinant pGem2 containing colonies, and the orientations of the inserts, were determined by restriction digest analysis of miniprep DNA (2-18). Recombinant M13 clones were selected using the blue-white colour selection (Yanish-Peron *et al.*, 1985).

## 2-27 Preparation of single-stranded DNA markers

pUC19 plasmid DNA digested with HpaII (Bresatec, Adelaide) was end-labelled with $\alpha$-$^{32}$P-dCTP using the Klenow fragment of DNA polymerase I as described by Maniatis *et al.* (1982). Prior to loading, the markers were denatured by heating at 80°C for 1 min with an equal volume of formamide loading solution (2-7), and snap-cooled on ice.

# CHAPTER 3

# MUTAGENESIS ANALYSIS OF A SELF-CLEAVING HAMMERHEAD STRUCTURE

## Introduction

At the commencement of this work, the sequences of only eight naturally occurring RNAs containing hammerhead structures had been determined. From these, the consensus hammerhead structure (Figure 1.1) was defined. Limited mutagenesis of the hammerhead structure had been published (Sampson *et al.*, 1987, Koizumi *et al.*, 1988a,b), but really there was limited information on the types of variation in the consensus hammerhead structure that permitted self-cleavage. Therefore, specific RNAs were designed to investigate further the structural requirements of the hammerhead structure for self-cleavage. The mutagenesis carried out in this Chapter was aimed to be conservative or non-disruptive, as it appeared from the natural variation (Hutchins *et al.*, 1986, Forster and Symons, 1987a, Keese and Symons, 1987b, Epstein and Gall, 1987) and previous analysis (Sampson *et al.*, 1987, Koizumi *et al.*, 1988a,b) that the structural requirements for self-cleavage were quite strict.

The approach used for the mutagenesis study involved T7 RNA polymerase transcription from synthetic DNA templates (Milligan *et al.*, 1987); hammerhead variants are easily synthesised by variation of the synthetic template. The hammerhead sequence from the plus sense RNA of the virusoid of lucerne transient streak virus (vLTSV) was used for this study as a system for transcribing the wild-type sequence had already been established (Forster and Symons, 1987b). Substitutions, deletions and insertions were introduced into the plus vLTSV hammerhead sequence by transcription from the appropriate variant oligodeoxynucleotide template.

The results showed 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. Since the completion of this work, further mutagenesis analyses have been published: the results from all works will be considered in the discussion.

Some of the transcription results (but not the analysis of purified RNA) of the mutants referred to in this chapter were from preliminary studies done prior to the commencement of my Ph.D. (and are indicated as such in Table 3.1 and referenced in the text).

## Methods

### 3-1 *In vitro* transcription from synthetic DNA templates

$^{32}$P-labelled RNAs were produced by oligodeoxynucleotide directed transcription using T7 RNA polymerase, as described in 2-10, with 0.5 mM UTP.

### 3-2 Self-cleavage reactions

If required, the full-length RNAs were isolated and, after heating at 80°C for one min in 1 mM 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, as described in 2-13. These two conditions were known to result in the efficient self-cleavage of other RNAs (Chapters 4 and 5).

## Results

The RNA used as the wild-type sequence for this mutagenesis study was the 58 base RNA used by Forster and Symons (1987b). 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 (Figure 3.1). As this structure has a stable stem III, it cleaves by a single-hammerhead structure (Forster and Symons, 1987a, Chapter 4). 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 (Figure 3.2, lane 1).

Eleven variants of the plus vLTSV hammerhead sequence were created by transcription of the appropriate synthetic template DNAs (3-1) and their capacity for self-cleavage during the transcription reaction assessed by polyacrylamide gel electrophoresis and autoradiography. The results are summarised in Table 3.1 and the transcription patterns for

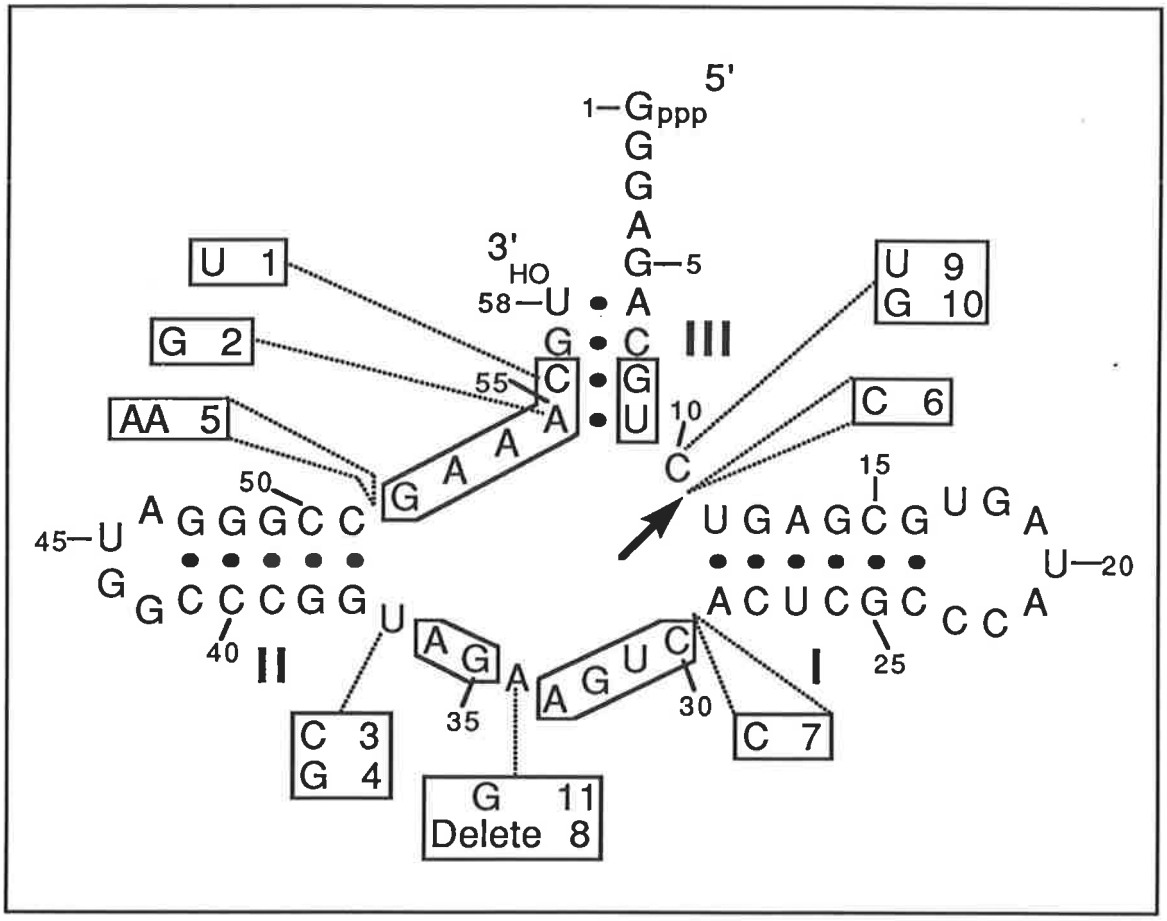



**Figure 3.1** Mutants of the hammerhead structure of the 58 base plus vLTSV RNA produced by transcription of an oligodeoxynucleotide template. The sequence is derived from nucleotides 164 to 216 of plus vLTSV (Forster and Symons, 1987a), except for the five 5'-terminal nucleotides which were derived 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 3.1. Stems are numbered I to III (after Forster and Symons, 1987a), the site of cleavage is indicated by an arrow, and bases conserved between most naturally occurring hammerhead structures are boxed.

**Table 3.1** % Self-cleavage of mutant plus vLTSV RNAs

| RNA | % Self-Cleavage | | |
|---|---|---|---|
| Wild-type or Mutant No. [a] | During Transcription [c] | Purified RNA [b] | |
| | | 37°C Buffer A [d] 1 h | 55°C Buffer B [e] 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 | - | - |

[a] Sequence variants as in Figure 3.1.

[b] 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.

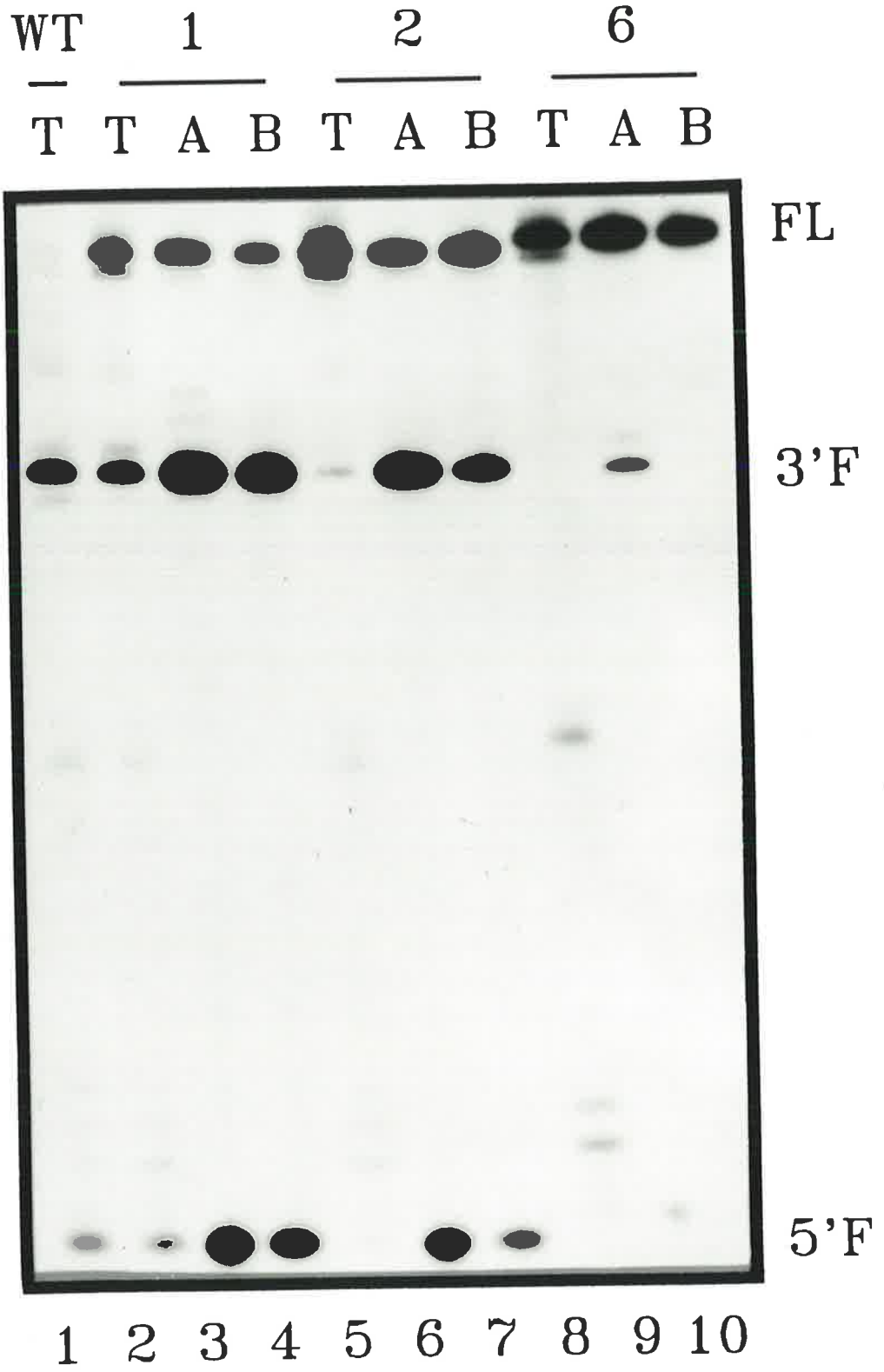
[c] Conditions during transcription were essentially, 6 mM $MgCl_2$, 1 mM spermidine, pH 7.5 (see 3-1).

[d] Buffer A : 50 mM $MgCl_2$, 0.5 mM EDTA, pH 9.0 (see 3-2).

[e] Buffer B : 10 mM $MgCl_2$, 0.5 mM EDTA, pH 8.0 (see 3-2).

# Transcription reaction results obtained prior to commencement of Ph.D.

**Figure 3.2** 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.


WT
1
2
6
T T A B T A B T A B
FL
3'F
5'F
1 2 3 4 5 6 7 8 9 10

three mutants are shown in Figure 3.2 (refer to Figure 3.1 for the numbering of the mutants). The full-length RNAs of those mutants that self-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 (3-2), after heating and snap-cooling (Figure 3.2, summarised in Table 3.1). These conditions were known to result in the efficient cleavage of other RNAs (see Chapters 4,5).

RNA sequencing (2-16) of several of the RNAs confirmed that self-cleavage occurred at the expected site (data not shown). RNA sequencing and 5' end nucleotide analysis (2-17) of the 3'-self-cleavage fragment of mutant 6 identified that the majority of self-cleavage (about 75%) occurred after the second C residue at the self-cleavage site, with about 25% of the cleavage occurring after the first C (results not shown).

## Discussion

### 3-3 Self-cleavage occurs in hammerhead structures with the conserved bases altered in stem III

The sequence conservation in the three base-paired stems of naturally occurring hammerhead structures is limited to four bases in stem III. Mutations that disrupt base-pairing of the conserved residues in stem III have been shown to result in the abolishment of self-cleavage (Sampson *et al.*, 1987; Koizumi *et al.*, 1988a,b). Mutants 1 and 2 (Figure 3.1) show that mutations of the conserved bases that maintain the capacity for base-pairing can permit self-cleavage, although with efficiencies lower than that of the wild-type sequence. Mutant 1 cleaved to 47% during the transcription reaction (Sheldon, 1987) and cleaved to high levels when incubated under the two Buffer conditions (Figure 3.2, lanes 2,3,4; Table 3.1). Mutant 2 cleaved less efficiently (8%) during the transcription reaction (Sheldon, 1987) than at 37°C in Buffer A (73%) or at 55°C in Buffer B (39%; Figure 3.2, lane 5,6,7; Table 3.1). Base-pairing within the stem III was possible in these mutants through G.U base-pairs, which have approximately the same stability as A.U base-pairs (Saenger, 1984). 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 higher $MgCl_2$ concentrations and higher pH conditions in the two Buffers compared with the transcription reaction conditions may have increased the stability of the hammerhead structure resulting in a higher efficiency of self-cleavage.

In another system (Koizumi *et al.*, 1988b), altering the conserved C.G base-pair in stem III to A.U did not disrupt self-cleavage. However, the same base-pair change had a greater disruptive effect on the self-cleavage of two other hammerhead structures, reducing self-cleavage to either 40 % (Koizumi *et al.*, 1988a) or 25 % (Ruffner *et al.*, 1990) of the wild-type efficiency. Similarly, changing the conserved C.G base-pair in stem III to a U.A base-pair decreased self-cleavage efficiency in two artificial systems (Koizumi *et al.*, 1988a, Ruffner *et al.*, 1990). However, the plus satellite RNA of barley yellow dwarf virus (sBYDV), which self-cleaves *in vitro*, naturally has a U.A base-pair at this site (Miller *et al.*, 1991). These examples demonstrate that the same base changes can have different effects in different hammerhead structures (that is, hammerhead structures with different non-conserved residues), and so it is not necessarily the base change *per se* that affects self-cleavage activity, but rather the base change in the context of the whole hammerhead structure. Consequently, caution should be applied when analysing results from mutation experiments.

Analysis of the naturally occurring hammerhead RNAs (for review: Bruening, 1989) indicates that there is no sequence or size conservation of stem I and II or of their loops, in fact the loops are not even required for self-cleavage (Uhlenbeck, 1987, Koizumi *et al.*, 1988b, Haseloff and Gerlach, 1988, Jeffries and Symons, 1989). Stem III therefore is unique in containing conserved residues (although some variation in these residues is tolerated) and therefore may be more intimately associated with the active site than the other stems.

## 3-4 Hammerhead structures with mismatches in the base-paired stem II can still self-cleave

Several naturally occurring hammerhead RNAs contain mismatches in stem II. Rakowski and Symons (1989) isolated a natural avocado sunblotch viroid (ASBV) variant with a base substitution in stem II such that Watson-Crick base-pairing is disrupted, and the formation of stem II made unlikely (Tinoco *et al.*, 1971). Dimeric plus ASBV RNA transcripts prepared *in vitro* containing this base change self-cleaved during transcription to approximately 25% of the level of the wild-type dimeric RNA (Rakowski and Symons, 1989). Presumably, the stability of stem II is maintained during *in vitro* transcription despite

the mismatch, possibly by the flanking non-hammerhead stem sequences. A sequence variant of the plus RNA of vLTSV (clone M3.10, Figure 7.9, Chapter 7), and the plus RNA of sBYDV (Miller *et al.*, 1991), have mismatches for the inner base-pair of stem II (the base-pair adjacent to the central single-stranded regions). In addition, sequence variants of the self-cleaving newt RNA have either one or two of the inner base-pairs of stem II mismatched (Epstein and Pabon-Pena, 1991). Ruffner *et al.* (1990) in an artificial system also found that base-pairing at this inner base-pair was not essential for self-cleavage. Presumably, the effect of these mismatches would be to increase the size of the interior single-stranded region, similar to the effect of U35 in the plus RNA of vLTSV (see below).

There is only one known example of a naturally occurring RNA with a disruption of base-pairing in stem I : the plus satellite RNA of arabis mosaic virus (sArMV) has an internal mismatch in stem I (Kaper *et al.*, 1988; Figure 1.1), however, as no self-cleavage studies have been done with this RNA, no information is available on the effect of this mismatch on self-cleavage.

### 3-5 Is U37 hydrogen-bonded to G52?

The plus vLTSV and plus sArMV hammerhead structures are unusual in containing a residue (U37 in the plus vLTSV hammerhead; Figure 3.1) between the conserved bases of the lower single-stranded region (bases 30-36 in plus vLTSV; Figure 3.1) and stem II (Forster and Symons, 1987a, Kaper *et al.*, 1988). Relative to the hammerhead structures of other RNAs, this extra base represents an insertion of a U. Whether this base in plus vLTSV is hydrogen-bonded to G52 or whether it is unpaired was investigated by substituting U37 for C and G residues (Figure 3.1, mutants 3 and 4, Table 3.1). 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 suggests that either bases 37 and 52 are not base-paired, perhaps because the tertiary structure of the hammerhead structure prevents this from occurring, or that an extra base-pair in this region does not disrupt the active hammerhead structure. Computational modelling of the plus vLTSV hammerhead structure has suggested that U37 does not interact with other bases (Mei *et al.*, 1989).

These results, in addition to the ability of hammerhead structures with mismatches in stem II to self-cleave, further indicates the structural flexibility in this region of the hammerhead structure.

## 3-6 Insertion and deletion in the hammerhead structure affects self-cleavage

An AA insertion was made between C51 and G52 (Figure 3.1, mutant 5), with the rationale that one A would base-pair with U37 to extend stem II by one base-pair and thereby remove U37 (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, Figure 3.1). It is possible that base-pairing between the inserted A and U37 would not occur, in which case there would be five unpaired bases in the upper, left-hand single-stranded region. Mutant 5 RNA did not self-cleave when transcribed from its DNA template (Sheldon, 1987; Table 3.1); 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 3.1). Presumably the high $Mg^{2+}$ concentration and high pH in Buffer A stabilised the active structure of mutant 5, whereas the conditions in the transcription mix (6 mM $Mg^{2+}$, pH 7.5) and Buffer B (10 mM $Mg^{2+}$, pH 8.0) 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, Figure 3.1, mutant 6) did not cleave during the transcription reaction (Sheldon, 1987), nor when incubated at 55°C in Buffer B, but did self-cleave to about 10% when incubated at 37°C in Buffer A (Figure 3.2, lanes 8,9,10, Table 3.1). RNA sequencing and 5' end nucleotide analysis of the 3'-self-cleavage fragment of mutant 6 RNA identified that the majority of self-cleavage (about 75%) occurred after the second C, with the remainder of the cleavage occurring after the first C (results not shown). A similar insertion in a full-length minus vLTSV RNA transcript also abolished self-cleavage during transcription (M2, Chapter 7, Figure 7.3, lane 4), as did deletion of the base 5' to the self-cleavage site in the same RNA (M3, Chapter 7, Figure 7.3, lane 6).

An RNA with a C inserted between A29 and C30 (Figure 3.1, mutant 7) cleaved to about 85% during the transcription reaction (Table 3.1), and an RNA with the non-conserved base A34 deleted (Figure 3.1, mutant 8) self-cleaved to about 12% during the

transcription reaction but self-cleaved more efficiently when the purified RNA was incubated under the two conditions (40% in Buffer A at 37°C and 15% in Buffer B at 55°C; Table 3.1). These results point to a degree of flexibility in the size of the lower single stranded region of the hammerhead structure.

Deletion of an A from the GAAAC sequence (bases 52-56, Figure 3.1) in the double-hammerhead structure of dimeric plus ASBV RNA transcripts abolished cleavage (Forster *et al.*, 1988) (and also in the minus ASBV RNA (Davies *et al.*, 1991) and minus vLTSV RNA (Chapter 7, Figure 7.3, lane 2)).

The results of the insertion and deletion variants presented, and the other variants mentioned, suggest 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 may be spatially removed from the critical centre of the active structure.

## 3-7 Substitution of two non-conserved bases : C10 and A34

The base 5' to the cleavage site is either a C or an A in all known natural hammerhead RNAs. RNAs made with the other two bases at this site (Figure 3.1, mutants 9 and 10) both self-cleaved. RNAs with a U at that site cleaved as efficiently as the wild type RNA during transcription (Sheldon, 1987); however, RNAs with a G at this site cleaved to only about 30% during the transcription reaction (Sheldon, 1987), 67% at 37°C in Buffer A, and 30% at 55°C in Buffer B (Table 3.1). Koizumi *et al.* (1988a), using gel purified RNAs based on the newt hammerhead sequence, also found that a U at this site resulted in efficient self-cleavage; however, no cleavage was obtained in RNAs with a G at this site. Ruffner *et al.* (1990) obtained self-cleavage at 5% of the wild-type rate in RNAs with a U, and 0.3% of the wild-type rate in RNAs with a G at that site. These differences in results emphasise again that the effect of a mutation on self-cleavage activity can be modified by the hammerhead sequence into which it is placed.

Koizumi and Ohtsuka (1991) substituted either G or I (inosine) for the base 5' to the self-cleavage site. Approximately a six-fold reduction in self-cleavage activity was obtained with I and about a 900-fold reduction with G, compared with the wild-type sequence. Further mutagenesis experiments revealed that the large decrease with G substitution was

mainly due to the effect of the 2'-amino group, rather than the ability of the G residue to form a base-pair with the conserved C of the lower single-stranded region (Koizumi and Ohtsuka, 1991). The authors suggest that the 2'-amino group may sterically inhibit the reaction. A methyl-cytidine residue at this site has also been found to abolish self-cleavage (Koizumi *et al.*, 1989, Odai *et al.*, 1990a), possibly also by steric inhibition.

Hammerhead structures containing A, C and U, but not G, residues at position 34 have been found in the natural RNAs studied so far (Forster and Symons, 1987a, Keese and Symons, 1987, Epstein and Gall, 1987, Davies *et al.*, 1990). The efficient self-cleavage of an RNA made with a G at this position (Figure 3.1, mutant 11, Table 3.1) 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. Presumably, the role of this base is in creating the correct spacing between the conserved bases, as deletion of this base reduced self-cleavage activity (mutant 8).

## 3-8 Altering conserved bases in the single-stranded regions

In this work, no mutations involving the conserved bases in the single-stranded regions of the hammerhead were examined. However, Ruffner *et al.* (1990) has since undertaken a thorough mutagenesis of these regions, and found that any mutation of these bases reduced the self-cleavage rate to 0 - 6 % of the wild-type rate.

Odai *et al.* (1990a) substituted the G residue of the CUGA motif with an inosine residue (I), and observed over a 20-fold decrease in rate compared with wild-type. Interestingly, thermal denaturation analysis suggested that removal of the 2-amino group from the G residue, by substitution with I, reduced the thermal stability of the complex, even though the residue is not included in a base-paired stem (Odai *et al.*, 1990a). This suggests that this base may be involved in tertiary interactions, or in complexing with $Mg^{2+}$, thereby stabilising the structure, consistent with the predictions of Mei *et al.* (1989). The formation of the active structure, by tertiary interaction and complexing with catalytically important divalent metal ions, is likely to be the role for the other conserved residues also. It appears unlikely that the conserved residues contribute any catalytically important reactive groups (Mei *et al.*, 1989).

### 3-9 Role of phosphate groups and 2'-hydroxyl groups

Recently, several papers have been published that have identified phosphate and 2'-hydroxyl groups that may be important in the hammerhead self-cleavage reaction. Cedergreen and coworkers have undertaken a series of deoxyribonucleotide substitution experiments, the results of which suggest that the 2'-hydroxyls of: (a) the conserved U in stem III, (b) the G of the CUGA motif and (c) the A at the 3'-end of the lower single-stranded region are involved in binding of $Mg^{2+}$ (Perreault *et al.*, 1990, Yang *et al.*, 1990, Perreault *et al.*, 1991). These results contrast, in part, with those published by Olsen *et al.* (1991), in which the 2'-hydroxyl of the A at the 3'-end of the lower single-stranded region could be replaced by either a 2'-deoxy, or a 2'-fluoro group with almost no loss of self-cleavage activity. The involvement of the 2'-hydroxyl of the conserved U in stem III must also be questioned, as the U was substituted with dT, which not only lacks the 2'-hydroxyl, but also possesses a methyl group. The presence of this large group so close to the self-cleavage site may have had some disruptive effect on the formation of the active structure.

The 2'-hydroxyl of the base 5' to the site of self-cleavage has, as expected, been demonstrated to be essential for the self-cleavage reaction (Koizumi *et al.*, 1989, Perreault *et al.*, 1990). Apart from this base, and those mentioned above, substitution of the 2'-hydroxyl of other residues in the single-stranded regions with either 2'-deoxy, or 2'-fluoro groups results in only minor decreases in self-cleavage activity (Perreault *et al.*, 1990, Yang *et al.*, 1990, Perreault *et al.*, 1991, Pieken *et al.*, 1991, Olsen *et al.*,1991). These results suggest that the majority of the 2'-hydroxyl groups in the central single-stranded regions play only minor roles in the formation of the active structure, and the self-cleavage reaction.

Ruffner and Uhlenbeck (1990) obtained a reduction in self-cleavage of hammerhead structures with phosphorothioate substitution 5' to: (a) the conserved A residue at the 3'-end of the lower single-stranded region, (b) the two unbase-paired A residues in the GAAAC motif, as well as (c) the phosphodiester bond at the site of self-cleavage. The reduction in self-cleavage activity with the substituted phosphates may be caused by either altered tertiary folding or altered coordination with the divalent metal ion (Ruffner and Uhlenbeck, 1990). Because of the unique presence of U37 in the plus vLTSV hammerhead structure (Figure 3.1), it would be interesting to determine whether the phosphate of the A at the 3'-end of the

lower single-stranded region is important, as was found by Ruffner and Uhlenbeck (1990), or whether in plus vLTSV, it is the phosphate of U37 that is important.

## 3-10 Conclusions

It is apparant that a mutation can have different effects in different hammerhead structures (with different sequences in the stems and non-conserved residues). Obviously, there are interactions within the hammerhead structure (including those between the RNA and the essential divalent cation), which cannot be predicted on the basis of the simplistic hammerhead secondary structural model. One effect of a mutation may be to stabilise an alternative inactive structure. If this occurred, self-cleavage activity could be reduced, but as a consequence of the RNA being directed to fold into an inactive structure, rather than any specific effect of the mutated base. This makes determination of a 'consensus' hammerhead structure based on the mutagenesis data presented here and elsewhere (Koizumi *et al.*, 1988a,b, Ruffner *et al.*, 1990) difficult. This is quite important, especially in the design of ribozymes to cleave target RNAs, either *in vitro* or *in vivo*, where the use of a 'consensus' hammerhead sequence (as determined by limited mutagenesis data, e.g. Ruffner *et al.*, 1990) may result in inefficient, and ineffective cleavage of the target.

Despite these complications, some broad conclusions about hammerhead structural requirements can be made. Certainly it appears that there is flexibility in the sequence requirements for self-cleavage *in vitro*. However, 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 *in vitro* self-cleavage. Therefore, *in vivo*, there may be selective pressure on the natural self-cleaving RNAs to maintain the conserved sequences and hence secondary structure.

Of the three stems, stem III has the most strict requirements, it contains conserved bases, and its stability is essential for self-cleavage (Chapters 4,5,6). Whilst the formation of the other stems is required, it appears that, especially in the case of stem II, some mismatches are tolerated. Mismatches of the base-pairs of stem II adjacent to the central single stranded region appear to have no disruptive effect on self-cleavage. Stem loops are not required in the formation of the active structure. Alteration of the upper single-stranded region, including the phosphate groups, generally decreases self-cleavage efficiency. The

lower single-stranded region, in contrast, is more tolerant to changes, both in size, and in some cases, the identity of the bases.

These generalisations lead to a picture of the hammerhead in which the stabilities of stems I and III are more important than the stability of stem II, and the lower single-stranded region has less strict requirements than the upper single-stranded region. This possibly suggests that the stems I and III and the upper single-stranded region form the core of the active site, with the lower single-region, and stem II, being less involved.

The folding of the RNA as directed by the hammerhead secondary structure, and tertiary interactions between the conserved residues, is believed to form an active structure that allows the coordination of a hydrated $Mg^{2+}$ ion, to stabilise a pentacoordinate intermediate (Mei *et al.*, 1989, van Tol *et al.*, 1990, Slim and Gait, 1991, Dahm and Uhlenbeck, 1991). The RNA structure appears to place a unique strain on the phosphodiester bond at the site of self-cleavage (Mei *et al.*, 1989). Nucleophilic displacement by the 2'-hydroxyl at the self-cleavage site on the adjacent 3'-phosphate occurs, resulting in the cleavage of the RNA and generating the 2',3'-cyclic phosphodiester and 5'-hydroxyl terminal groups. It is also possible, that $Mg^{2+}$ could be involved in the self-cleavage reaction, by abstracting the proton from the 2'-hydroxyl group at the self-cleavage site, to initiate the nucleophilic attack on the phosphodiester linkage (Dahm and Uhlenbeck, 1991, Koizumi and Ohtsuka, 1991). In addition to this, $Mg^{2+}$ appears to act partly as a counter ion to neutralise the negative charges on the phosphodiester backbone of the RNA, and so stabilise the interactions between different parts of the RNA molecule (Dahm and Uhlenbeck, 1991). The high $MgCl_2$ concentration that stimulated the self-cleavage of certain vLTSV variant RNAs (for example, mutants 2,5,6,8,10) presumably acted by increasing the stability of the hammerhead structure, thereby allowing the self-cleavage reaction to occur.

# CHAPTER 4

# DOUBLE-HAMMERHEAD SELF-CLEAVAGE OF A 40 BASE NEWT-LIKE RNA

## Introduction

The stem III of the single-hammerhead structure of the self-cleaving newt RNA consists of a two base-pair stem and a two base loop, and is of low stability compared with other hammerhead structures (Figure 1.1). Shortly before commencement of my Ph.D., a double-hammerhead structure was proposed as the active structure of the newt RNA. The double-hammerhead structure is formed by the interaction of two hammerhead sequences, to create a more stable, six base-pair, stem III, whilst maintaining the other features of the single-hammerhead structure, namely the 13 conserved residues and three stems.

As self-cleavage by a double-hammerhead structure would involve the interaction of two hammerhead sequences, then, if only one hammerhead sequence were contained on each RNA molecule, the reaction would be a bimolecular reaction, and therefore, the efficiency of self-cleavage would be dependent on the concentration of the RNA. The efficiency of single-hammerhead self-cleavage, on the other hand, as it is a unimolecular reaction, would be independent of RNA concentration. Therefore, one simple way to test the validity of the double-hammerhead model in relation to the newt RNA was to determine whether the rate of self-cleavage of a short RNA containing the sequence of one newt hammerhead was dependent on the concentration of RNA.

This Chapter reports on the series of experiments, which revealed that a 40 base RNA (termed nCG RNA), containing the approximate sequence of the newt hammerhead, self-cleaved as a bimolecular double-hammerhead structure. In addition, the 5'-self-cleavage fragment of this RNA could act in *trans* to mediate the self-cleavage of a full-length RNA by the formation of a partial double-hammerhead structure.

## Methods

### 4-1 Preparation of RNAs

RNAs were produced by oligodeoxynucleotide directed transcription using T7 RNA polymerase (2-10). Non-radioactive transcription reactions contained an equimolar

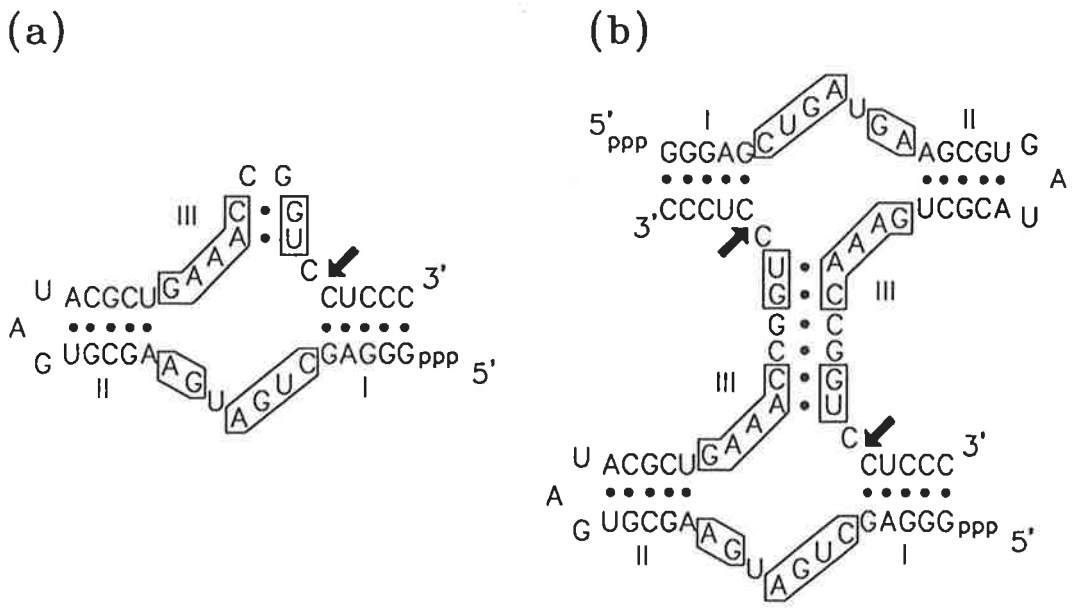



**Figure 4.1** Sequence of the 40 base nCG RNA generated by T7 RNA polymerase transcription of a synthetic oligodeoxynucleotide template. **(a)** Drawn as a single-hammerhead structure. **(b)** Drawn as a double-hammerhead structure. Certain residues have been altered compared with the hammerhead sequence from the RNA transcript of the newt satellite 2 sequence (Figure 1.1) to conform to the T7 promoter requirements and to reduce the possibility of alternative secondary structures. Stems are numbered I to III (after Forster and Symons, 1987a), sites of cleavage and base-pairs are indicated by arrows and dots, respectively, and bases conserved between most naturally occurring hammerhead structures are boxed.

concentration of NTPs, whereas, radioactive transcriptions differed by containing 0.025 mM UTP to generate a low concentration of RNA (2-10).

Unlabelled 5'-self-cleavage fragment RNA was prepared for use in self-cleavage reactions, by large scale self-cleavage reactions of unlabelled 40 base RNA, in Buffer A (see below) at 37°C for 2 h.

### 4-2 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, as described in 2-13.

## Results

### 4-3 Preparation of nCG RNA

The aim of the experiments described in this chapter was to determine whether the active structure for *in vitro* self-cleavage of the newt RNA was a single- or a double-hammerhead. This was approached by transcribing a 40-mer RNA that contained the approximate sequence of a single newt hammerhead from the appropriate synthetic oligodeoxynucleotide template using T7 RNA polymerase (Milligan *et al.*, 1987, 2-10). The promoter requirements for the T7 RNA polymerase system (Milligan *et al.*, 1987) did not permit the preparation of an RNA identical to the newt hammerhead sequence (Figure 1.1); in addition, the sequence of stem II was modified to minimise the possibility of alternative secondary structures forming. Figure 4.1 shows this 40-mer 'newt-like' RNA (termed nCG RNA, consistent with the nomenclature used in Chapter 5) drawn as (a) single- and (b) double-hammerhead structures.

Transcription from the synthetic DNA template (2-10) yielded a doublet of bands that migrated on a 20 % polyacrylamide, 7 M urea gel at approximately the position expected for the full-length product. Enzymic sequence analysis (2-16) determined that the lower band

was the full-length 40 base RNA (results not shown). Non-template encoded addition of nucleotides at the 3'-end of the transcript is a common finding with transcription systems (Milligan *et al.*, 1987).

The full-length nCG RNA did not self-cleave during the transcription reaction, and the 40 base full-length RNA was isolated from the gel (2-12), and used in the self-cleavage reactions described below.

## 4-4 Preliminary self-cleavage of nCG RNA

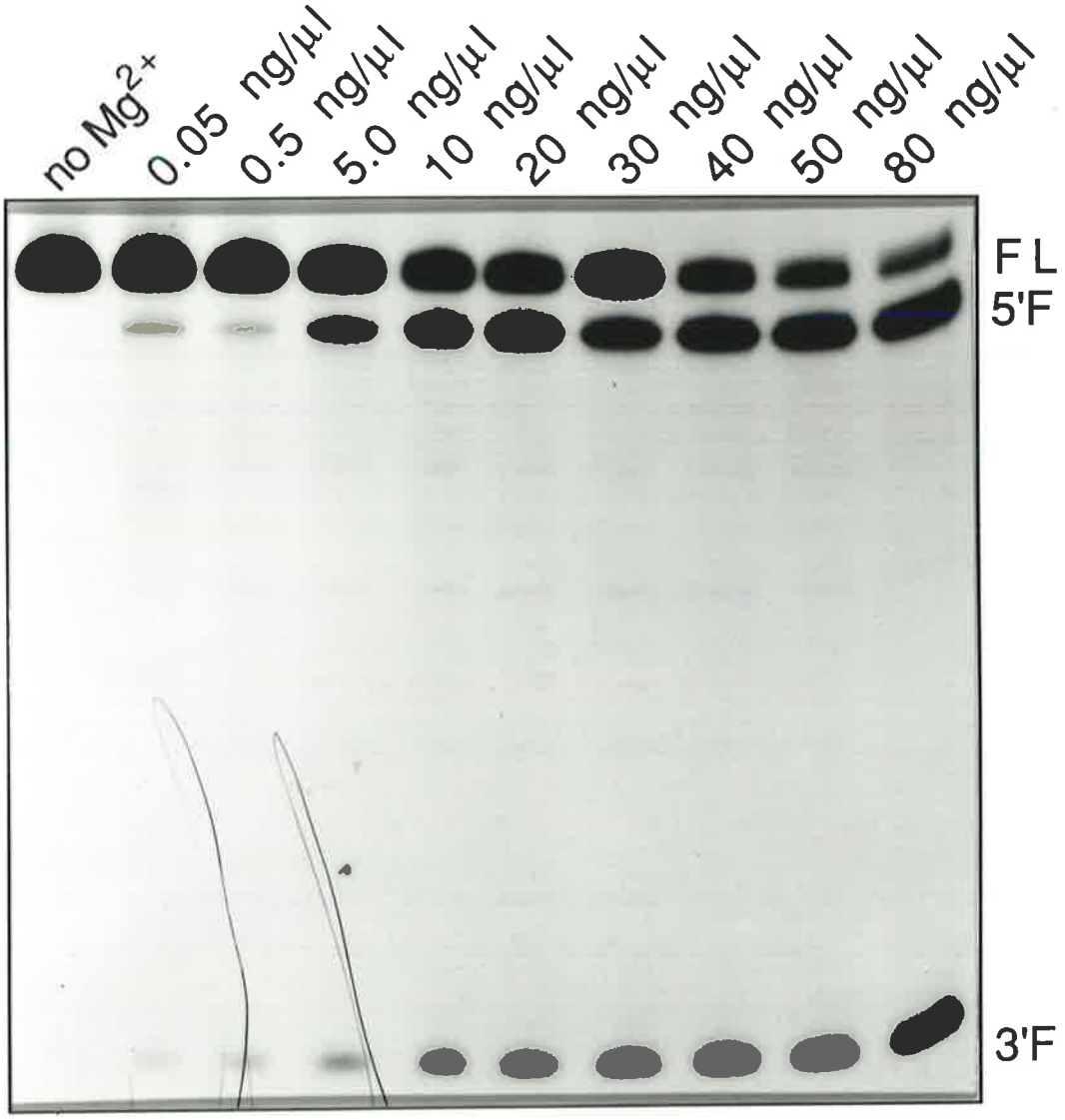
Preliminary experiments were undertaken at two concentrations of purified RNA (0.05 ng/µl and 50 ng/µl) to determine whether self-cleavage occurred, and the optimum conditions for the reaction. The lack of self-cleavage of the newt-like RNA during transcription, even though concentrations of about 30 ng/µl were generated, was consistent with the poor self-cleavage of purified nCG RNA when incubated under similar conditions (50 ng/µl RNA in 10 mM $MgCl_2$, pH 7.5, 37°C for 2 h, results not shown). A range of other magnesium concentrations, pH conditions, and temperatures of incubation were tested. Two conditions were found to give approximately the same high level of self-cleavage, with minimal non-specific RNA breakdown; 50 mM $MgCl_2$, pH 9.0 (Buffer A) at 37°C, and 10 mM $MgCl_2$, pH 8.0 (Buffer B) at 55°C (data not shown). When incubated under these conditions, the 40 base nCG RNA self-cleaved specifically to generate a 35 base 5'-fragment and a 5 base 3'-fragment. Direct RNA sequence analysis (2-16) and end-nucleotide analysis (2-17) established that self-cleavage had occurred at the expected bond, indicated by the arrow in Figure 4.1 (results not shown).

## 4-5 Self-cleavage of the nCG RNA approximates to a bimolecular reaction

Initially, only one reaction condition was used to investigate the dependence of self-cleavage on RNA concentration (10 mM $MgCl_2$, pH 8.0 (Buffer B) at 55°C). RNAs were incubated at 9 different RNA concentrations, spanning a concentration range of 1600-fold, from 0.05 ng/µl to 80 ng/µl. As can be seen in Figure 4.2, the extent of self-cleavage after 2 h increased with increasing RNA concentration.

A time course was carried out for three concentrations of RNA, 0.05 ng/µl, 5 ng/µl and 50 ng/µl. The graph of self-cleavage efficiency versus time for RNA incubated at 55°C

**Figure 4.2** RNA self-cleavage of nine different concentrations of nCG RNA, as indicated. RNAs in 1 mM EDTA were heated at 80°C for 1 min and snap-cooled on ice, then incubated in 10 mM $MgCl_2$, 0.5 mM EDTA, 50 mM Tris-HCl, pH 8.0 at 55°C for 2 h, as described in 4-2. 'No $Mg^{2+}$' lane: RNA was incubated in 10 mM EDTA, 50 mM Tris-HCl, pH 8.0 at 55°C for 2 h. RNAs were electrophoresed on a 20 % polyacrylamide, 7 M urea gel, and detected by autoradiography. FL, full-length nCG RNA; 5'F, 5'-self-cleavage fragment; 3'F, 3'-self-cleavage fragment.


no $Mg^{2+}$
0.05 ng/µl
0.5 ng/µl
5.0 ng/µl
10 ng/µl
20 ng/µl
30 ng/µl
40 ng/µl
50 ng/µl
80 ng/µl
F L
5'F
3'F

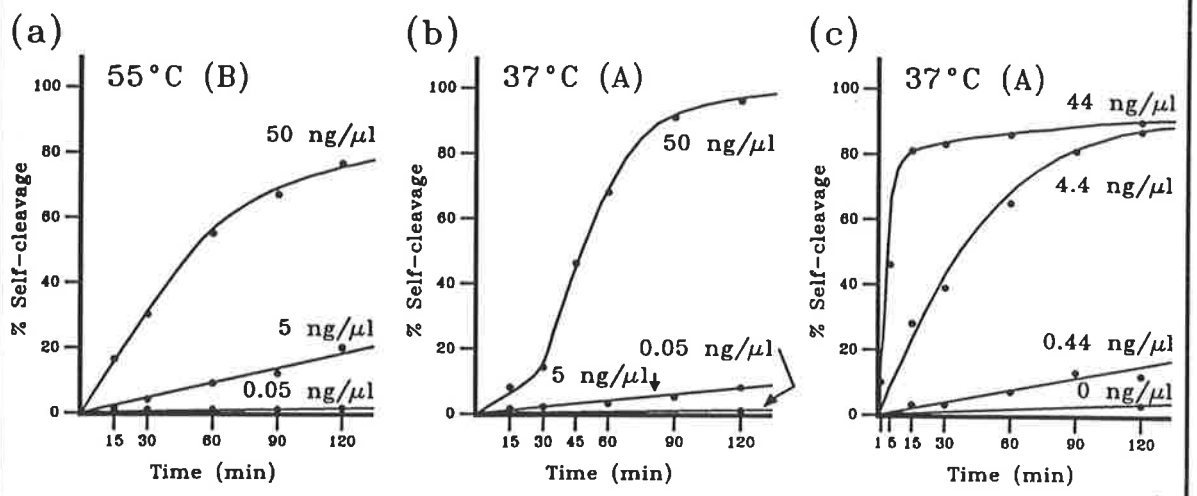



**Figure 4.3** Effect of RNA concentration on the extent (%) of self-cleavage of the 40 base nCG RNA as a function of time. (**a**) The 40 base nCG RNA at concentrations of 0.05, 5.0, 50 ng/μl, incubated at 55°C in Buffer B. (**b**) As for (a), except that the RNAs were incubated at 37°C in Buffer A. (**c**) The 35 base 5'-self-cleavage fragment of the nCG RNA at 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.)

in Buffer B (Figure 4.3a) shows, as before, that the extent of self-cleavage increased with increasing concentration of RNA. Cleavage of the lowest concentration of this RNA (0.05 ng/μl) 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 initial rate of self-cleavage (ng $\mu l^{-1} min^{-1}$) at a concentration of 50 ng/μl of RNA was approximately 80 times that at an RNA concentration of 5 ng/μl; the second order rate equation for a bimolecular reaction predicts a 100-fold difference in rate. Hence, the kinetics for the self-cleavage reaction of the 40 base nCG RNA approximate to those of a bimolecular reaction, as predicted by the double-hammerhead model.

## 4-6 Self-cleavage under a different condition

The same concentration range of RNA was incubated under another reaction condition. The graph of the cleavage reaction carried out at 37°C in 50 mM $MgCl_2$, pH 9.0 (Buffer A), is given in Figure 4.3b. It differs from the 55°C graph (Figure 4.3a) 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.

## 4-7 Cleavage of the nCG RNA can be catalysed by the 5'-self-cleavage fragment

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 4.3c 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 (Figure 4.4a).

On the basis of these results, the sigmoidal curve obtained at 37°C (Figure 4.3b) can be interpreted as follows. The proposed nCG single-hammerhead structure (with three stems: Figure 4.1a) does not form due to the low stability of stem III and consequently no single-hammerhead self-cleavage occurs. Inactive structures containing stems I and II, but

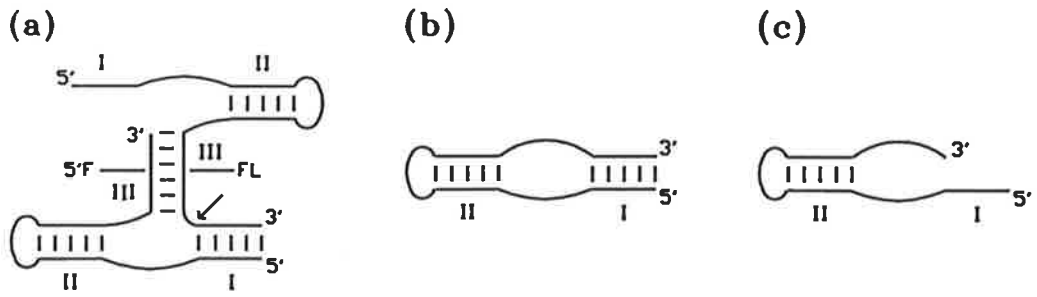



**Figure 4.4** Schematic diagrams of nCG full-length (FL) and 5'-fragment (5'F) secondary structures. (**a**) Proposed partial double-hammerhead structure formed by interaction of one full-length RNA with one 5'-fragment, capable of catalysing the cleavage of the full-length RNA. (**b**) Proposed structure of the full-length RNA 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 are numbered I to III (after Forster and Symons, 1987a), sites of cleavage and base-pairs are indicated by arrows and dots, respectively, and bases conserved between the virusoid, ASBV, plus sTRSV and newt RNAs are boxed.

not stem III (Figure 4.4b) (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 stem I) and so would not be capable of forming such stable structures (Figure 4.4c). As a consequence, the formation of a partial double-hammerhead structure by the interaction of a full-length RNA and a 5'-fragment (Figure 4.4a), resulting in the cleavage of the full-length RNA, would occur more readily as it is easier to form than the standard double-hammerhead structure. 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 (Figure 4.3b).

The rate of self-cleavage of 0.05 ng/µl of full-length RNA when incubated with varying concentrations of 5'-fragment was roughly dependent on the concentration of the 5'-fragment. The increase in the initial reaction rate was approximately 10 fold for the increase in the 5'-fragment concentration from 0.44 to 4.4 ng/µl and 6-fold from 4.4 to 44 ng/µl. The lower than expected increase in rate from 4.4 to 44 ng/µl (Figure 4.3c), possibly indicates that the full-length RNA was becoming saturated with the 5'-fragment.

## Discussion

### 4-8 Double-hammerhead mediated self-cleavage of the 40 base nCG RNA

The results presented in this Chapter provide evidence for the involvement of the double-hammerhead structure in the *in vitro* self-cleavage of the 40 base nCG RNA. The very low level of self-cleavage of the nCG RNA at 0.05 ng/µl (Figure 4.3a,b,c) demonstrates that a single-hammerhead structure, if it occurred at all, was a very minor contributor to nCG self-cleavage. The efficient self-cleavage of higher concentrations of RNA, that roughly followed second order reaction kinetics (Figure 4.3a), demonstrated that a bimolecular double-hammerhead structure was responsible for the self-cleavage of this RNA. Hence, it appears that nCG RNA is unable to form a single-hammerhead structure

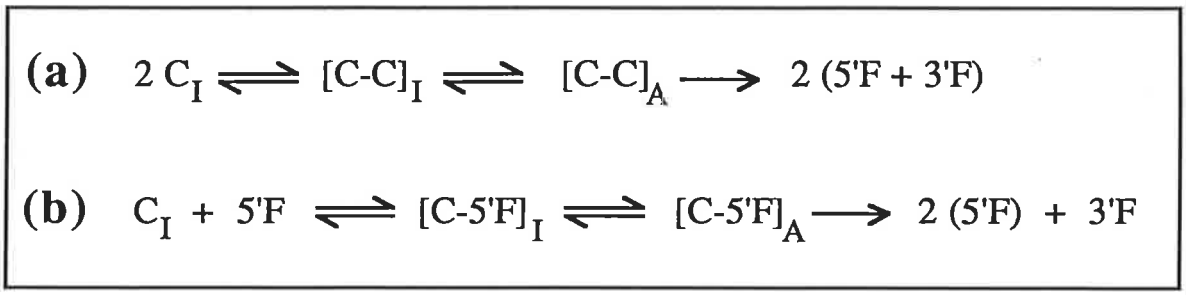



**Figure 4.5** Proposed interaction between inactive and active conformations of the full-length nCG RNA (C) and the 5'-self-cleavage fragment (5'F), and the pathways leading to self-cleavage of the nCG RNA. (**a**) Self-cleavage by double-hammerhead ($[C\text{-}C]_A$) structures. (**b**) Self-cleavage of the nCG RNA catalysed by the 5'-fragment (5'F). 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. $[C\text{-}5'F]_A$ and $[C\text{-}5'F]_I$ represent active and one or more inactive conformations, respectively, of the full-length RNA:5'-fragment complex.

that allows it to self-cleave, however, the double-hammerhead structure is sufficiently stable to allow self-cleavage.

## 4-9 Self-cleavage of nCG RNA can occur by two different pathways

On the basis of the work presented here, and from other results (Forster and Symons, 1987a, Forster *et al.*, 1987), it seems likely that Figure 4.5 describes the reaction pathways for the self-cleavage of the newt-like RNA. In order for self-cleavage to occur, one of the active structures, either double-hammerhead ($[C\text{-}C]_A$), or partial double-hammerhead ($[C\text{-}5'F]_A$) must form. It seems likely that there are three steps involved in the formation of the active structure. Firstly, the structures that the RNAs possess at the start of the reaction ($C_I$ and $5'F_I$), must be unfolded, so that secondly, two RNAs can interact to form a bimolecular complex ($[C\text{-}C]_I$ and $[C\text{-}5'F]_I$). Thirdly, structural alteration of the bimolecular complex must occur to form the active structure ($[C\text{-}C]_A$ and $[C\text{-}5'F]_A$). The first two steps probably occur together, possibly cooperatively, and are represented by the first step in the equations in Figure 4.5. The conformational change to form an active bimolecular structure is represented by the second step of the equations in Figure 4.5.

## 4-10 The relative stabilities of active and inactive structures affect the extent of the self-cleavage reaction, and by which pathway self-cleavage occurs

Which pathway the cleavage reaction followed was dependent on the reaction conditions used. In Buffer B at 55°C, the reaction followed pathway (a) (Figure 4.5a), however, in Buffer A at 37°C, the majority of the self-cleavage appeared to occur by pathway (b) (Figure 4.5b). This presumably reflects the different relative stabilities of the double-, or partial double-hammerhead and inactive structures, under the conditions used.

The proportion of RNA molecules that form either of the active structures ($[C\text{-}C]_A$ and $[C\text{-}5'F]_A$), depends on, apart from RNA concentration, the relative stabilities of inactive structures (monomolecular : $C_I$ and $5'F_I$, and bimolecular : $[C\text{-}C]_I$ and $[C\text{-}5'F]_I$), compared with the active structures under the reaction conditions used. If a proportion of the RNA is folded into relatively very stable inactive structures, then it will never participate in the reaction, i.e., the equilibrium of the reaction will be to the left-hand side.

Viewing the self-cleavage reaction in the manner described above allows interpretation of the self-cleavage profiles in Figure 4.3. In Figure 4.3a, the self-cleavage reaction did not exactly follow second order kinetics, possibly because a proportion of the RNA, is folded into relatively stable inactive structures, and does not participate in the reaction. In Figure 4.3b, it appears that the high relative stabilities of monomolecular inactive structures resulted in poor self-cleavage at low RNA concentrations. At higher RNA concentrations (50 ng/µl), the rate of cleavage was similarly slow until the generation of the less stable 5'-fragment allowed the formation of partial double-hammerhead structures, at which stage the reaction rate increased markedly, as the active structure became easier to form.

In Figure 4.3c, self-cleavage of full-length RNA required the interaction of the full-length RNA with a 5'-self-cleavage fragment. A very low concentration of the full-length RNA was used, to eliminate the possibility of self-cleavage mediated by a standard double-hammerhead structure, formed by the interaction of two full-length RNAs. The self-cleavage of the full-length RNA appears to be dependent on the concentration of the 5'-fragment RNA. This is presumably because the chance of a productive interaction between full-length and 5'-fragment was greater at higher concentrations of 5'-fragment. That the reaction so closely follows the expected kinetics (rate proportional to [nCG RNA]x[5'F RNA]), indicates that stable inactive structures that might have perturbed the kinetics (as in Figure 4.3b) did not form.

The reason why the two different reaction conditions caused the RNA to follow different reaction pathways is the greater stability of inactive monomolecular structures (and other structures as well) in Buffer A at 37°C compared with Buffer B at 55°C. Presumably, the higher $Mg^{2+}$ concentration and lower temperature resulted in increased stability of the structures formed, and perhaps decreased the transitions from one structure to another.

## 4-11 Formation of the active structure is rate-limiting

Forster and Symons (1987b) found that quantitative cleavage of a 52-mer containing the plus vLTSV hammerhead sequence occurred in less than one minute. From this, it appears that once the active structure is formed, self-cleavage is rapid. In Figure 4.3b, the rate of self-cleavage at 50 ng/µl is slow initially, until generation of the 5'-fragment allows

more rapid formation of the active structure, at which time the self-cleavage rate increases. Hence, it appears that the rate limiting step in the self-cleavage reactions described here, is the formation of the active structures.

## 4-12 Relevance to other systems

Koizumi *et al.* (1988a) used a *trans* hammerhead system that was based on the newt hammerhead sequence. From the results presented in this Chapter, it would be expected that the "substrate" RNA would be cleaved only if it interacted with another substrate RNA as well as the "enzyme" RNA, to form a partial hammerhead structure. The relatively high RNA concentrations used, and the long incubation times (23 h), make it possible that self-cleavage of the substrate RNA did occur by the interaction of three RNAs, rather than by the two RNA single-hammerhead structure presented in the paper (Koizumi *et al.*, 1988a).

Since the completion of this work, Epstein and Pabon-Pena (1991) have reported apparant single-hammerhead self-cleavage in certain monomeric and dimeric newt transcripts. It appears that in these cases, non-hammerhead sequences can allow the stabilisation of the single-hammerhead structures in the newt transcripts. This is discussed further in Chapter 6.

# CHAPTER 5

# STABILISING THE SINGLE-HAMMERHEAD STEM III CONVERTS A DOUBLE-HAMMERHEAD REACTION INTO A SINGLE-HAMMERHEAD REACTION

## Introduction

Chapter 4 provided evidence for the involvement of a double-hammerhead structure in the self-cleavage of the 40 base nCG RNA, by demonstrating that, under certain reaction conditions, the kinetics for the self-cleavage reaction of the nCG RNA approximated to those expected for a bimolecular reaction. Hence, the single-hammerhead structure of the nCG RNA, with its weak stem III, was unable to mediate self-cleavage, however, double-, or partial double-hammerhead structures, both with more stable stem IIIs, were able to mediate self-cleavage.

In this Chapter, the double-hammerhead mediated self-cleavage of the nCG RNA was converted to a single-hammerhead reaction by increasing the size of stem III and/or of its loop, thereby stabilising the stem III of the single-hammerhead structure, and enabling a single-hammerhead structure to form.

## Methods

Preparation of RNAs and self-cleavage reactions were as in Chapter 4.

## Results

The approach used for the conversion of self-cleavage of the nCG RNA (Figure 4.1) 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. The RNAs, like the nCG RNA in Chapter 4, were generated by transcription with T7 RNA polymerase using synthetic DNA templates. 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 this type of transcription reaction was taken to indicate single-hammerhead cleavage. This was further

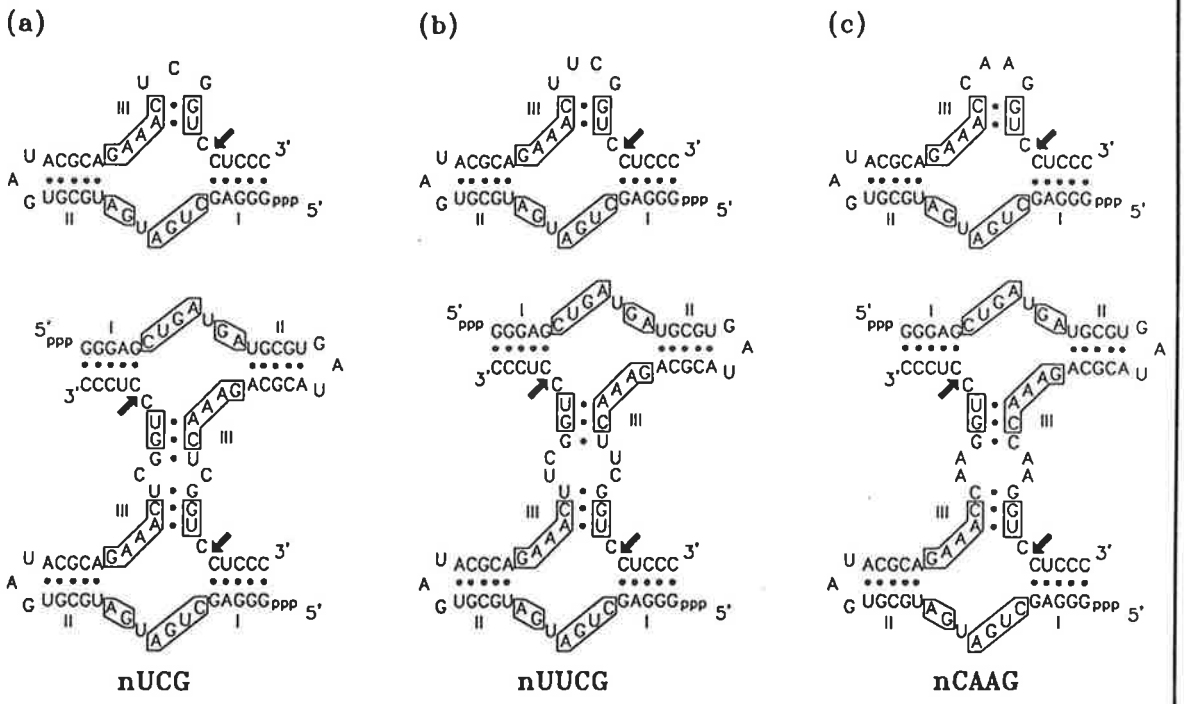



**Figure 5.1** Sequence of RNAs generated by T7 RNA polymerase transcription of synthetic DNA templates drawn as both single- and double-hammerhead structures. **(a)** nUCG RNA, **(b)** nUUCG RNA, **(c)** nCAAG RNA. The RNAs are based on the nCG sequence (Figure 4.1a) but in addition to the bases inserted into 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 are numbered I to III (after Forster and Symons, 1987a), sites of cleavage and base-pairs are indicated by arrows and dots, respectively, and bases conserved between most naturally occurring hammerhead structures are boxed.

verified by the efficient cleavage of low concentrations (0.05 ng/µl) of the purified full-length RNA when incubated with $MgCl_2$.

Little, or no, cleavage during the transcription reaction, however, does not preclude the possibility of single-hammerhead cleavage, as the RNA may have folded preferentially into inactive structures 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 4-2), 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 on, or independent of, the RNA concentration.

## 5-1 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 two 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 5.1a. 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 was folded into inactive structures 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 indicate that inactive structures are more stable, under the conditions used, than the hammerhead 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 5.1b.

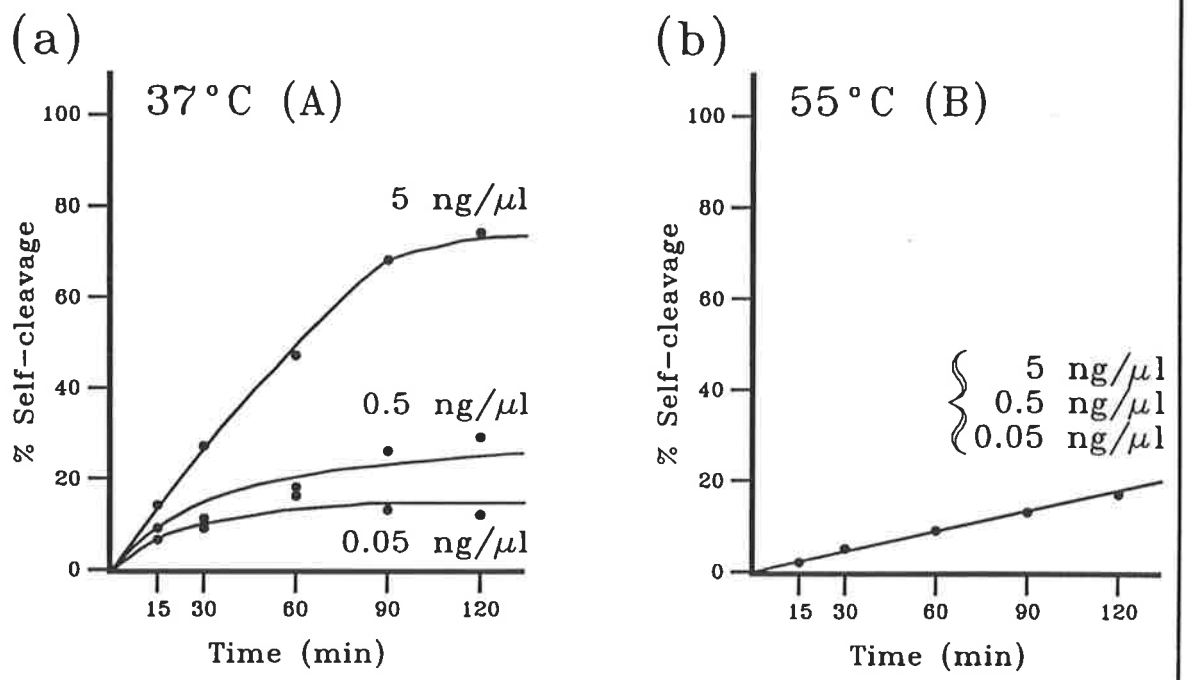



**Figure 5.2** Effect of nUUCG concentration on the extent (%) of self-cleavage as a function of time. The 42 base nUUCG RNA (Figure 5.1b) was incubated at concentrations of 0.05, 0.5 and 5.0 ng/μl (**a**) at 37°C in Buffer A, and (**b**) at 55°C in Buffer B.

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 (Figure 5.2a) the extent of self-cleavage of nUUCG RNA 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 (Figure 5.2b).

The lack of concentration dependent cleavage at 55°C can be explained by considering that the nUUCG double-hammerhead structure was not able to form due to the presence of the destabilising interior loop in stem III (Figure 5.1b). The concentration independent cleavage that occurred indicates that the single-hammerhead structure was stable. At 37°C it appears that both single- and double-hammerhead structures were stable and therefore there was a proportion of single-hammerhead cleavage with an additional amount of double-hammerhead cleavage which was 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, Figure 5.1c).

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.

## 5-2 RNAs with a three base-pair stem III and three or four base loop can self-cleave by a single-hammerhead structure

Tuerk *et al.* (1988) reported that RNA base-paired stems closed by a C-G base pair and with loops of sequence UUCG are 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 (Tuerk *et al.*, 1988). 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 (Figure 5.3a-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 (Figure 5.4a, 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).

nUUCG(GC) RNA, in contrast, cleaved poorly during the transcription reaction (Figure 5.4a, 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 (Figure 5.4b), 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 (Figure 5.4c). 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 (Figure 5.4c). 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 destabilisation of stems by switching the top base-pair or altering the sequence of the loop (Tuerk *et al.*, 1988) 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 three base-pair stem III and a three base loop of sequence UCG (termed nUCG(CG), Figure 5.3d) 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

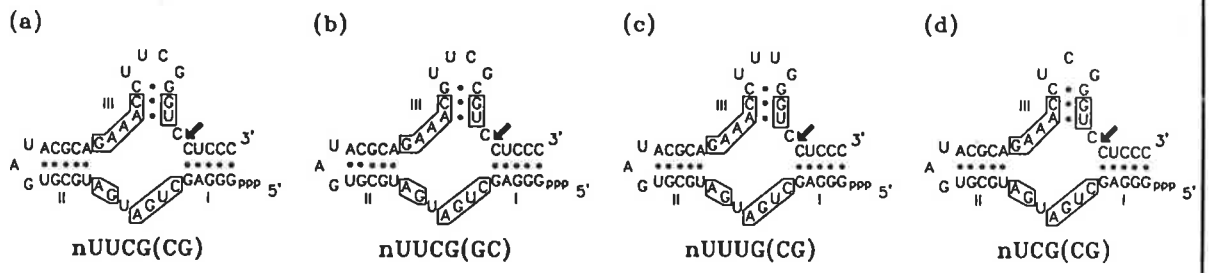
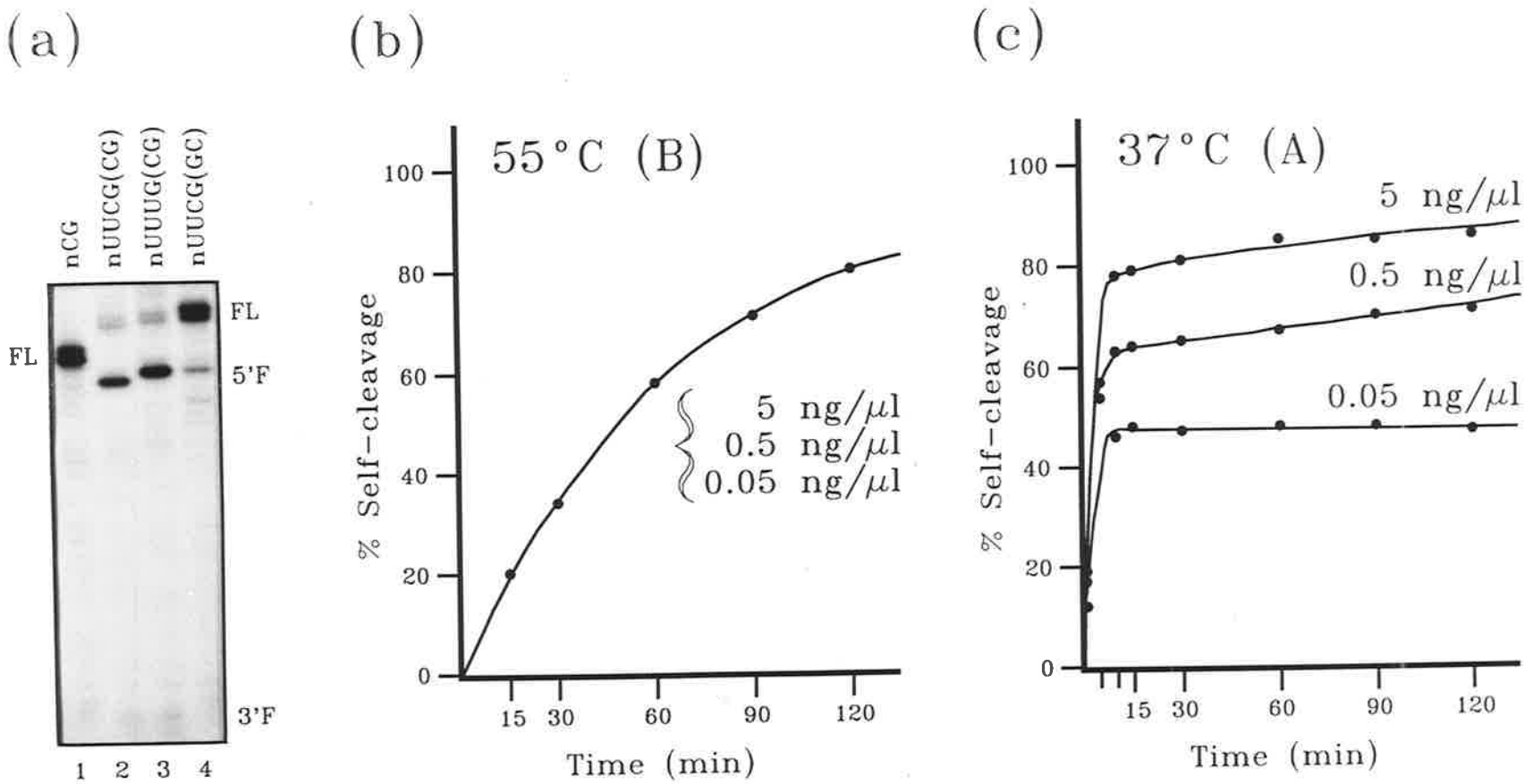



**Figure 5.3** Single-hammerhead structures of RNAs with stem IIIs of three base-pairs and increased loop size relative to the nCG RNA, generated by T7 RNA polymerase transcription of synthetic DNA templates. The RNAs are based on the nCG sequence (Figure 4.1a) but in addition to the bases inserted into 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 are numbered I to III (after Forster and Symons, 1987a), sites of cleavage and base-pairs are indicated by arrows and dots, respectively, and bases conserved between most naturally occurring hammerhead structures are boxed.

**Figure 5.4 (adjacent page)** 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 nCG (Figure 4.1) 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) (Figure 5.3a) template. **Lane 3**, transcription of nUUUG(CG) (Figure 5.3c) template. **Lane 4**, transcription of nUUCG(GC) (Figure 5.3b) 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.


(a)
nCG
nUUCG(CG)
nUUUG(CG)
nUUCG(GC)
FL
FL
5'F
3'F
1 2 3 4
(b)
55 °C (B)
% Self-cleavage
100
80
60
40
20
0
5 ng/μl
0.5 ng/μl
0.05 ng/μl
15 30 60 90 120
Time (min)
(c)
37 °C (A)
% Self-cleavage
100
80
60
40
20
0
5 ng/μl
0.5 ng/μl
0.05 ng/μl
15 30 60 90 120
Time (min)

non-concentration 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 three base-pair stem III and a three or four base loop.

## Discussion

### 5-3 Stabilising the single-hammerhead stem III converts a double-hammerhead reaction into a single-hammerhead reaction

The results reported in this Chapter demonstrate that the double-hammerhead self-cleavage reaction of the nCG RNA (Figure 4.1) can be converted into a single-hammerhead reaction by enlarging stem III and/or its loop to give a more stable single-hammerhead structure.

The work in this Chapter, and Chapter 4 have demonstrated that the stability of stem III is an important factor 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. These 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. Ruffner *et al.* (1989) have also demonstrated that a single-hammerhead structure containing a three base-pair stem III with a three base loop was capable of self-cleavage.

Comparison of the self-cleavage profiles in Figure 5.2b and Figure 5.4b reveals that nUUCG(GC) self-cleaves to a much higher extent than nUUCG in Buffer B at 55°C. Hence, under these conditions, more efficient self-cleavage occurred in the nUUCG(GC) single-hammerhead structure, with three base-pairs in stem III, than in the nUUCG single-

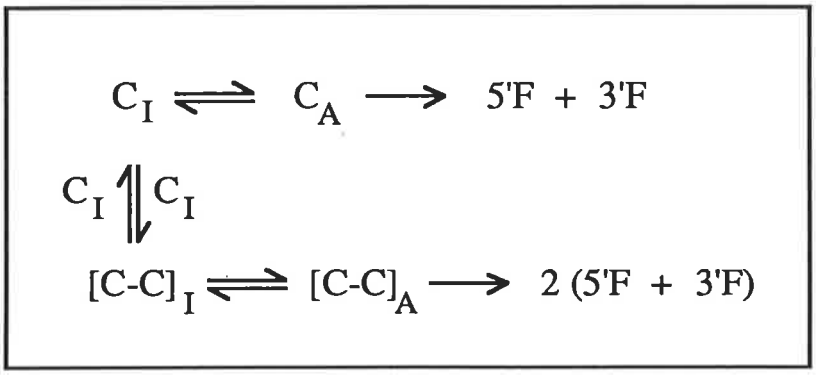



**Figure 5.5** Proposed interaction between inactive and active conformations of the various RNAs and the pathways leading to their self-cleavage. Self-cleavage occurs by both single-hammerhead ($C_A$) and double-hammerhead ($[C\text{-}C]_A$) structures. Self-cleavage generates a 5'-fragment (5'F) and a 3'-fragment (3'F), A and I indicate active and one or more inactive structures, respectively.

hammerhead, with two base-pairs in stem III. Presumably, this reflects the greater stability of the nUUCG(GC) single-hammerhead, due to the more stable stem III.

## 5-4 The relative stabilities of inactive and active structures determine the pathway and extent of the self-cleavage reaction

It seems likely that equations similar to those presented in Chapter 4 represent the self-cleavage of the variant RNAs in this Chapter. The RNAs in this Chapter, however could self-cleave by both single- and double-hammerhead structures, depending on the conditions used (Figure 5.5). In order for self-cleavage to occur, the inactive structures (represented by monomer $C_I$ and dimer $[C\text{-}C]_I$ in Figure 5.5) undergo transformation to an active structure, either single-hammerhead ($C_A$; Figure 5.5) or double-hammerhead ($[C\text{-}C]_A$; Figure 5.5). Which pathway the cleavage reaction of any particular RNA follows depends on the relative stabilities of the single-, or double-hammerhead and inactive structures under the conditions used. These results are interesting as they demonstrate that an RNA can self-cleave by two different (but related) structures.

Consistent with the nCG RNA (Chapter 4), the results from the variant RNAs suggest that the rate limiting step of the self-cleavage reaction is the formation of the active structures ($C_A$ or $[C\text{-}C]_A$; Figure 5.5). At 37°C, RNAs with a three base-pair stem III (nUCG(CG) and nUUCG(GC); Figure 5.3c,d) appear to form the active structure rapidly as the majority of the self-cleavage occurred in less than fifteen minutes (Figure 5.4c; 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 (Figure 5.4b; and results not shown), indicating that the transition from inactive to active structures occurred more slowly.

During the transcription reaction, nUUCG(CG) and nUUUG(CG) self-cleaved to approximately 95%, whereas nUUCG(GC) self-cleaved to only about 5% (Figure 5.4a, 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 have formed preferentially to the active structure as the nascent RNA emerged 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 were refolded into a mixture of active and inactive structures. The fraction of RNA that did not cleave presumably was folded into stable inactive structures.

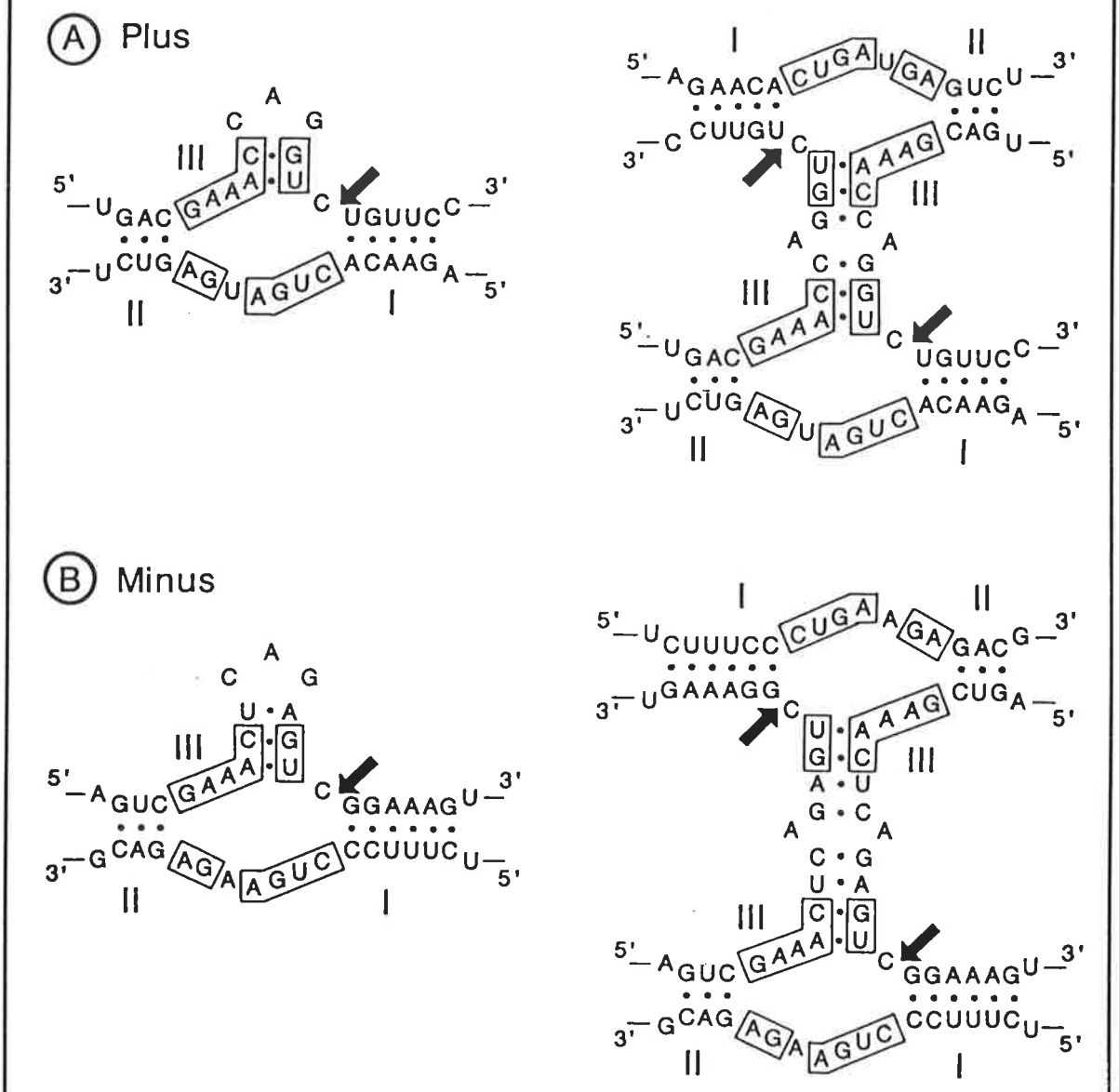



**Figure 6.1** Single- and double-hammerhead structures of the self-cleaving RNAs of plus (**A**) and minus (**B**) ASBV (Forster *et al.*, 1988). Base-paired stems are numbered I to III after Forster and Symons (1987a), sites of cleavage are indicated by arrows and nucleotides conserved between most naturally occurring self-cleaving RNAs are boxed.

# CHAPTER 6

# ALTERNATIVE HAMMERHEAD STRUCTURES IN THE *IN VITRO* SELF-CLEAVAGE OF ASBV RNAs

## Introduction

The single-hammerhead structures of plus and minus ASBV, like that of the newt RNA (Chapter 4) appear theoretically unstable due to the presence of weak stem IIIs with sterically constraining loops (Figure 6.1; Hutchins *et al.*, 1986, Forster and Symons, 1987b). The minus ASBV single-hammerhead stem III contains a three base-pair stem with a three base loop, and the plus contains a two base-pair stem with a three base loop. Double-hammerhead structures, which contain theoretically more stable stem IIIs, have been proposed to mediate self-cleavage of these RNAs (Figure 6.1; Forster *et al.*, 1988). The double-hammerhead structure of minus ASBV contains eight base-pairs with an interior loop of two bases, and plus ASBV double-hammerhead structure contains six base-pairs with an interior loop of two bases.

It has been demonstrated previously that double-hammerhead structures mediated self-cleavage of dimeric plus and minus ASBV RNA transcripts during the *in vitro* transcription reaction (Forster *et al.*, 1988, Davies *et al.*, 1991). The observation that monomeric plus and minus ASBV RNA transcripts did not self-cleave during *in vitro* transcription (Forster *et al.*, 1988, Davies, 1988) was consistent with the requirement of double-hammerhead structures for these RNAs, as the efficiency of formation of a double-hammerhead structures during the transcription reaction, by the interaction of two monomeric RNAs, would be expected to be low (Forster *et al.*, 1988, Davies, 1988). On the basis of these results, it appeared that both plus and minus ASBV RNAs required double-hammerhead structure for self-cleavage (Forster *et al.*, 1988, Davies, 1988).

Other evidence, however, indicated that minus ASBV RNA transcripts might be capable of single-hammerhead self-cleavage. Self-cleavage of a dimeric minus ASBV RNA transcripts at the first self-cleavage site only, generates two fragments, 5'E and M/3'E (see Figure 6.3A). M/3'E (previously called 3'P, Hutchins *et al.*, 1986) contains one complete hammerhead sequence, and the stem I sequence immediately 3' to the first self-cleavage site. As such, it cannot form an intramolecular double-hammerhead structure, however, it

underwent efficient self-cleavage (Hutchins *et al.*, 1986). Similarly, Forster *et al.* (1987) obtained efficient *trans* self-cleavage of a partial monomeric minus ASBV RNA, when incubated with another partial minus monomeric RNA. In order for a double-hammerhead structure to be formed with these RNAs, interaction of four RNAs would have to occur. Considering the efficiency of the reaction (Forster *et al.*, 1987), this seems unlikely. In these two examples, it seems likely that self-cleavage of minus ASBV RNAs was occurring by single-hammerhead structures.

In addition, on the basis of the results in Chapter 5, it would be predicted that minus ASBV with its three base-pair stem III and three base loop would be capable of single-hammerhead self-cleavage. Indeed, Ruffner *et al.* (1989) obtained apparant single-hammerhead self-cleavage using two short RNA oligonucleotides with sequence based on the minus ASBV hammerhead sequence, further indicating that minus ASBV RNA should be capable of single-hammerhead self-cleavage.

In this Chapter, the *in vitro* self-cleavage of plus and minus monomeric and dimeric ASBV RNA transcripts was explored further, to investigate whether double-hammerhead structures were required for plus and minus ASBV self-cleavage, or whether self-cleavage could occur by single-hammerhead structures. The full-length, uncleaved transcription products were purified for a variety of plus and minus dimeric and monomeric RNAs, and incubated under self-cleavage conditions. The results obtained indicate that generally, minus ASBV can self-cleave by a single-hammerhead structure, but plus ASBV requires a double-hammerhead structure. However, a number of other factors were found to affect the type of structure involved in the *in vitro* self-cleavage reaction.

## Materials

### 6-1 Plasmid constructions of ASBV cDNA clones

The original ASBV Sau3A monomeric and dimeric cDNA clones was constructed by C. Hutchins. From these clones, C. Davies constructed the mutated dimeric cDNA clones and the BstNI and HphI monomers, and generously provided them for my use. Descriptions of the clones are given, unless published elsewhere.

Plus and minus dimeric Sau3A clones in the plasmid vectors pSP64 and pSP65 (Figures 6.4A, 6.5C, 6.7B,C). The construction of the plasmids containing dimers of the wild-type

ASBV cDNA cloned at the Sau3A position (terminal ASBV nucleotides 153 and 154) in both plus and minus orientations, in the transcription vectors pSP64 and pSP65, respectively, have been described (Hutchins *et al.*, 1986). The construction of similar clones, in the plus orientation, that are mutant in either or both of the plus ASBV hammerhead sequences (the conserved GAAAC sequence mutated to GAAC) have also been described (Forster *et al.*, 1988).

Dimeric BstNI clones in the plasmid vector pGem1 (Figures 6.3A, 6.5D). ASBV cDNA dimeric clones in pGem1, in the minus orientation, cloned at the BstNI site of ASBV cDNA, that are mutated in either, neither, or both minus ASBV hammerhead sequences (the GAAAC sequence mutated to GAAC) were described by Davies *et al.* (1991). Note that during the cloning procedure, the BstNI site (CC/AGG) between the ligated monomers was destroyed, and a StyI site (C/CAAGG) was created (Figures 6.3A, 6.5D).

Plus and minus monomeric Sau3A clones in the plasmid vector pSP64 (Figures 6.5B, 6.7D,E). Wild-type monomer sized fragments were excised using Sau3A from a Sau3A monomer clone in phage M13mp93 DNA and ligated into dephosphorylated BamHI digested pSP64. The sequence and orientation of the inserts was established by subcloning into M13, followed by dideoxy sequencing.

Monomeric HphI clones in the plasmid vector pGem1 (Figures 6.5E, 6.7F). Monomeric cDNA resulting from the digestion of the dimeric Sau3A clone in pSP64 with HphI was treated with the exonuclease function of T4 DNA polymerase to remove the single-base 3' overhang, resulting in the loss of one base-pair. The fragment, with terminal ASBV nt 119 and 121, was then ligated into dephosphorylated SmaI digested pGem1. The sequence of the insert was confirmed and the orientation determined by subcloning into M13 and dideoxy sequencing.

## Methods

### 6-2 *In vitro* transcription from linearised plasmid templates

Plasmid DNA was prepared as described in 2-18. Clones were digested with the appropriate restriction enzyme (as indicated in each Figure or in the text) and transcribed with either T7 or SP6 RNA polymerase (2-11). RNA transcripts were separated on a 7 M urea, 5% polyacrylamide gel, and full-length transcripts were excised from the gel and eluted (2-12).

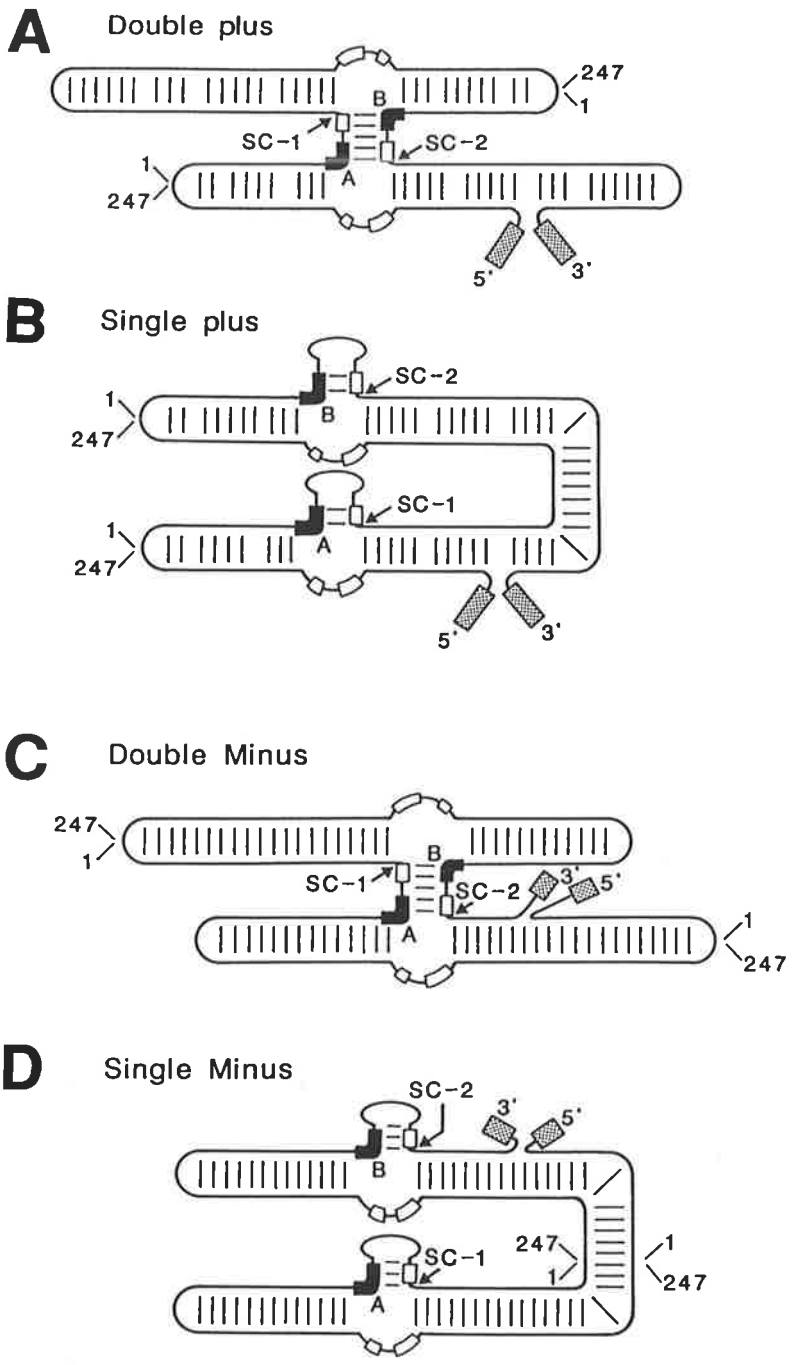



**Figure 6.2** Schematic representations of a dimeric plus ASBV RNA transcribed from wild-type Sau3A dimeric cDNA clone in pSP64 vector (Figure 6.3A), and a dimeric minus ASBV RNA transcribed from wild-type BstNI dimeric cDNA clone in pGem1 vector (Figure 6.2A), folded to contain double- (**A** plus, **C** minus) and single- (**B** plus, **D** minus) hammerhead structures. Self-cleavage sites, labelled SC-1 and SC-2, are indicated by arrows; stippled boxes indicate vector sequences at 5'- and 3'-ends; closed boxes indicate GAAAC sequences (Figure 6.1) labelled A and B; open boxes indicate remaining conserved nucleotides (Figure 6.1). Base-pairing is represented by lines between RNA strands. Sequence numbered after Symons (1981).

### 6-3 *In vitro* self-cleavage of purified RNAs

Gel purified full-length RNA transcripts in 1 mM EDTA, pH 6, were heated at 80°C for 1 min, snap-cooled on ice and then incubated in 50 mM Tris-HCl, pH 8.0, 10 mM $MgCl_2$ (or 50 mM $MgCl_2$), 0.5 mM EDTA at 37°C for 1 h (refer to 2-13). Omitting the heating and snap-cooling step had little effect on the efficiency of self-cleavage, but was routinely included, to provide a uniform starting material for the reactions.

## Results

### 6-4 Dimeric plus and minus ASBV RNAs both self-cleave by double-hammerhead structures during *in vitro* transcription

Dimeric plus and minus ASBV RNA transcripts, generated *in vitro* by SP6 RNA polymerase transcription of the dimeric Sau3A or BstNI cDNA templates, respectively (Figure 6.4A, 6.3A), have previously been shown to self-cleave during the transcription reaction by double-hammerhead structures (Forster *et al.*, 1988, Davies *et al.*, 1991). The requirement for double-hammerhead structures was demonstrated by the use of single-base mutations in which the conserved GAAAC sequences just 5' to the self-cleavage sites, SC-1 and SC-2 (Figures 6.1, 6.3A, 6.4A), were mutated to GAAC (deletion of one A residue) either separately, or together to give a double mutant (this mutation abolishes self-cleavage *in vitro* (Forster *et al.*, 1988); see Figure 6.3B, lanes 2,3 and Figure 6.4B, lanes 2,3). The full-length plus and minus RNA transcripts are shown diagramatically in Figure 6.2, folded into double- (Figure 6.2A,C) and single- (Figure 6.2B,D) hammerhead structures. As can be interpreted from Figure 6.2, abolishment of self-cleavage at the self-cleavage site over 250 bases away from the mutated GAAAC sequence was indicative of double-hammerhead mediated self-cleavage. Inhibition of self-cleavage at the self-cleavage site just 3' to the mutated GAAAC sequence would have been indicative of single-hammerhead mediated self-cleavage (Forster *et al.*, 1988).

During the transcription reaction, the efficiency of self-cleavage of both plus and minus dimeric RNAs mutated at one site was about 50% (Forster *et al.*, 1988, Davies *et al.*, 1991; Figure 6.3B, lanes 4,6, Figure 6.4B, lanes 4,6); presumably the residual uncleaved RNA was folded into inactive conformations that did not permit self-cleavage. In this Chapter, the full-length mutated plus and minus RNAs were purified and incubated under

self-cleavage conditions (10 mM $MgCl_2$, pH 8.0, 37°C for 1 h) to establish the structures involved in the self-cleavage of the purified RNAs.

## 6-5 Purified dimeric minus ASBV RNA self-cleaves by a single-hammerhead structure

The full-length mutated dimeric minus ASBV RNA transcripts were purified and incubated in 10 mM $MgCl_2$, pH 8.0, at 37°C for 1 h. Figure 6.3B, lanes 5 and 7, show the self-cleavage pattern for the WT/M RNA (GAAAC-(B) mutated) and the M/WT RNA (GAAAC-(A) mutated), respectively. As is clearly evident, a different self-cleavage profile occurred with both purified RNAs from that of the RNAs during the transcription reactions (Figure 6.3B, lane 4,6). Purified WT/M RNA (GAAAC-(B) mutated) self-cleaved at SC-1, resulting in the cleavage products 5'E and M/3'E (Figure 6.3B, lane 5), in contrast to the production of 5'E/M and 3'E during transcription (Figure 6.3B, lane 4). Similarly, purified M/WT RNA (GAAAC-(A) mutated) self-cleaved at SC-2, yielding the cleavage products 5'E/M and 3'E (Figure 6.3B, lane 7), whereas during transcription, self-cleavage occurred at SC-1, yielding 5'E and M/3'E (Figure 6.3B, lane 6). As expected, self-cleavage was abolished at both sites in purified RNA with both GAAAC sequences mutated (Figure 6.3B, lane 3).

Hence, the results showed that single-hammerhead RNA self-cleavage occurred in purified dimeric minus ASBV RNAs. The possibility that the apparent single-hammerhead self-cleavage was actually the result of a *trans* reaction between two wild-type hammerhead sequences from two dimeric RNAs is very unlikely since the self-cleavage reactions were conducted at very low concentrations of RNA (approximately 0.3 nM; 50 pg/µl).

In addition to the single-hammerhead self-cleavage, a small amount of double-hammerhead self-cleavage occurred as indicated by the presence of trace amounts of 5'E/M and 3'E in the WT/M reaction (Figure 6.3B, lane 5) and of 5'E and M/3'E in the M/WT self-cleavage reaction (Figure 6.3B, lane 7).

Presumably, conformational changes occurred during the post-transcriptional treatment of the purified RNA that allowed some of the RNA to fold into a single-hammerhead structure and a much smaller fraction into a double-hammerhead structure. In

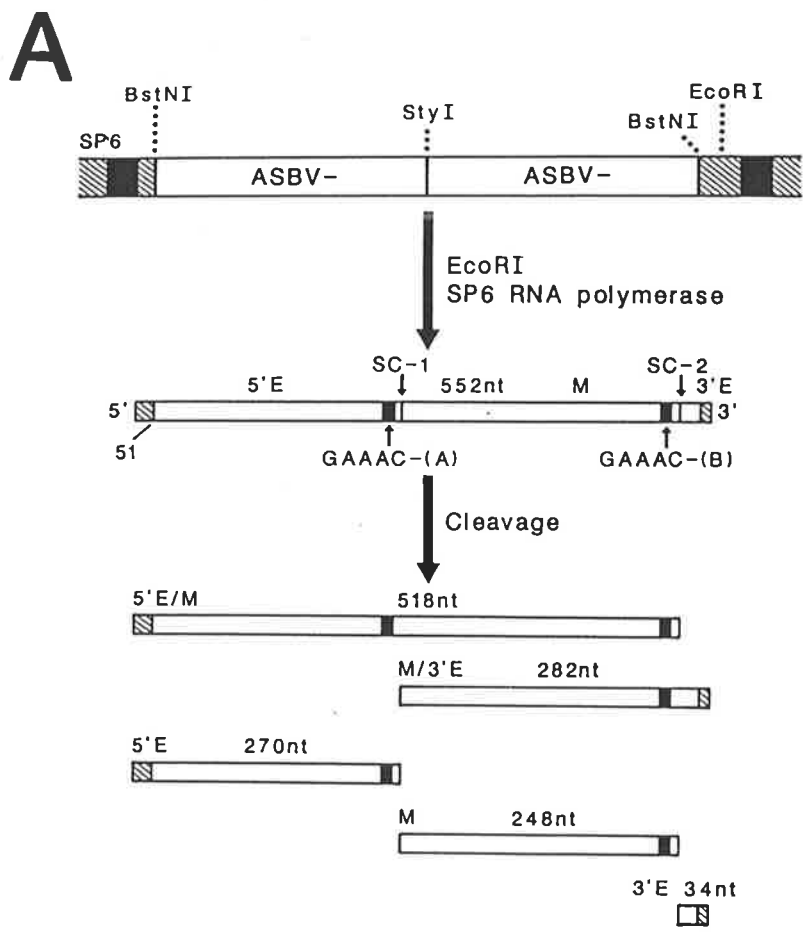
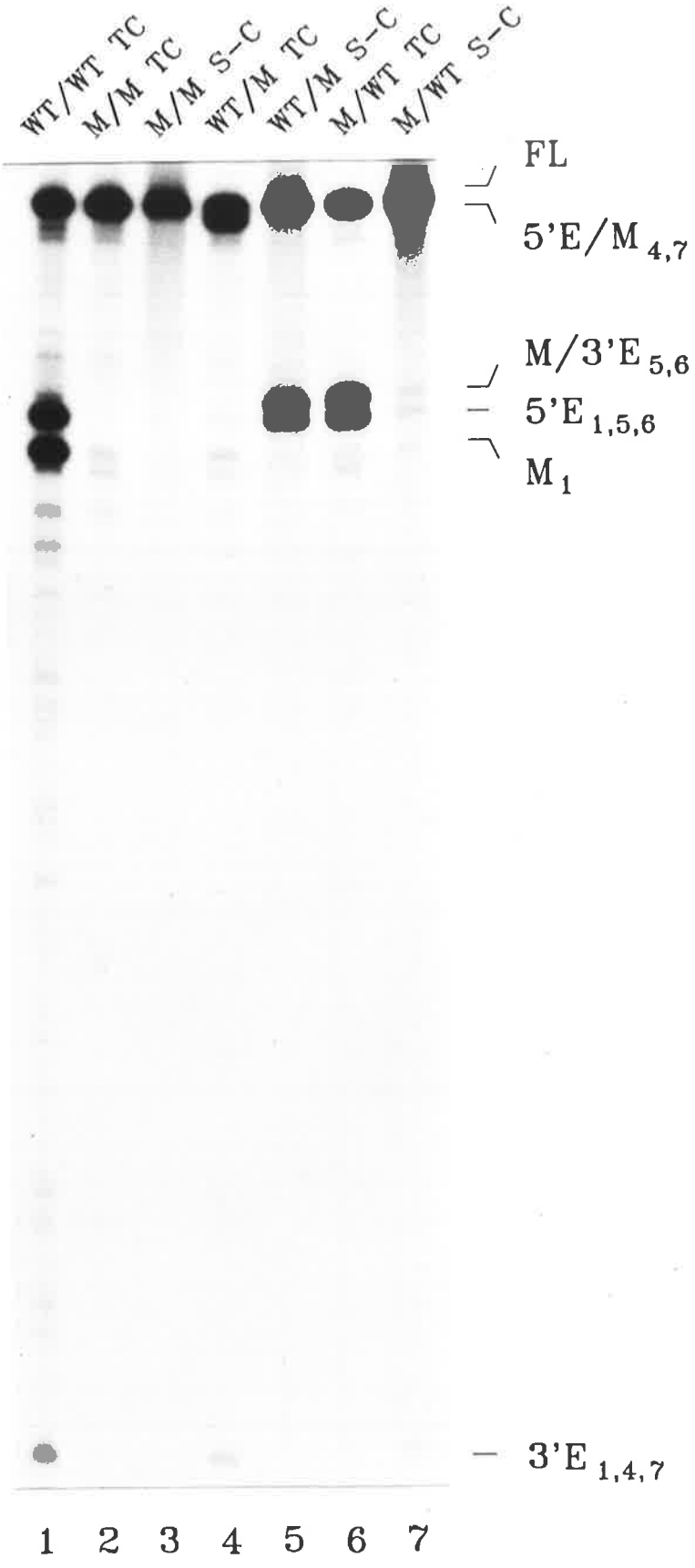



**Figure 6.3** Synthesis and self-cleavage of wild-type and mutant dimeric minus ASBV RNA transcripts. (**A**) Diagram of plus wild-type BstNI dimeric cDNA clone of ASBV in pGem1 vector and the SP6 RNA polymerase products generated by transcription of the vector linearised with EcoRI. Self-cleavage at both sites (SC-1 and SC-2, arrowed) of the full-length transcript (FL) gave rise to a 5'-end fragment (5'E), a monomer fragment (M) and a 3'-end fragment (3'E). Also shown are the products when self-cleavage occured only at SC-1 (5'E and M/3'E), or only at SC-2 (5'E/M and 3'E). Hatched boxes indicate vector sequences at 5'- and 3'-ends of both the cDNA clone and RNA transcripts; closed boxes, GAAAC sequences (Figure 6.1) labelled A and B and indicated by arrows; large closed box, SP6 RNA polymerase promoter. Relevant restriction sites in the cDNA clone are indicated. ASBV sequence is numbered after Symons (1981). (**B**) (Adjacent Page) Analysis of the SP6 RNA polymerase transcription (TC) reactions and of the self-cleavage (S-C) reactions of purified products by electrophoresis on a 5% polyacrylamide, 7 M urea gel and autoradiography. The positions of the products are indicated on the right-hand side of the gel and correspond to those in (A); the subscript numbers refer to the lanes in which the bands occur. **Lane 1**; transcript of the wild-type BstNI dimeric template linearised with EcoRI (WT/WT TC). **Lane 2**; as for lane 1, but both template GAAAC sequences (A and B) are mutated to GAAC (M/M TC). **Lane 3**; purified full-length M/M RNA (mutant at both GAAAC sequences) incubated in 10 mM $MgCl_2$, pH 8.0 (refer to 6-3) (M/M S-C). **Lane 4**; as for lane 2 but only GAAAC-(B) mutated (WT/M TC). **Lane 5**; as for lane 3 but with purified full-length WT/M RNA (WT/M S-C). **Lane 6**; as for lane 2 but only GAAAC-(A) mutated (M/WT TC). **Lane 7**; as for lane 3 but with purified full-length M/WT RNA (M/WT S-C). Note that bands $3'E_4$ and $3'E_7$ are weak in the Figure but obvious on the original autoradiogram.

B


WT/WT TC
M/M TC
M/M S-C
WT/M TC
WT/M S-C
M/WT TC
M/WT S-C
FL
5'E/M 4,7
M/3'E 5,6
5'E 1,5,6
M 1
3'E 1,4,7
1 2 3 4 5 6 7

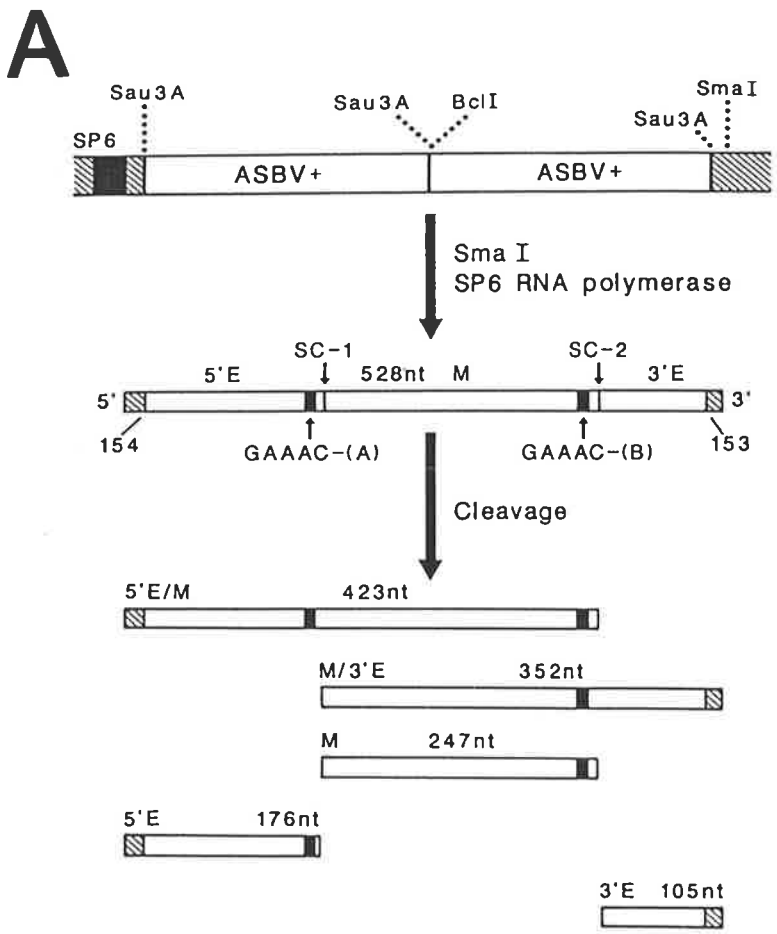
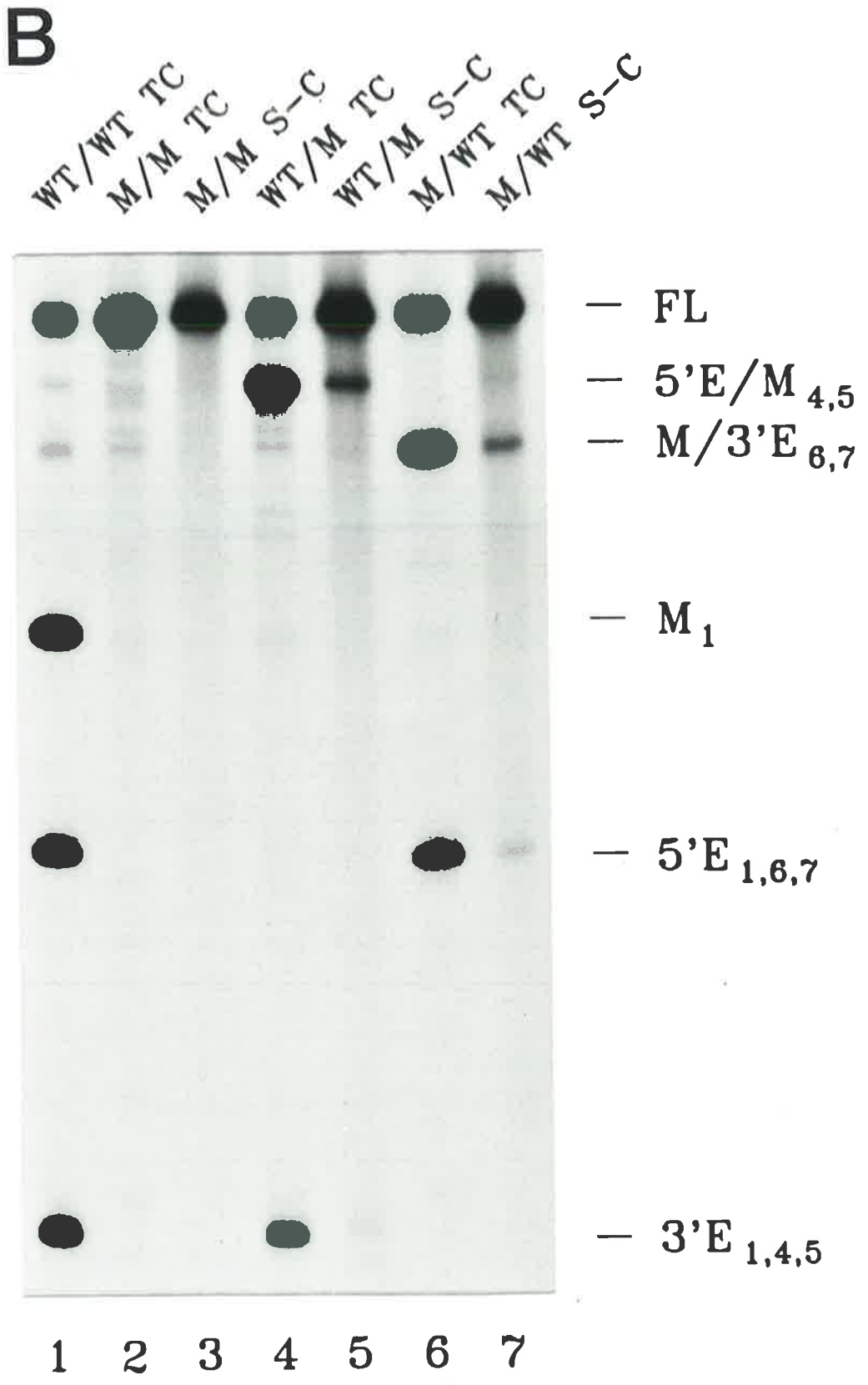



**Figure 6.4** Synthesis and self-cleavage of wild-type and mutant dimeric plus ASBV RNA transcripts. (**A**) Diagram of plus wild-type Sau3A dimeric cDNA clone of ASBV in pSP64 vector and the SP6 RNA polymerase products generated by transcription of the vector linearised with SmaI. Self-cleavage at both sites (SC-1 and SC-2, arrowed) of the full-length transcript (FL) gave rise to a 5'-end fragment (5'E), a monomer fragment (M) and a 3'-end fragment (3'E). Also shown are the products when self-cleavage occurred only at SC-1 (5'E and M/3'E), or only at SC-2 (5'E/M and 3'E). Hatched boxes indicate vector sequences at 5'- and 3'-ends of both the cDNA clone and RNA transcripts; closed boxes, GAAAC sequences (Figure 6.1) labelled A and B and indicated by arrows; large black box, SP6 RNA polymerase promoter. Relevant restriction sites in the cDNA clone are indicated. ASBV sequence is numbered after Symons (1981). (**B**) (Adjacent Page) Analysis of the SP6 RNA polymerase transcription (TC) reactions and of the self-cleavage (S-C) reactions of purified products by electrophoresis on a 5% polyacrylamide, 7 M urea gel and autoradiography. The positions of the products are indicated on the right-hand side of the gel and correspond to those in (A); the subscript numbers refer to the lanes in which the bands occur. **Lane 1**; transcript of the wild-type Sau3A dimeric template linearised with SmaI (WT/WT TC). **Lane 2**; as for lane 1, but both template GAAAC sequences (A and B) are mutated to GAAC (M/M TC). **Lane 3**; purified full-length M/M RNA (mutant at both GAAAC sequences) incubated in 10 mM $MgCl_2$, pH 8.0 (refer to 6-3) (M/M S-C). **Lane 4**; as for lane 2 but only GAAAC-(B) mutated (WT/M TC). **Lane 5**; as for lane 3 but with purified full-length WT/M RNA (WT/M S-C). **Lane 6**; as for lane 2 but only GAAAC-(A) mutated (M/WT TC). **Lane 7**; as for lane 3 but with purified full-length M/WT RNA (M/WT S-C). Note that bands $5'E_7$ and $3'E_5$ are weak in the Figure but obvious on the original autoradiogram.


B
WT/WT TC
M/M TC
M/M S-C
WT/M TC
WT/M S-C
M/WT TC
M/WT S-C
FL
5'E/M 4,5
M/3'E 6,7
M 1
5'E 1,6,7
3'E 1,4,5
1 2 3 4 5 6 7

contrast, during the transcription reaction the nascent RNA presumably folded preferentially into a double-hammerhead structure.

## 6-6 Purified dimeric plus RNA self-cleaves by a double-hammerhead structure

The self-cleavage products obtained from purified plus dimeric transcripts, incubated under self-cleavage conditions, were the same as those obtained during the transcription reaction for both plus RNAs in which the first GAAAC sequence (GAAAC-(A)) only was mutated (M/WT; Figure 6.4B, lanes 6,7) and in which the second GAAAC sequence (GAAAC-(B)) only was mutated (WT/M; Figure 6.4B, lanes 4,5), indicating that self-cleavage occurred by a double-hammerhead structure under these conditions also. As expected, self-cleavage was abolished at both sites in purified RNA with both GAAAC sequences mutated (Figure 6.4B, lane 3). Therefore, in contrast to dimeric minus RNA, the dimeric plus RNA self-cleaved by a double-hammerhead structure both during the transcription reaction and as purified full-length RNA.

## 6-7 Purified monomeric minus ASBV RNAs can self-cleave by a single-hammerhead structure

In view of the results obtained with dimeric minus transcripts where self-cleavage occurred by a double-hammerhead structure during transcription but by a single-hammerhead structure with purified full-length RNAs (Figure 6.3B), it was of interest to investigate the self-cleavage of monomeric minus RNAs. The expectation was that, like the purified dimeric minus RNA transcripts, the monomeric minus RNAs should be capable of self-cleavage by a single-hammerhead structure.

Four linearised plasmid templates were used for the production of minus monomeric ASBV RNAs. Three restriction enzyme sites within the ASBV cDNA were used in the construction of the clones, to give different transcriptional start sites within the ASBV sequence (Figure 6.5A). Transcription from all four monomeric templates yielded only full-length products, and no self-cleavage products (Figure 6.5F, lanes 1,3,5,7; previously reported in Davies, 1988). The EcoRI linearised Sau3A monomeric minus clone produced a 303 nt monomeric RNA transcript termed Sau(-)m (Figure 6.5B), the BclI truncated Sau3A

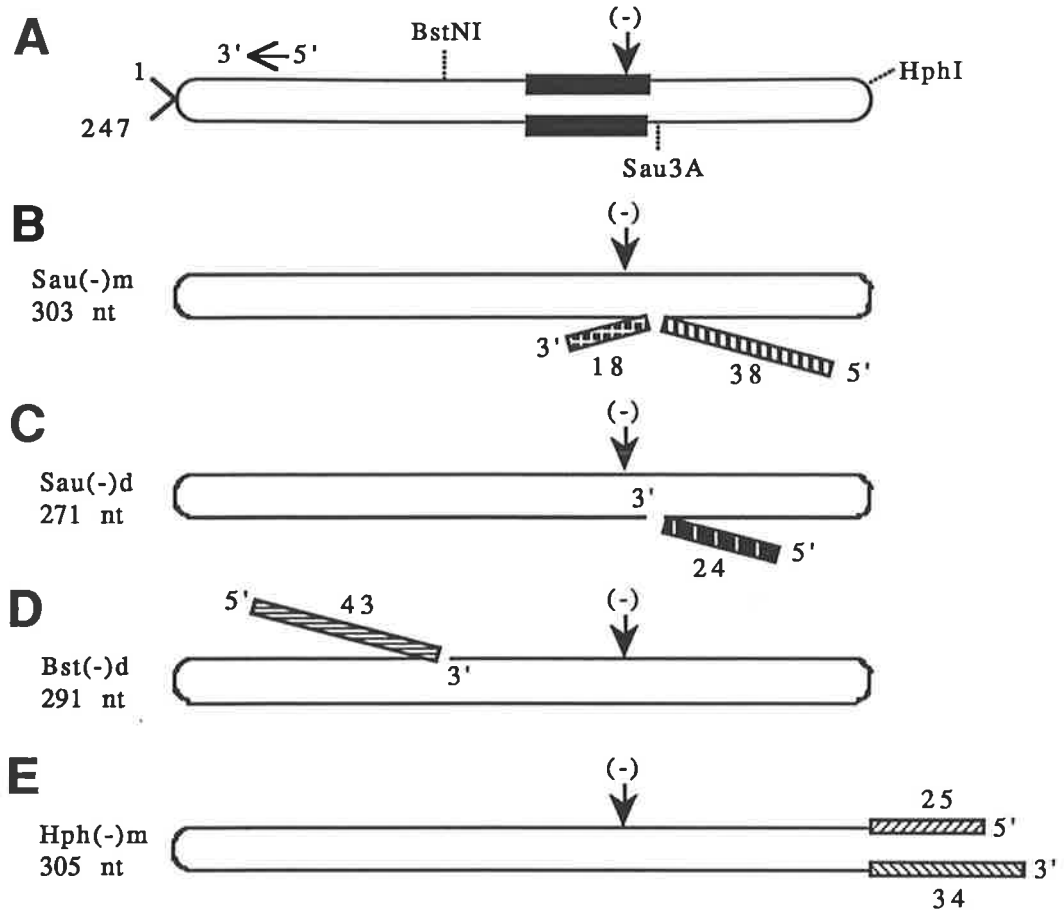
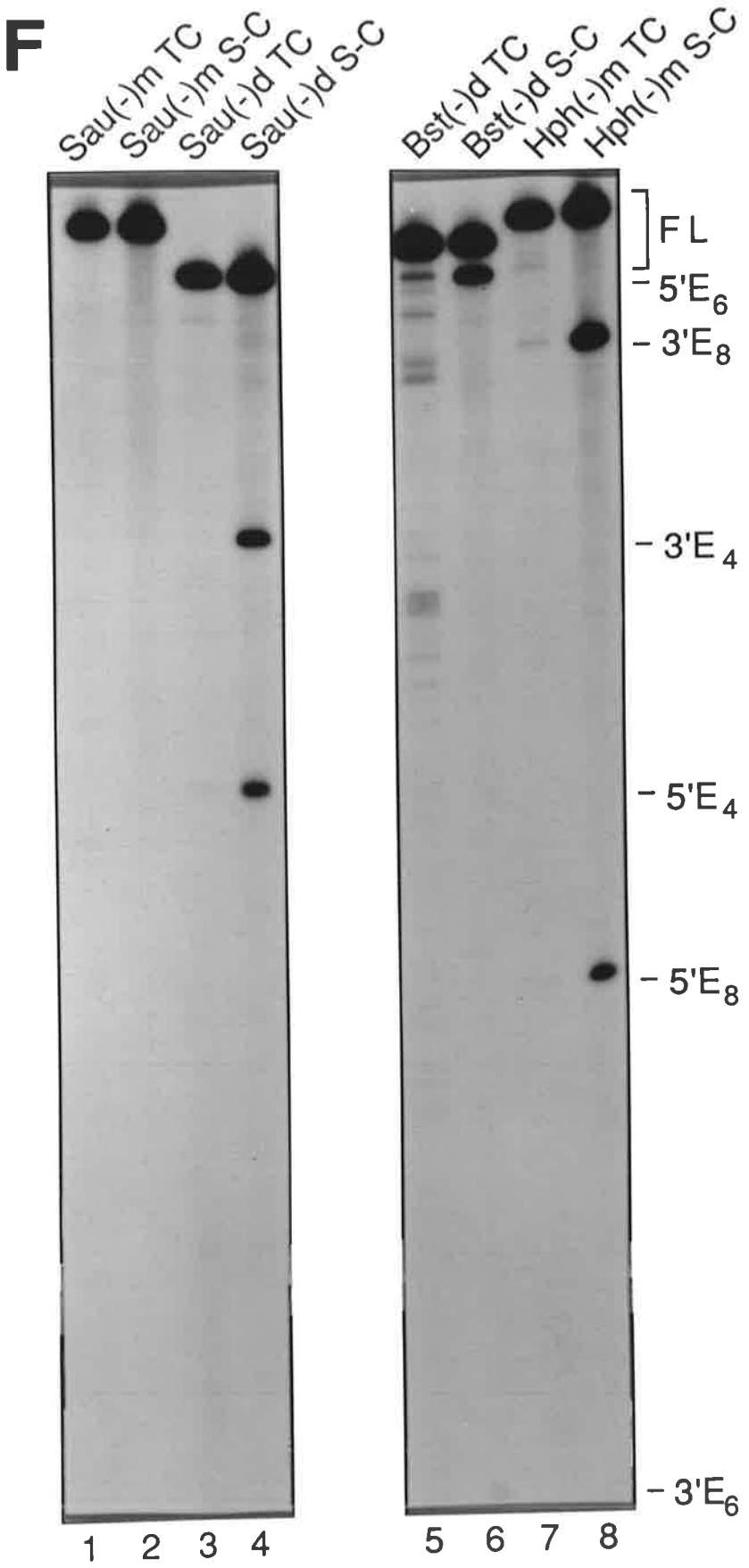



**Figure 6.5** Monomeric minus ASBV RNA transcripts, and their ability to self-cleave during transcription and as purified RNAs. **(A)** Schematic diagram of the minus RNA of ASBV showing position of restriction enzyme sites used in the construction of the cDNA clones used as templates in (B)-(E). The sequence (represented by the continuous line) is in the 3' to 5' direction. The sequences composing the minus hammerhead structure are indicated by the black rectangles. The site of self-cleavage of the minus ASBV RNA is indicated by the vertical arrow. The numbering of the ASBV sequence is as in Symons (1981). **(B)**-**(E)** Diagrams of monomeric minus RNA transcripts synthesised by transcribing: **(B)** an EcoRI linearised Sau3A monomeric clone in pSP64 with SP6 RNA polymerase; **(C)** a BclI linearised dimeric Sau3A clone in pSP65 with SP6 RNA polymerase; **(D)** a StyI linearised dimeric BstNI clone in pGem1 with SP6 RNA polymerase; **(E)** an EcoRI linearised HphI monomeric clone in pGem1 with T7 RNA polymerase. Vector sequences are indicated by the filled rectangles, different fillings represent different sequences. The size of each RNA transcript, and the number of bases of vector sequences are given. **(F)** (Adjacent Page) Analysis of RNA transcripts produced during transcription of the minus monomeric templates described above (TC, **Lanes 1,3,5,7**), and of purified full-length RNAs incubated in 10 mM $MgCl_2$, 0.5 mM EDTA, pH 8.0 (refer to 6-3) (S-C, **Lanes 2,4,6,8**). Products were resolved by electrophoresis on a 5% polyacrylamide, 7 M urea gel and autoradiography, **Lanes 1 and 2**, Sau(-)m (Figure 6.5B). **Lanes 3 and 4**, Sau(-)d (Figure 6.5C). **Lanes 5 and 6**, Bst(-)d (Figure 6.5D). **Lanes 7 and 8**, Hph(-)m (Figure 6.5E). The positions of the products are indicated on the right-hand side of the gel; the subscript numbers refer to the lanes in which the bands occur.


F
Sau(-)m TC
Sau(-)m S-C
Sau(-)d TC
Sau(-)d S-C
Bst(-)d TC
Bst(-)d S-C
Hph(-)m TC
Hph(-)m S-C
F L
5'E6
3'E8
3'E4
5'E4
5'E8
3'E6
1 2 3 4
5 6 7 8

dimeric minus clone produced a 271 nt monomeric RNA transcript termed Sau(-)d (Figure 6.5C), the BstNI dimeric clone truncated with StyI produced a 291 nt monomeric RNA transcript termed Bst(-)d (Figure 6.5D), and the HphI monomeric clone linearised with EcoRI produced a 305 nt monomeric RNA transcript termed Hph(-)m (Figure 6.5E).

The four full-length minus monomeric RNA transcripts were purified, and each incubated in 10 mM $MgCl_2$, pH 8.0, at 37°C for 1 h (6-3). This treatment resulted in the efficient self-cleavage of three of the four RNA transcripts; Sau(-)d, Bst(-)d and Hph(-)m (Figure 6.5F, lane 4,6,8); however, Sau(-)m self-cleaved poorly (1-2%) (Figure 6.5F, lane 2, bands visible on original autoradiogram, and in other experiments).

The efficient self-cleavage of three of the four RNAs indicated that minus monomeric RNAs were capable of single-hammerhead self-cleavage. The low concentration of RNA used in these experiments (approximately 0.5 nM; 50 pg/µl), makes it unlikely that the self-cleavage observed was due to intermolecular double-hammerhead self-cleavage. Therefore, in contrast to the situation during the transcription reaction, where presumably the RNAs folded preferentially into inactive structures, purified monomeric minus RNAs were capable of single-hammerhead mediated self-cleavage. The different ASBV termini in the Sau(-)d, Bst(-)d and Hph(-)m RNA transcripts (dictated by the restriction enzyme sites used in the construction of the template cDNA clones; Figure 6.5A) did not appear to affect the ability of the RNA transcripts to form single-hammerhead structures.

Interestingly, Sau(-)d, which underwent efficient self-cleavage (Figure 6.5F, lane 4), and Sau(-)m, which self-cleaved poorly (Figure 6.5, lane 2), differed only in the identity of the 5' and 3' vector sequences at the termini of the transcripts. Sau(-)m possesses 18 nt of 3' vector sequence, whereas Sau(-)d has no 3' vector sequences, in addition, the 5' vector sequences of the two RNAs are different (Figure 6.5B,C). An RNA transcript produced from the Sau3A monomeric clone linearised with SmaI, rather than EcoRI, had only three vector encoded residues on the 3'-end, in contrast to the 18 of Sau(-)m, but otherwise was identical. This RNA also self-cleaved poorly under the conditions used (data not shown). Therefore, it appears that the 5' vector sequence, and/or the three 3'-vector encoded residues, of Sau(-)m were responsible for the reduced self-cleavage efficiency compared with Sau(-)d, presumably by directing the RNA to fold into inactive structures.

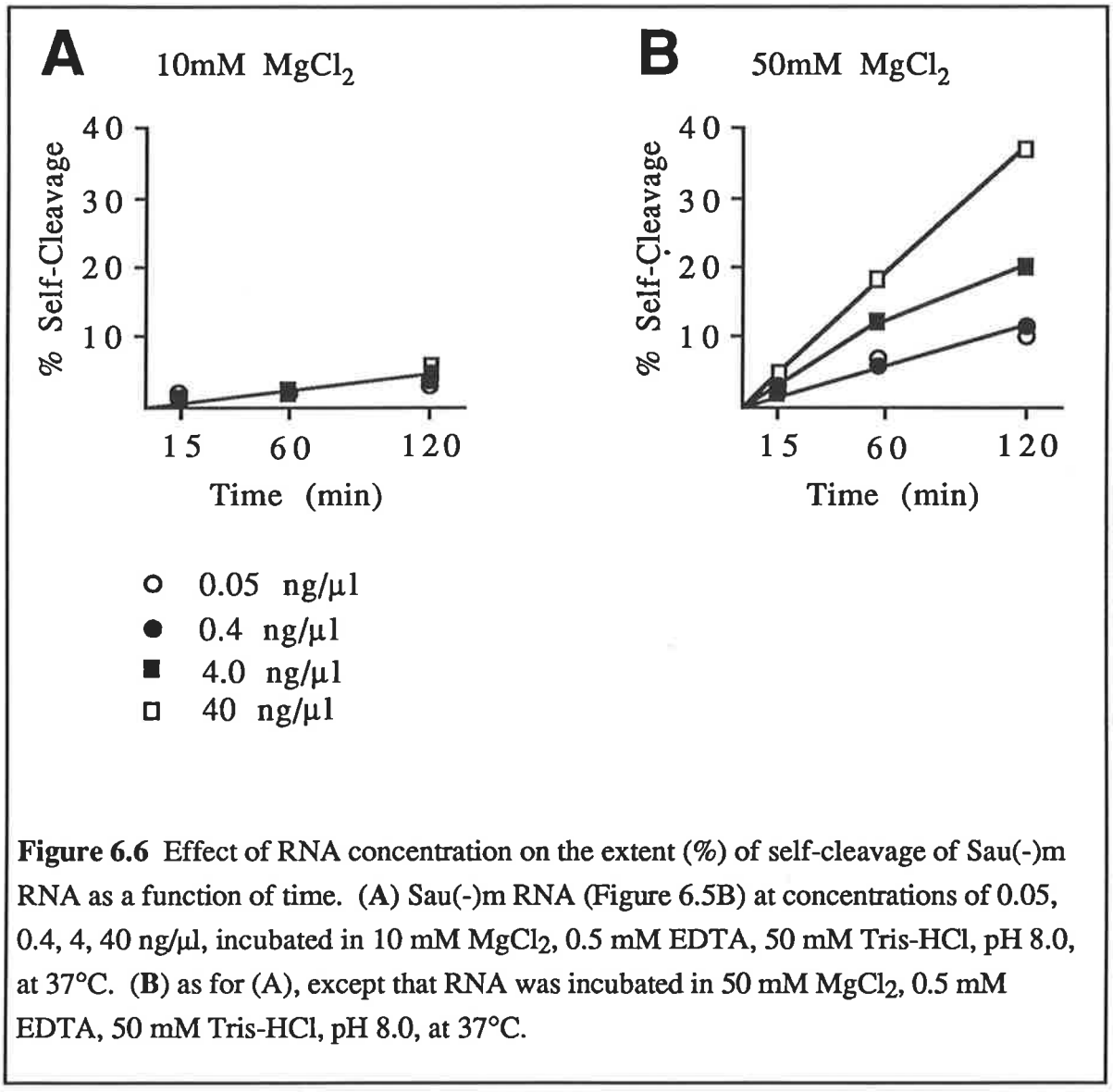



**Figure 6.6** Effect of RNA concentration on the extent (%) of self-cleavage of Sau(-)m RNA as a function of time. (**A**) Sau(-)m RNA (Figure 6.5B) at concentrations of 0.05, 0.4, 4, 40 ng/µl, incubated in 10 mM $MgCl_2$, 0.5 mM EDTA, 50 mM Tris-HCl, pH 8.0, at 37°C. (**B**) as for (A), except that RNA was incubated in 50 mM $MgCl_2$, 0.5 mM EDTA, 50 mM Tris-HCl, pH 8.0, at 37°C.

The ASBV cDNA from the Sau3A monomeric clone was re-subcloned into M13 and sequenced, to confirm that it had the correct sequence (data not shown).

The presence of alternative structures (including active single-hammerhead structures and inactive structures) in an RNA containing a partial sequence of vLTSV has been demonstrated by non-denaturing gel electrophoresis (Forster *et al.*, 1987). Similar experiments (results not shown) indicated that the structural differences between "active" Sau(-)d and "inactive" Sau(-)m were not sufficiently marked that they could be resolved on non-denaturing gels.

The very low level of self-cleavage of Sau(-)m RNA was further explored by incubating higher concentrations of the RNA under the same self-cleavage conditions (10 mM $MgCl_2$, pH 8.0, 37°C). Figure 6.6A shows the self-cleavage profiles for four concentrations of Sau(-)m RNA. As is evident there was low efficiency of self-cleavage even at high RNA concentrations. The same range of concentrations of RNA was incubated with a higher $MgCl_2$ concentration: a time course of the self-cleavage reaction undertaken in 50 mM $MgCl_2$, pH 8.0, at 37°C is shown for four RNA concentrations in Figure 6.6B. Very low concentrations (0.05 ng/µl) of Sau(-)m RNA self-cleaved to about 10 %, after 2 h. This level of self-cleavage was increased with higher concentrations of RNA. The curves for the two lowest RNA concentrations, 0.05 and 0.4 ng/µl, are the same. This may represent the basal level of concentration independent single-hammerhead self-cleavage, and the increased self-cleavage at the higher concentrations of RNA, most likely is due to double-hammerhead self-cleavage.

Hence, the presence of extra/different vector sequences on Sau(-)m compared with Sau(-)d has markedly reduced the efficiency of formation of the single-hammerhead structure. Presumably, in 10 mM $MgCl_2$, inactive structures formed by the RNA are more stable than the single-hammerhead structure. Higher $MgCl_2$ concentrations (50 mM) apparantly stabilised the single-hammerhead structure, and enhanced the interaction between RNAs to form double-hammerhead structures, so that a greater efficiency of self-cleavage occurred.

In summary, the results from this section have indicated that purified minus monomeric RNAs were capable of single-hammerhead mediated self-cleavage, although the presence of vector sequences affected the formation of the active structure in Sau(-)m.

Further, the results are consistent with single-hammerhead self-cleavage of isolated dimeric minus ASBV RNAs (Figure 6.3B).

## 6-8 Monomeric plus ASBV RNAs self-cleave poorly by single-hammerhead structures

Dimeric plus ASBV RNA transcripts self-cleaved by a double-hammerhead structure both during transcription and as purified full-length RNA (Figure 6.4B). Here, the self-cleavage of monomeric plus ASBV RNA transcripts was investigated, to assess whether, like the dimeric plus RNAs, the monomeric plus RNAs self-cleaved by double-hammerhead structures.

Five linearised plasmid templates, with the ASBV cDNA sequence cloned at two different sites (Sau3A and HphI, Figure 6.7A), were used for the production of plus monomeric RNAs. The RNA transcripts produced from these templates are shown schematically in Figure 6.7B-F. Two templates were derived from a Sau3A dimeric clone; by digestion with BclI to produce a template for a monomeric RNA transcript termed Sau(+)dBcl (Figure 6.7B), and by digestion with DdeI, to produce a template for a partial monomeric RNA transcript termed Sau(+)dDde (Figure 6.7C). Similarly a Sau3A monomeric clone was linearised with EcoRI, to generate a template for a monomeric RNA transcript termed Sau(+)mEco (Figure 6.7D), and with DdeI, to generate a template for a partial monomeric RNA transcript termed Sau(+)mDde (Figure 6.7E). An HphI monomeric clone was linearised with EcoRI to generate a template for a monomeric RNA transcript termed Hph(+)mEco (Figure 6.7F).

As with the minus monomeric transcripts, no plus monomeric RNA self-cleaved during the transcription reaction (results not shown). The full-length products from the transcription reaction (of size indicated in Figure 6.7) were purified, and a range of different RNA concentrations were incubated under two $MgCl_2$ concentrations.

Low efficiency of self-cleavage was obtained with all five RNAs when incubated in 10 mM $MgCl_2$, pH 8.0, at 37°C. There was a slightly higher efficiency of self-cleavage at the higher RNA concentrations, but the maximum extent was about 4 % (results not shown).

Higher efficiencies of self-cleavage were obtained when the purified transcripts were incubated in 50 mM $MgCl_2$, pH 8.0, at 37°C. The results are presented graphically in

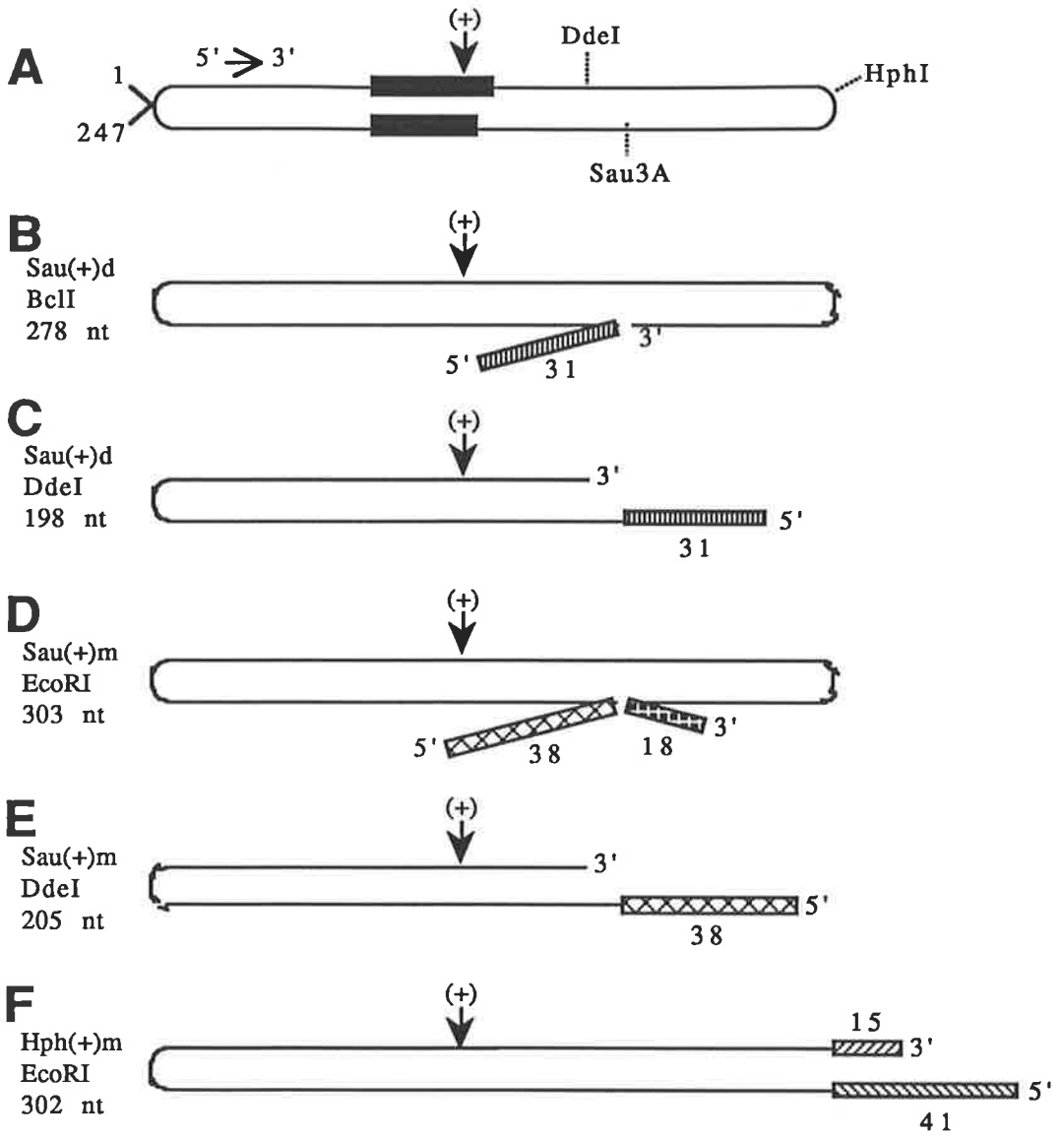



**Figure 6.7** Diagrams of monomeric and partial monomeric plus ASBV RNA transcripts. (**A**) Schematic diagram of the plus RNA of ASBV showing position of the Sau3A and HphI restriction enzyme sites used in the construction of the clones used as templates in (B)-(F), and the DdeI restriction enzyme site used in the linearisation of the template DNAs in (C) and (E). The sequence (represented by the continuous line) is in the 5' to 3' direction. The sequences composing the plus hammerhead structure are indicated by the black rectangles. The site of self-cleavage of the minus ASBV RNA is indicated by the horizontal arrow. The numbering of the ASBV sequence is as in Symons (1981). (**B**)-(**F**) Diagrams of monomeric (**B,D,F**), or partial monomeric (**C,E**) plus ASBV RNA transcripts synthesised by transcription with SP6 RNA polymerase of: (**B**) a BclI linearised Sau3A dimeric clone in pSP64; (**C**) a DdeI linearised Sau3A dimeric clone in pSP64; (**D**) an EcoRI linearised Sau3A monomeric clone in pSP64; (**E**) a DdeI linearised monomeric Sau3A clone in pSP64; (**F**) an EcoRI linearised HphI monomeric clone in pGem1. Vector sequences are indicated by the filled rectangles, different fillings represent different sequences. The size of each RNA transcript, and the number of bases of vector sequences is given.

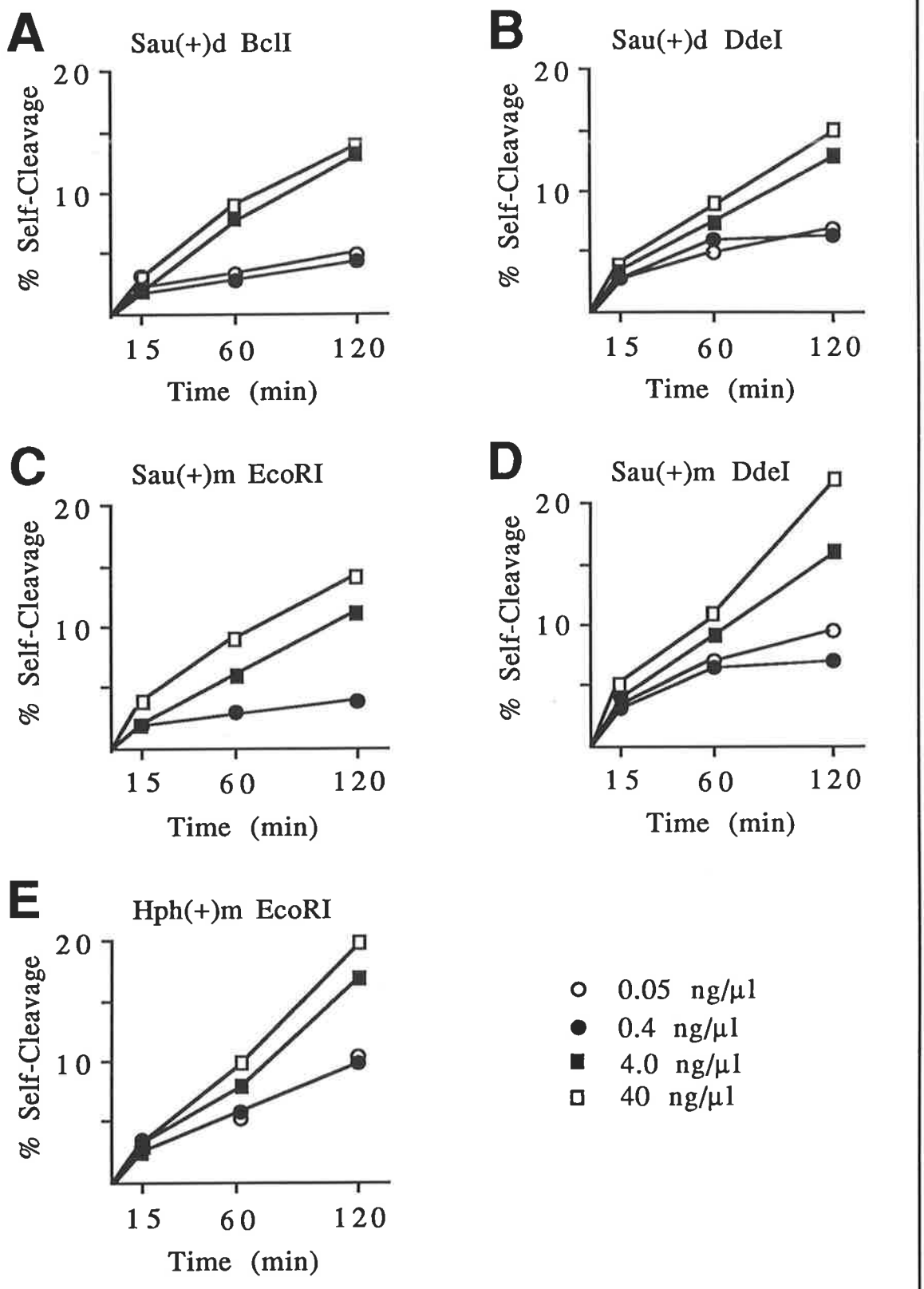



**Figure 6.8** Effect of RNA concentration on the extent (%) of self-cleavage of monomeric and partial-monomeric plus ASBV RNA transcripts as a function of time. (**A**) Sau(+)dBClI RNA (Figure 6.7B) at concentrations of 0.05, 0.4, 4, 40 ng/µl, incubated in 50 mM $MgCl_2$, 0.5 mM EDTA, 50 mM Tris-HCl, pH 8.0, at 37°C. (**B**) As for (A), except that RNA was Sau(+)dDdeI (Figure 6.7C). (**C**) As for (A), except that RNA was Sau(+)mEcoRI (Figure 6.7D). (**D**) As for (A), except that RNA was Sau(+)mDdeI (Figure 6.7E). (**E**) As for (A), except that RNA was Hph(+)dEcoRI (Figure 6.7F).

Figure 6.8. The self-cleavage profiles for all five RNAs were similar. Generally, there was little difference between the extent of self-cleavage at the two lowest concentrations of RNAs. This, presumably, reflects self-cleavage by a single-hammerhead structure, as it occurred at very low concentrations of RNA, and was independent of RNA concentration. At higher concentrations of RNA, a greater extent of self-cleavage was observed, this additional self-cleavage presumably was due to formation of double-hammerhead structures.

Therefore, under high $MgCl_2$ concentrations, self-cleavage by both single-, and double-hammerhead structures was able to occur in monomeric plus ASBV RNA transcripts. Presumably, the high $MgCl_2$ concentration acted both to stabilise the single-hammerhead structure, and also, by acting as a counter ion, encouraged the interaction between RNA molecules to allow double-hammerhead formation and self-cleavage.

The low efficiency of self-cleavage, even at high $MgCl_2$ concentrations, reflects both the low stability of the single-hammerhead structure (due to the weak stem III), and the difficulty of formation of the double-hammerhead structure by the interaction of two RNAs. It is likely that at the start of the self-cleavage reaction the RNA transcripts were folded into secondary structures, probably similar to the rod-like structure formed by the natural viroid (Symons, 1981). These structures would have to be unfolded to allow intermolecular interaction, to form double-hammerhead structures. The low efficiency of double-hammerhead self-cleavage presumably reflects the relatively high stability of the preformed monomolecular structures. It was thought that Sau(+)mDdeI and Sau(+)dDdeI, because they lack one third of the ASBV sequence, may have been able to interact to form the double-hammerhead structure more efficiently than the larger RNAs. At the highest concentration of RNA used, Sau(+)mDde self-cleaved to a greater extent than Sau(+)mEco, indicating that the double-hammerhead structure does form more efficiently in Sau(+)mDde. However, no similar effect was seen with Sau(+)dDde, compared to Sau(+)dBcl.

Pairwise comparison between the different RNAs allowed the effect of both 3' and 5' vector sequences on self-cleavage to be examined. Sau(+)dDde and Sau(+)mDde differ in sequence by only a few bases in the 5'-vector sequence, however, Sau(+)mDde self-cleaved more efficiently than Sau(+)dDde at high RNA concentrations. In contrast, the presence of 18 3'-vector residues appears to have had little effect on the efficiency of

Sau(+)mEco compared with Sau(+)dBcl. The low efficiency of self-cleavage generally, however, precluded any detailed analysis.

## Discussion

The aim of the experiments described in this Chapter was to determine whether *in vitro* self-cleavage of RNA transcripts containing the plus and minus ASBV sequences occurred by single-hammerhead or double-hammerhead structures. Determination of the single- or double-hammerhead route in the dimeric RNA transcripts involved the use of single base deletion mutants, whereby conversion of the conserved GAAAC sequence in the hammerhead structure to GAAC completely eliminated self-cleavage at one of the two sites. Inhibition of self-cleavage at the site just 3' to the mutated GAAAC demonstrated single-hammerhead self-cleavage, whereas inhibition of self-cleavage at the other self-cleavage site (over 250 bases from the mutated GAAAC site) indicated double-hammerhead mediated self-cleavage (Forster *et al.*, 1988; and Figures 6.1, 6.2).

In the monomeric plus and minus ASBV RNA transcripts studied in this work, determination of the type of hammerhead structure mediating self-cleavage, was based on the fact that the formation of a double-hammerhead structure requires the interaction of two RNAs. At very low concentrations of RNA, such interactions would be unlikely. Hence, self-cleavage at low RNA concentrations, can be presumed to be mediated by single-hammerhead structures. As double-hammerhead self-cleavage requires the interaction of two monomeric RNAs, self-cleavage by this structure can be interpreted if it occurs at greater efficiency at higher RNA concentrations. (However, both single- and double-hammerhead self-cleavage may be possible at high RNA concentrations.)

### 6-9 Minus ASBV RNA transcripts can self-cleave by single-hammerhead structures

The results presented in this Chapter demonstrate that dimeric minus ASBV RNA transcripts self-cleaved by double-hammerhead structures during transcription, but predominantly by single-hammerhead structures when the purified RNA was incubated under self-cleavage conditions (Figure 6.3B). The ionic and pH conditions were similar in both reactions (during transcription: 6 mM $MgCl_2$, pH 7.5; self-cleavage conditions: 10 mM

$MgCl_2$, pH 8.0). Presumably therefore, the active structures formed were dependent on whether the entire RNA was available for folding at one time (as with the purified RNAs), or whether there was sequential appearance of the RNA, so that subsets of the RNA sequence became available for folding before the remainder of the sequence (as occurred during transcription). During transcription, the formation of the double-hammerhead structure, in preference to the single-hammerhead structure, may have been promoted by the order in which the sequences became available for folding as the nascent RNA emerged from the RNA polymerase.

Both during transcription, and after purification, self-cleavage went to 50 % or less, hence, in addition to the formation of either single- or double-hammerhead structures, a proportion of the RNAs were folded into inactive structures. Presumably, these inactive structures were too stable to convert to either single- or double-hammerhead structures during the course of the reactions. However, as the purified RNA was derived from RNA that was folded into inactive structures during transcription, it appears that the purification procedure resulted in the denaturation of the RNA, so that it was able to fold into single-hammerhead structures (and a small amount of double-hammerhead structure) when incubated under self-cleavage conditions.

The four minus monomeric RNA transcripts did not self-cleave during transcription (Figure 6.5F), indicating they were folded into inactive structures that were too stable to interconvert with active structures. As nearly the entire transcript sequence for all four of the monomeric minus RNAs must be synthesised before the complete minus hammerhead sequence is produced, there is potential for alternative structures to form, before the possibility of formation of the single-hammerhead structure. The efficient self-cleavage of three of the four purified minus monomeric RNA transcripts when incubated under self-cleavage conditions at low RNA concentrations (Figure 6.5F), is consistent with the ability of the minus dimeric RNA to self-cleave by a single-hammerhead structure.

Therefore, as predicted, the minus ASBV single-hammerhead structure with its three base pair stem III, and three base loop, was capable of mediating *in vitro* self-cleavage.

## 6-10 Plus ASBV RNA requires a double-hammerhead structure for self-cleavage

In contrast to the dimeric minus RNAs, dimeric plus ASBV RNA transcripts underwent self-cleavage by double-hammerhead structures both during transcription, and when the purified full-length RNAs were incubated under self-cleavage conditions (Figure 6.4B). Monomeric, and partial monomeric plus ASBV RNA transcripts self-cleaved poorly under conditions (10 mM $MgCl_2$, pH 8.0) which resulted in the efficient single-hammerhead self-cleavage of most monomeric minus RNAs, indicating a poor ability for the plus transcripts to form single-hammerhead structures. The plus monomeric and partial monomeric RNA transcripts were, however, capable of a low level of single-hammerhead self-cleavage, as well as double-hammerhead self-cleavage, in the presence of a higher concentration of $MgCl_2$ (50 mM; Figure 6.8). Presumably, the high $MgCl_2$ concentrations increased the stability of the single-hammerhead structures and, furthermore, promoted interaction between RNAs, to allow the formation of double-hammerhead structures. Inactive structures will also have been stabilised, in fact, these structures accounted for the majority of the RNAs present in the reaction. It is possible that under high $Mg^{2+}$ concentrations, plus dimeric RNA transcripts would also exhibit some single-hammerhead self-cleavage.

Therefore, it appears that the plus ASBV single-hammerhead structure, containing a two base-pair stem III with a three base loop, is unable to mediate self-cleavage, except under conditions of high $MgCl_2$ concentrations.

## 6-11 A stable stem III is required for self-cleavage

The stem IIIs of the single-hammerhead structure of plus and minus ASBV and newt RNAs are of low theoretical stability relative to the other natural hammerhead structures (Figure 1.1), and in particular, those of plus ASBV and newt RNAs were considered unlikely to form (Hutchins *et al.*, 1986, Forster and Symons, 1987b). Both plus dimeric ASBV RNA transcripts and the newt RNA transcripts undergo self-cleavage *in vitro* (Hutchins *et al.*, 1986, Epstein and Gall, 1987), despite their single-hammerhead structures containing stem IIIs that were considered unlikely to form (Hutchins *et al.*, 1985, Forster and Symons, 1987b). Hence it appeared that either the formation of stem III in the single-

hammerhead structure was not required for self-cleavage of these RNAs, or an alternative structure, the double-hammerhead structure which provides more stable stem IIIs, was involved in self-cleavage.

The results presented in Chapters 4, 5, and this Chapter clearly favour the latter explanation. In Chapter 4, self-cleavage of nCG RNA (resembling the newt RNA) occurred with bimolecular reaction kinetics, indicating the involvement of the double-hammerhead structure. Very little self-cleavage occurred at low nCG RNA concentrations, indicating that the single-hammerhead structure was not able to mediate self-cleavage of the nCG RNA. In Chapter 5, the double-hammerhead mediated self-cleavage reaction of the nCG RNA was converted to a single-hammerhead reaction by increasing the size of the nCG stem III and/or its loop (hence, increasing the theoretical stability of its stem III), thereby enabling a single-hammerhead structure to form. It was demonstrated that the minimum stem III requirement for single-hammerhead self-cleavage of nCG-like RNAs was either a two base-pair stem, with a four base loop, or a three base-pair stem with a three base loop. The ability of the minus ASBV RNA transcripts to self-cleave by single-hammerhead structures, and the low ability of the plus RNA transcripts to do so, is consistent with the results of the short RNAs.

Therefore, it appears that those RNAs (plus ASBV and newt RNAs) with single-hammerhead stem IIIs of lowest theoretical stability are either not capable of self-cleavage by single-hammerhead structures, or self-cleave very poorly. They are, however, capable of self-cleavage by double-hammerhead structures, which have theoretically more stable stem IIIs. Hence, the results presented indicate that a stable stem III is required for self-cleavage of these RNAs. Consistent with this, minus ASBV RNAs, and other RNAs (e.g., plus and minus vLTSV RNAs) with more stable single-hammerhead stem IIIs, are capable of single-hammerhead self-cleavage.

It has been proposed that conserved bases of the hammerhead structure (including those in stem III) play no direct role in the catalytic activity of the hammerhead structure (Mei *et al.*, 1989). Therefore, presumably, stem III has a structural role in holding the RNA in the correct secondary structure, so that tertiary interactions can occur to form the active structure. In addition, it is possible that one or more of the conserved bases of stem III are involved in coordination with the essential $Mg^{2+}$ ion (see section 3-8).

## 6-12 The formation of inactive structures can affect self-cleavage

As discussed in Chapters 4 and 5, the formation of alternative inactive structures in short RNA oligonucleotides containing only hammerhead sequences with no flanking sequences, could disrupt self-cleavage, for example, the nCG (Chapter 4) and variant RNAs (Chapter 5, particularly nUCG). This has also been reported extensively in the literature (for example, Ruffner *et al.*, 1989, Jeffries and Symons, 1989, Heus *et al.*, 1990, Fedor and Uhlenbeck, 1990). In the larger RNAs examined in this Chapter, non-hammerhead sequences were also found to promote the formation of inactive structures.

One monomeric minus RNA, Sau(-)m, self-cleaved poorly under conditions that resulted in the efficient single-hammerhead self-cleavage of a very similar monomeric minus RNA (Sau(-)d; Figure 6.5F). The two RNAs differed only in the vector sequences present at the 5'- and 3'-termini of the transcripts (Figure 6.5B,C). Presumably, the vector sequences in the Sau(-)m RNA promoted the folding of the RNA into inactive structures, either by assisting the stabilisation of the inactive structures, and/or by interacting directly with the hammerhead sequence, thereby preventing the formation of the single-hammerhead structure. Analysis of the 5'-vector sequences of Sau(-)m RNA did not reveal any obvious regions which might base-pair with the hammerhead sequences and hence result in the disruption of the formation of the single-hammerhead structure.

The folding of hammerhead containing RNAs, and other self-cleaving RNAs, into inactive structures, thereby excluding the formation of the active structure, has also been found in many systems to have a major impact on the ability of RNAs to self-cleave. For example, sequences flanking the plus vLTSV hammerhead sequence, in partial monomeric plus vLTSV RNA transcripts, prevented the formation of the hammerhead structure, and so self-cleavage did not occur (Forster and Symons, 1987b). When these sequences were deleted, the remaining hammerhead containing RNA self-cleaved efficiently (Forster and Symons, 1987b). Similarly, sequences of the genomic and anti-genomic RNAs of HDV (which self-cleaves by the axehead / pseudoknot structure, see Chapter 1) interfered with the self-cleavage of these RNAs, and self-cleavage occurred more efficiently when these sequences were deleted (Kuo *et al.*, 1988, Wu *et al.*, 1989, Perrotta and Been, 1990). Interestingly, in one case, the 5'-vector sequences of an *in vitro* HDV RNA transcript

actually enhanced self-cleavage, apparantly by interacting with a 16 nucleotide sequence that was otherwise inhibitory to self-cleavage (Belinsky and Dinter-Gottlieb, 1991).

Further, as discussed above, the formation of inactive structures may be promoted by the order in which the RNAs become available for folding, e.g., the minus monomeric RNAs during transcription. Once formed, even though these inactive structures may not be of greater stability than the active structures, they may be too stable to interconvert with the active structures under the conditions of the reaction.

Therefore, the ability to form an active hammerhead structure is dependent not only on the combined stability of its constitutive structural elements (including stem III as demonstrated by Chapters 4, 5 and this Chapter). It also requires that the RNA does not form inactive structures preferentially to the active structures. This may occur if the relative stability of the inactive structures is greater than that of the active structure(s), or if the RNA folds preferentially into inactive structures due to the order in which the RNA becomes available for folding.

## 6-13 A very stable stem I in the newt hammerhead structure allows single-hammerhead self-cleavage

Since the completion of this work, Epstein and Pabon-Pena (1991) have published a series of experiments on the self-cleavage of monomeric and dimeric newt satellite 2 transcripts. Certain constructs apparantly permitted single-hammerhead self-cleavage of the newt RNA. This is in contrast to the results using a short RNA containing the approximate sequence of the newt hammerhead (nCG RNA, Chapter 4). From analysis of the different constructs used by Epstein and Pabon-Pena (1991) that permitted single-hammerhead self-cleavage, and comparison with those constructs that did not permit single-hammerhead self-cleavage, it appears that single-hammerhead self-cleavage was dependent on non-hammerhead sequences extending from stem I. Indeed, whereas transcripts containing only a single hammerhead domain did not self-cleave at low concentrations (Epstein and Pabon-Pena, 1991, in agreement with Chapter 4), eleven additional newt nucleotides on the 5'-end, and 33 nucleotides on the 3'-end did permit self-cleavage at low RNA concentrations (Epstein and Pabon-Pena, 1991). These extra sequences (Epstein *et al.*, 1986, Epstein and Gall, 1987) permit six additional base-pairs extending from stem I. This is very interesting,

as it suggests that self-cleavage can occur by a single-hammerhead structure with a stem III of very low stability, if it is compensated for by the another stem being very stable. It appears that the newt RNA transcripts are effectively locked into a stable hairpin structure containing stem II and a very stable stem I. The formation of stem III may occur transiently in these RNAs, resulting in self-cleavage. This may explain the long $t_{1/2}$ of the single-hammerhead self-cleavage (about 100 min, Epstein and Pabon-Pena, 1991), compared with the $t_{1/2}$ of double-hammerhead self-cleavage in related constructs, lacking the stem I extension (less than 5 min, Epstein and Pabon-Pena, 1991).

Interestingly, in those constructs in which only single-hammerhead self-cleavage occurred, even at high concentrations of RNA, it appears that the formation of a double-hammerhead structure was actually prevented. Analysis of the newt transcript sequence (Epstein *et al.*, 1986, Epstein and Gall, 1987) reveals the ability to form a highly base-paired structure, consisting of 54 base-pairs, out of a 156 nucleotides (70 % of nucleotides base-paired) extending from stem I of the single-hammerhead structure. This would exclude the interaction of a second hammerhead sequence with the first, whether it is from another RNA, or from the other half of the dimeric transcript.

The newt RNA, therefore, provides an interesting example where non-hammerhead sequences help to stabilise the single-hammerhead structure that would otherwise be of too low stability to form.

## 6-14 Self-cleavage of plus and minus ASBV RNAs *in vivo* may occur by double-hammerhead structures

ASBV is believed to be replicated by a rolling circle mechanism (Branch and Robertson, 1984, Hutchins *et al.*, 1985) possibly involving *in vivo* self-cleavage of multimeric replicative intermediates to form monomeric RNAs (Hutchins *et al.*, 1986, Forster *et al.*, 1988, see Chapter 7). It is possible that self-cleavage *in vivo* would more closely resemble the self-cleavage of dimeric RNAs during *in vitro* transcription than the *in vitro* self-cleavage of gel-purified dimeric or monomeric RNAs. This is because sequential production and folding of the greater than unit-length RNAs occurs during transcription, mimicking multimeric RNA synthesis during rolling circle replication. As both dimeric plus

and minus RNAs self-cleaved by double-hammerhead structures during *in vitro* transcription of dimeric RNAs, it is possible that self-cleavage *in vivo* occurs by these structures also.

The plus ASBV hammerhead sequence exists in two parts, each part is opposite the other in the rod-like structure of ASBV (see Figure 6.7A). Theoretically, it requires only a relatively small structural change from the rod-like structure to form the plus single-hammerhead structure. The same situation exists for the minus hammerhead sequence (Figure 6.5A). In contrast, a major structural change from the vLTSV rod-like structure is required to form either the plus or minus vLTSV single-hammerhead structures (Forster and Symons, 1987a), and similarly for the other virusoids. It is possible that the requirement for a double-hammerhead structure to mediate the self-cleavage of plus ASBV has evolved to prevent self-cleavage of monomeric circular plus RNA (the mature viroid) *in vivo*, which potentially could form a single-hammerhead structure rather easily. vLTSV, and the other virusoids (whose single-hammerhead structures are theoretically stable; Figure 1.1), would not have needed to evolve such a mechanism, as the circular monomeric RNA is effectively prevented from self-cleaving by the large structural rearrangment from the rod-like structure required to form the single-hammerhead structures.

# CHAPTER 7

# BIOLOGICAL EFFECTS OF DISRUPTING *IN VITRO* SELF-CLEAVAGE OF THE MINUS RNA OF vLTSV

## Introduction

Chapters 3, 4, 5 and 6 have involved characterisation of the structural requirements for the *in vitro* RNA self-cleavage reaction mediated by the hammerhead structure. In this Chapter the possible involvement of the hammerhead self-cleavage reaction *in vivo*, as part of the replication process of vLTSV, was investigated.

vLTSV is believed to replicate by a symmetrical rolling circle mechanism (Figure 1.2a). Both plus and minus monomeric RNAs (as well as multimeric series) are detectable by Northern analysis, both in RNAs extracted from purified virions, and RNAs extracted directly from the plant (Hutchins *et al.*, 1985). The monomeric (circular) RNAs are believed to be the templates in the symmetrical rolling circle mechanism (see section 1-4). The hammerhead structures that mediate the *in vitro* self-cleavage of plus and minus vLTSV RNAs (Forster and Symons, 1987a) have been suggested to play a role *in vivo* in the processing of the plus and minus multimeric replicative intermediates to monomer linear RNAs. These are then thought to be circularised *in vivo*, possibly by a plant ligase.

The other two virusoids, vVTMoV and vSCMoV, are believed to replicate by an asymmetrical rolling circle mechanism (Figure 1.2b). Northern analysis reveals multimeric series, containing monomeric forms, of the plus RNA only, whereas the minus RNA exists predominantly as high molecular weight RNA (Hutchins *et al.*, 1985, Davies *et al.*, 1990). This correlates with the ability of the plus vSCMoV RNA, but not the minus vSCMoV RNA to self-cleave *in vitro* (Davies *et al.*, 1990; the *in vitro* self-cleavage abilities of the full-length plus and minus vVTMoV RNAs have not been investigated). Therefore, the proposed rolling circle mechanism of vLTSV differs from that of vVTMoV and vSCMoV in that monomerisation of the multimeric minus vLTSV RNAs occurs *in vivo*, but not of the multimeric minus RNAs of vVTMoV and vSCMoV.

In view of the proposed role of the hammerhead self-cleavage reaction in the *in vivo* processing of vLTSV minus RNAs to monomer size (Forster and Symons, 1987a), it would be expected that vLTSV molecules that were mutated in the minus hammerhead sequences so

that their minus RNAs were unable to self-cleave *in vitro*, would be unable to produce monomeric minus RNAs *in vivo*. Presumably, therefore, they would be unable to replicate by a symmetrical rolling circle mechanism. Possibly, these mutated vLTSV molecules would not be capable of replication at all, or alternatively, they might be replicated by an asymmetrical rolling circle mechanism, involving multimeric minus RNA (as is believed to occur with vVTMoV and vSCMoV).

With the knowledge derived from the mutagenesis of the hammerhead structure described in Chapter 3, and the references therein, mutations were chosen and introduced into a monomeric cDNA clone of the Australian isolate of vLTSV (vLTSV-A). The introduced mutations were shown to disrupt the *in vitro* self-cleavage of the minus RNA transcripts derived from the mutated clones, but had no effect on the *in vitro* self-cleavage of the plus RNA transcripts.

Double-stranded cDNA from the mutated vLTSV-A cDNA clones was coinoculated with the New Zealand strain of LTSV (LTSV-N) onto *Nicotiana clevlandii*. (Although not shown previously, it was considered likely that LTSV-N would support vLTSV-A.) The results from these infectivity studies showed that, contrary to expectations, the virusoid progeny from the mutated vLTSV-A inoculum contained monomer sized minus RNAs. DNA sequencing of cDNA clones generated from the progeny virusoid RNAs revealed that the majority of the progeny RNAs retained the original introduced mutations. These results suggest that the presence of mutations that disrupted *in vitro* self-cleavage of minus vLTSV RNA transcripts did not abolish the generation of monomeric minus vLTSV RNAs *in vivo*. An intriguing finding was that at least half of the progeny cDNA clones generated from plants inoculated with the mutated vLTSV-A cDNA contained other base changes distributed throughout the vLTSV-A sequence. However, only about 20 % of the progeny cDNA clones generated from wild-type vLTSV-A inoculated plants contained base changes. The large number of the base changes may reflect an interesting adaptive response on the part of the mutated virusoids.

## Materials

### 7-1 LTSV-N

A virusoid-free isolate of the New Zealand strain of LTSV (LTSV-N) was generously provided by Dr. R. L. S. Forster (Plant Sciences Division, Department of Scientific and Industrial Research, Private Bag, Auckland, N. Z.). It was passaged in *Chenopodium quinoa* by C. Davies and stored as dried leaf material in a desiccating environment at 4°C.

A stock of LTSV-N virus solution was prepared and was tested to ensure that it was free of vLTSV-N. Three plants were infected with a slurry of dried leaf material (see above) in sodium phosphate buffer pH 7.2; these plants were subsequently used to infect thirty plants, by the two step inoculation procedure described below (7-4). After ten days, virion particles were purified (7-5) and stored at 4°C. The presence of the virus was assayed by an Ouchterlony diffusion test of LTSV-N antibodies against purified virions (7-6). The virus was demonstrated to be virusoid free, by Northern hybridization analysis of purified viral RNA (7-7) with a plus vLTSV specific probe (7-8). In addition PCR (2-24) using the two plus vLTSV specific primers (2-4 and see 7-9) did not generate a band of the correct size, further confirming that the virus was free of the virusoid.

## Methods

### 7-2 *In vitro* mutagenesis of a vLTSV-A cDNA clone

The mutations (M1, M2 and M3; see Figure 7.1) were introduced into a monomeric vLTSV-A M13mp18 cDNA clone by oligonucleotide-directed M13 mutagenesis (2-21), using three 20-mer oligonucleotides (2-4). Positive plaques were selected for each construct as described (2-21), and the presence of the mutation in the cDNA clones was confirmed by dideoxy sequencing (2-22). HindIII/EcoRI double-stranded cDNA fragments containing vLTSV-A cDNA plus flanking M13 vector sequences, were excised from HPLC-purified M13 RF DNA (2-19) for each of the three mutated (M1, M2 and M3) and unmutated (wild-type) vLTSV M13mp18 cDNA clones. The cDNA fragments of approximately 375 bp were ligated into HindIII and EcoRI digested, de-phosphorylated pGem2 (Figure 7.2) and transformed into *E. coli* MC1061 (2-26). Large scale plasmid DNA was prepared from the appropriate colonies and purified by HPLC (2-18).

## 7-3 Preparation of mutated and wild-type vLTSV-A cDNA inoculum

Double-stranded mutated (M1, M2 and M3) and unmutated (wild-type) monomeric vLTSV-A cDNAs to be used for inoculation were prepared by digestion of the appropriate pGem2 clones with SalI. This produced approximately 324 base-pair (the size depended on the mutation present) double-stranded cDNAs, with four nucleotide 5'-overhangs, containing the vLTSV-A sequence with no vector sequences attached. The vLTSV-A cDNAs were separated from the plasmid DNAs by agarose gel electrophoresis and were electroeluted (2-25), and their concentrations determined by U.V. spectroscopy.

## 7-4 Inoculation procedure

Two to three week old *Nicotiana clevlandii* plants were stored in the dark for about 16 h (this is believed to increase the infectivity rate, R. H. Symons, pers. comm.), prior to inoculation by a two step procedure. In the first stage of inoculation, three plants were lightly dusted with carborundum powder and gently rubbed with virus inoculum (10 µl of virus solution (7-1) per plant, distributed over three leaves). The plants were grown for about 10 days in a room illuminated for 15 h/day at 23°C.

In the second stage, leaves from the first stage inoculated plants were ground in 0.1 M sodium phosphate, pH 7.2, to use as the virus inoculum for 25 carborundum dusted plants (three leaves each). Groups of five plants were then inoculated with one of four double-stranded monomeric vLTSV-A cDNAs (M1, M2, M3 or wild-type). 5 µl/leaf of 50 ng/µl vLTSV cDNA (see 7-3) in 0.1 M sodium phosphate, pH 7.2 was inoculated onto three leaves per plant - the same leaves that were inoculated with the virus inoculum. One group of five plants had no further treatment ("virus only" inoculated plants). All plants were grown for 10 days (as above).

Extreme precautions were taken to ensure that no contamination of virusoid cDNA occurred, including growing the first stage inoculated plants in a separate compartment from the second stage inoculated plants. Gloves were used and changed between sets of inoculations. At all stages, wild-type vLTSV-A inoculations were carried out after mutated vLTSV-A inoculations.

## 7-5 Virus purification

10 days after inoculation the plants were harvested; they were placed in the dark the previous night to keep carbohydrate levels as low as possible (R. H. Symons, pers. comm.). Once harvested, the leaves were placed on ice, and were crushed between the rollers of a sap extractor (Erich Pollahne, Germany) with 1.5 volumes of buffer (70 mM sodium phosphate, pH 7.2, 1 mM EDTA, 0.1 % thioglycollic acid). 0.5 volumes of chloroform was mixed with the slurry, and placed at 4°C for 30 min. The phases were separated by centrifugation in a Sorvall HB-4 rotor at 10,000 rpm at 4°C for 15 min. The upper phase was removed and recentrifuged. The virus in the supernatent was pelleted by centrifugation in a Beckmann Ti50 rotor at 45,000 rpm at 4°C for 90 min. The pellet was resuspended in 70 mM sodium phosphate, pH 7.2, 1 mM EDTA and centrifuged in a Beckmann JA-21 rotor at 10,000 rpm at 4°C for 15 min. The supernatent was layered on a 1 ml 20 % (w/v) sucrose cushion and centrifuged in Beckmann Ti-50 rotor at 45,000 rpm at 4°C for 90 min. The pellet was resuspended in 20 mM Tris-HCl, pH 7.5, 1 mM EDTA, 10 mM NaCl and clarified by centrifugation in a Beckmann JA-21 rotor at 10,000 rpm at 4°C for 15 min. The supernatent (containing the virus) was stored at 4°C.

## 7-6 Ouchterlony antibody diffusion test

0.175 g agarose was dissolved in 1.25 ml 0.2 M sodium phosphate, pH 7.4 and 21 ml $H_2O$ by heating the mixture, which was then allowed to cool. 0.25 ml 1 % $NaN_3$ was added and 10 ml of the solution was poured onto a glass slide. Holes were punched using a gel cutting implement, an LTSV-N antibody (raised in rabbits; generously provided by the late Dr. R. I. B. Francki, Department of Plant Pathology, Waite Institute, University of Adelaide) was placed in one hole and virus sample in a second hole. They were allowed to diffuse at 37°C overnight. The gel was then dried onto the glass slide and stained with 0.5 % Coomassie brilliant blue R, 45 % ethanol, 10 % acetic acid and destained in 45 % ethanol, 10 % acetic acid. The presence of LTSV in the solution was indicated by a blue precipitin line between the virus and antibody holes.

## 7-7 Viral RNA extraction

RNA was extracted from purified virions by heating at 60°C for 10 min in the presence of 10 mM Tris-HCl, pH 9, 1 mM EDTA, 2 % SDS, followed by a

phenol/chloroform extraction and ethanol precipitation. The RNA was resuspended in 1 mM EDTA.

### 7-8 Northern hybridization analysis of vLTSV RNAs

Northern hybridization analysis was carried out as described below, based on the method of Thomas (1980), unless otherwise indicated in the text.

Nucleic acid samples were electrophoresed on 1.9 % agarose, 1.2 M formaldehyde gels (14 x 14 x 0.3 cm) containing 10 mM sodium phosphate, pH 7.4 for 2.5 h at 30 mA. Nucleic acid samples were prepared for loading by heating for 5 min at 65°C and then at room temperature for 5 min in the presence of 50 % formamide, 2.4 M formaldehyde, 0.5 mM EDTA, 10 mM sodium phosphate, pH 7.4, followed by the addition of 5 x glycerol load (2-7). Nucleic acids were transferred from the gel to nitrocellulose by capillary action overnight and then baked *in vacuo* for 2 h at 80°C. Filters that were to be probed for minus sequences were washed at 90°C for 10 min in $H_2O$, after baking and before prehybridization, to remove loosely bound nucleic acid (Hutchins *et al.*, 1985).

Prehybridization and hybridization of the filters were carried out by one of two methods. In early experiments, the filter was sealed in a plastic bag and incubated in a temperature controlled shaking water-bath, in later experiments, the filter was placed on a piece of nylon mesh in a glass bottle, and rotated in a hybridization oven (Scientronic). Prehybridization solution contained 5 x SSC, 50 mM sodium phosphate, pH 6.5, 5 mM EDTA, 0.2 % SDS, 50 % deionised formamide, 1 x Denhart's solution and 250 µg/ml salmon sperm DNA. The hybridization solution contained one part 50 % dextran sulphate to four parts prehybridization solution, in addition to the $^{32}$P-labelled RNA probe. Prehybridization and hybridization were at 55°C and 65°C respectively, for 16 - 24 h. 0.1 ml of prehybridization or hybridization solution was used per $cm^2$ of the filter.

The $^{32}$P-labelled RNA probe for the detection of plus vLTSV sequence was prepared by transcription from the HindIII linearised pGem2 wild-type vLTSV full-length clone with SP6 RNA polymerase (generates a minus full-length transcript and two self-cleavage fragments; Figure 7.2B). The probe for detection of the minus vLTSV sequence was prepared by transcription of the EcoRI linearised clone with T7 RNA polymerase, to generate a full-length plus sense transcript and two self-cleavage fragments (Figure 7.2C).

The products were purified by denaturing polyacrylamide gel electrophoresis (2-12). $0.05x10^6$ cpm of probe per $cm^2$ of filter was used and the probe solution was heated in 50 % formamide, 0.5 mM EDTA at 80°C for one min and snap-cooled on ice, prior to the addition to the hybridization solution.

After hybridization, the filter was washed three times at room temperature for 15 min in 2 x SSC, 0.1 % SDS, and three times at 65°C for 20 min in 0.1 x SSC, 0.1 % SDS. The filter was then blotted dry and autoradiographed.

## 7-9 Construction of the progeny vLTSV cDNA clones in M13mp18 and pGem2

cDNA clones in M13mp18 were prepared from encapsidated virusoid RNA using vLTSV specific primers. First strand cDNA was prepared from total viral RNA (using M-MLV RNase $H^-$ reverse transcriptase (2-23)), using a vLTSV plus strand specific primer (primer 1; complementary to nts 246 - 267 (2-4)) which binds to a region of relatively low base-pairing (see Figure 7.6). Vent DNA polymerase, using a PCR protocol (2-24), was used to produce the second strand of the cDNA and to amplify the double-stranded cDNA. Vent DNA polymerase has a higher fidelity than Taq DNA polymerase ($2.4 \times 10^{-5}$ errors/base-pair compared with $8.9 \times 10^{-5}$, Cariello *et al.*, 1991), and this was considered advantageous for this project. The reverse primer covered nts 267 - 289 ( 2-4)). The blunt-ended (2-24) 324 bp cDNAs were ligated into SmaI digested M13mp19 (2-26). Single-stranded M13 phage DNA was prepared as described in 2-20.

cDNA clones in the plasmid vector pGem2 were prepared from the M13mp18 cDNA clones, as follows. M13 single-stranded DNA prepared (2-20) from clones of interest, was used as the template for PCR amplification (2-24) of the vLTSV cDNA sequence, using the primers described above. The blunt ended double-stranded cDNAs were then ligated into SmaI digested pGem2 (2-26).

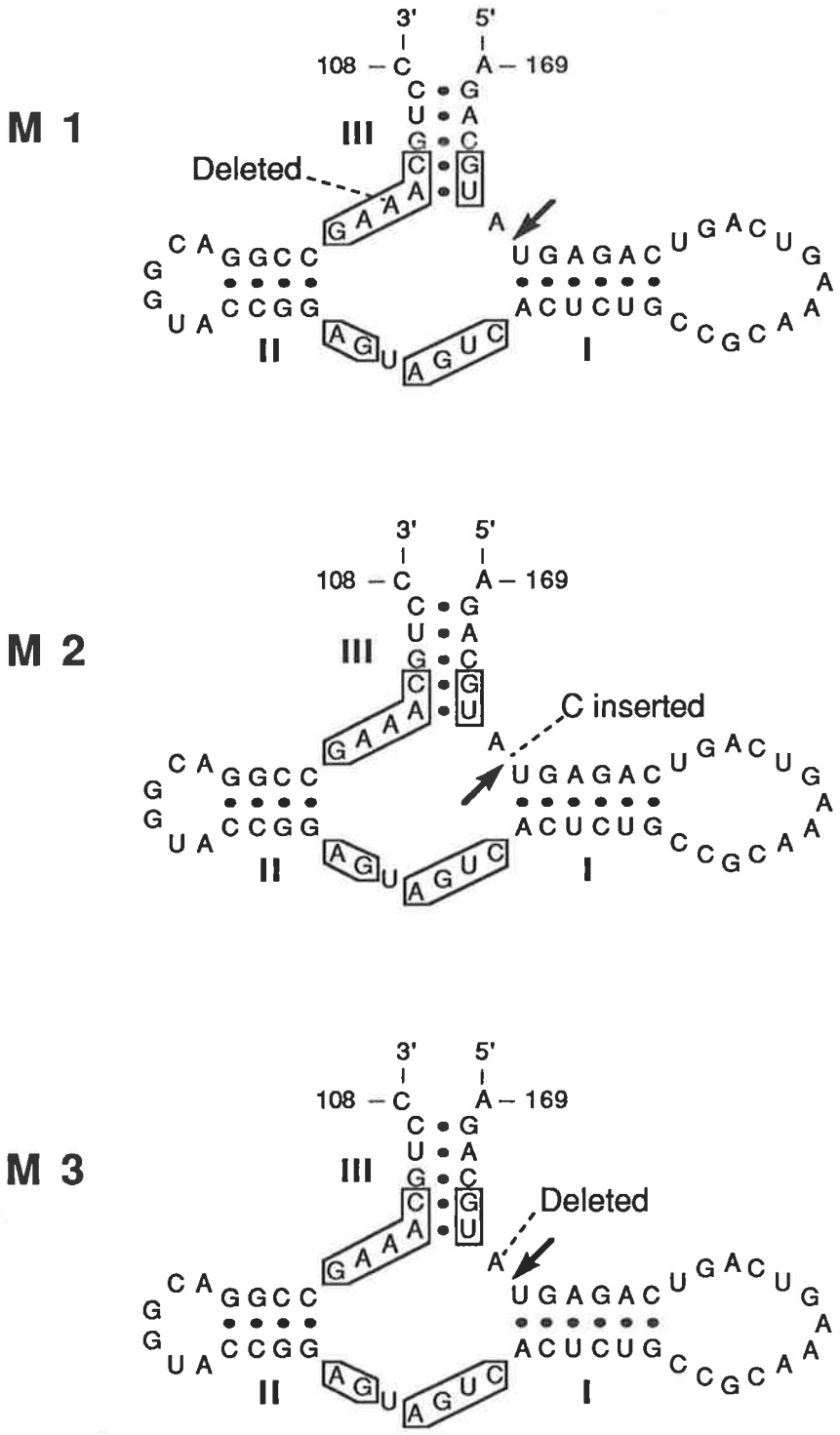



**Figure 7.1** The minus hammerhead structure of vLTSV-A, indicating the positions of the mutations (M1, M2 and M3) used to abolish *in vitro* self-cleavage of minus vLTSV-A RNA transcripts. Nucleotides conserved between most naturally occurring hammerhead structures are boxed. Arrows indicate the sites of *in vitro* self-cleavage in the wild-type molecule. Stems are numbered according to the convention of Forster and Symons (1987a) and vLTSV-A is numbered according to Keese *et al.* (1983). Note that the minus vLTSV-A sequence is shown; the plus sequence contains the complementary mutations.

## Results

### 7-10 Construction of mutated vLTSV-A cDNA clones

As the first stage of this project, single base mutations (described below) were introduced into a monomeric vLTSV-A cDNA clone (described below). The mutations were intended to disrupt the *in vitro* self-cleavage of the minus RNA transcripts generated from the mutated cDNA clones. Double-stranded cDNA copies of the mutated virusoid sequences were then to be used to inoculate *N. clevlandii* along with the LTSV-N helper virus.

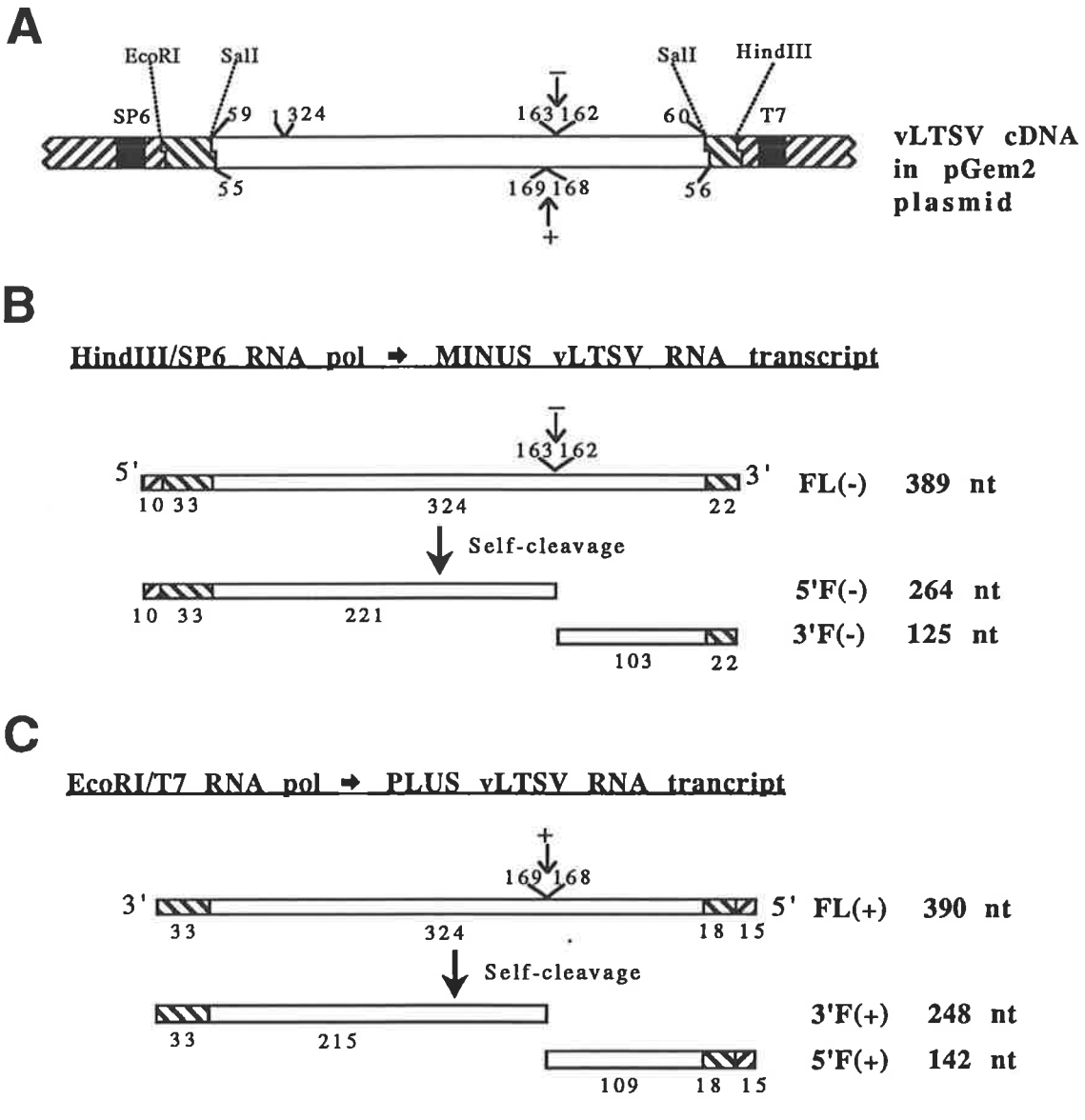
No infectivity studies of mutated virusoids have been undertaken previously, but it was anticipated that, as demonstrated with viroids (e.g., Owens *et al.*, 1986, Hammond and Owens, 1987), single-base mutations may abolish infectivity. Therefore, to increase the likelihood that at least one mutated virusoid sequence would be infectious, three different single base mutations were introduced separately into an existing full-length monomeric vLTSV-A cDNA clone in M13mp18 (cloned in the SalI site, terminal vLTSV-A residues 55 and 56; kindly donated by Dr. D. Mitchell), to create three mutated cDNA clones (7-2). The mutated and unmutated (wild-type) vLTSV-A cDNAs were then subcloned into the high copy number plasmid vector pGem2 (7-2; Figure 7.2), both to allow transcriptional analysis of the mutated sequences and to permit isolation of μg quantities of vLTSV-A insert cDNA, to use for inoculation.

The introduced mutations are shown within the minus vLTSV-A hammerhead structure in Figure 7.1, and their locations in the rod-like structure of vLTSV-A are shown in Figure 7.6. The first mutation (M1) has previously been used to abolished self-cleavage in dimeric plus (Forster *et al.*, 1988) and minus (Davies *et al.*, 1991; see Chapter 6) ASBV RNAs. The second mutation (M2) was the same as that used to abolish self-cleavage during transcription of a plus vLTSV hammerhead RNA (Chapter 3, mutant 6). The effect of mutation 3 (M3) on *in vitro* self-cleavage had not previously been tested.

### 7-11 The effect of the introduced mutations on *in vitro* self-cleavage of plus and minus vLTSV-A RNA transcripts

The effects of the introduced mutations on the *in vitro* self-cleavage of both plus and minus RNA transcripts derived from the mutated cDNA clones were examined. It was intended that the introduced mutations would disrupt the *in vitro* self-cleavage of the minus

**Figure 7.2** Diagram of the full-length monomeric vLTSV-A cDNA clone in pGem2, and the RNA transcripts derived from it. **(A)** The vLTSV-A cDNA clone in pGem2 was constructed by ligating the HindIII/EcoRI fragment from a full-length vLTSV M13mp18 cDNA clone (terminal vLTSV nucleotides 55 and 56) into HindIII/EcoRI digested pGem2 plasmid DNA. The terminal vLTSV nucleotides and relevant restriction endonuclease sites are indicated. The sites of self-cleavage of the plus and minus sequence are indicated by the arrows. **(B)** Schematic diagram of minus RNA transcripts produced following digestion of the wild-type vLTSV-A cDNA clone with HindIII, and transcription with SP6 RNA polymerase. The full-length RNA transcript (FL(-)) is 389 nt (65 nt of vector derived sequence). The wild-type vLTSV-A RNA transcript self-cleaves between nucleotides 162 and 163, to yield a 5'-fragment (5'F(-)) of 264 nt and a 3'-fragment (3'F(-)) of 125 nt. **(C)** Schematic diagram of plus RNA transcripts produced following digestion of the wild-type vLTSV-A cDNA clone with EcoRI, and transcription with T7 RNA polymerase. The full-length RNA transcript (FL(+)) is 390 nt (66 nt of vector derived sequence). The wild-type vLTSV-A RNA transcript self-cleaves during the transcription reaction to generate 142 nt 5'-fragment (5'F(+)) and a 248 nt 3'-fragment (3'F(+)). The number of nucleotides derived from M13mp18 and pGem2 vector sequences is indicated.

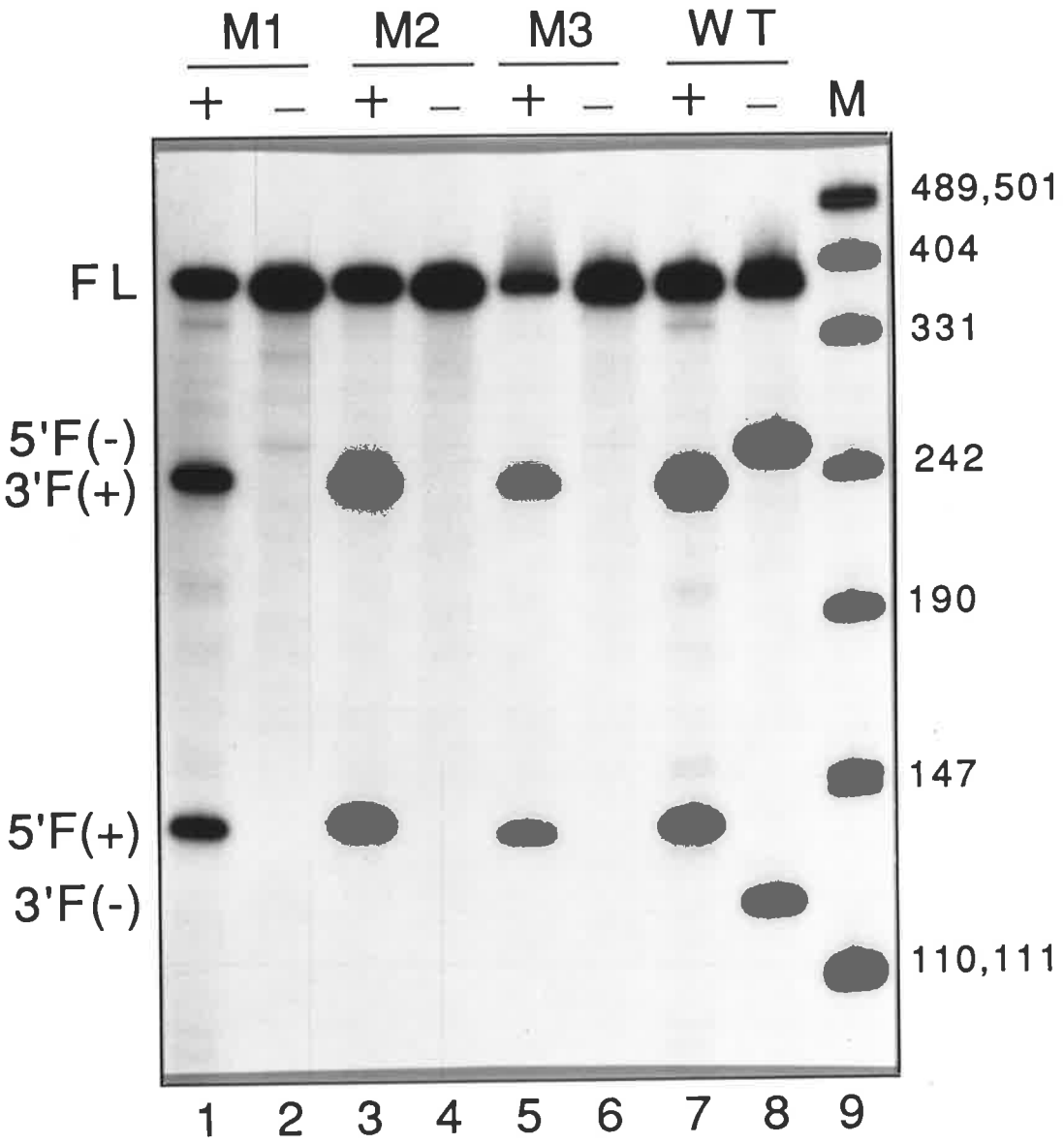

A
EcoRI
SalI
SalI
HindIII
SP6
59
1324
163 162
60
T7
55
169 168
56
+
vLTSV cDNA in pGem2 plasmid
B
HindIII/SP6 RNA pol → MINUS vLTSV RNA transcript
163 162
5'
3'
10 33
324
22
FL(-) 389 nt
Self-cleavage
10 33
221
5'F(-) 264 nt
103
22
3'F(-) 125 nt
C
EcoRI/T7 RNA pol → PLUS vLTSV RNA trancript
+
169 168
3'
5'
33
324
18 15
FL(+) 390 nt
Self-cleavage
33
215
3'F(+) 248 nt
109
18 15
5'F(+) 142 nt
M13mp18 vector sequence
pGem2 vector sequence
SP6 or T7 RNA polymerase promoter
vLTSV sequence

RNA transcripts derived from the mutated vLTSV-A cDNA clones, but have no effect on the *in vitro* self-cleavage of plus RNA transcripts. Plasmid DNA templates for the production of plus and minus mutant and wild-type RNA transcripts were prepared by digestion of the appropriate pGem2 clones with EcoRI or HindIII, and were transcribed with T7 or SP6 RNA polymerases, respectively (Figure 7.2, 2-11). The self-cleavage abilities of the mutated and wild-type RNA transcripts during the transcription reaction were analysed by denaturing polyacrylamide gel electrophoresis (Figure 7.3).

As expected, the wild-type RNA transcripts of both plus and minus sense self-cleaved to yield, in addition to the uncleaved full-length transcript, the 5'- and 3'-self-cleavage fragments (Figure 7.3, lanes 7,8). The sizes of the products were those expected by self-cleavage at the appropriate sites (Figure 7.2), as estimated by the migration of the single-stranded DNA markers (Figure 7.3, lane 9). The plus sense monomeric RNA transcripts, transcribed from the three mutated cDNA clones, self-cleaved to the same extent as the monomeric wild-type plus RNA transcript (Figure 7.3, lanes 1,3,5), indicating that, as expected, none of the three mutations had an effect on the self-cleavage efficiency of the plus hammerhead structure. In contrast, as intended, self-cleavage of all three mutated minus RNA transcripts was eliminated during *in vitro* transcription, and only the full-length transcript was present (Figure 7.3, lanes 2,4,6).

The results from the previous Chapters have indicated that some RNAs not capable of self-cleavage during transcription were capable of self-cleavage when purified and incubated under self-cleavage conditions. Hence, the full-length mutated and wild-type minus RNA transcripts were purified from the gel (2-12), and incubated under three self-cleavage conditions (2-13): (1) 5 mM $MgCl_2$, 0.5 mM EDTA, Tris-HCl, 7.5, 25°C for 1 h (the conditions used by Forster and Symons (1987a) for the self-cleavage of partial vLTSV RNA transcripts), (2) 10 mM $MgCl_2$, 0.5 mM EDTA, Tris-HCl, 8.0, 37°C for 1 h, and (3) 50 mM $MgCl_2$, 0.5 mM EDTA, Tris-HCl, 8.0, 37°C for 1 h (conditions (2) and (3) are those used in Chapter 6). Wild-type RNA transcripts self-cleaved to approximately the same extent under all three conditions (approximately 30 %, results not shown). There was no self-cleavage of M2 transcripts under any condition, and only a trace of self-cleavage of M3 under conditions (2) and (3), and none under condition (1) (results not shown). About 1 - 2 % self-cleavage of M1 occurred under all three conditions (results not shown). Condition

**Figure 7.3** Plus and minus vLTSV-A RNAs generated during transcription from wild-type and mutated full-length vLTSV-A cDNA clones in pGem2 (Figure 7.2). **Lane 1.** Transcription of the full-length vLTSV-A cDNA clone containing the M1 mutation, linearised with EcoRI and transcribed with T7 RNA polymerase, to generate the plus RNA transcript. **Lane 2.** As in lane 1, except that the clone was linearised with HindIII, and transcribed with SP6 RNA polymerase, to generate the minus RNA transcript. **Lanes 3 and 4.** As for lanes 1 and 2, except that the cDNA clone contained the M2 mutation. **Lanes 5 and 6.** As for lanes 1 and 2, except that the cDNA clone contained the M3 mutation. **Lanes 7 and 8.** As for lanes 1 and 2, except that the cDNA clone contained the wild-type vLTSV-A sequence. **Lane 9.** Single-stranded $^{32}$P-labelled HpaII pUC19 DNA markers (2-27), the sizes of which are given on the right hand side of the figure. The sizes of the full-length RNA transcripts (FL), and the 5'-, and 3'-self-cleavage fragments (5'F and 3'F) are as in Figure 7.2.


M1
M2
M3
WT
+ − + − + − + − M
FL
5'F(-)
3'F(+)
5'F(+)
3'F(-)
489,501
404
331
242
190
147
110,111
1 2 3 4 5 6 7 8 9

(1) seems the most likely of the three conditions to resemble those within the plant (Forster and Symons, 1987a). Under this condition, two of the mutated minus RNA transcripts (M2 and M3) did not self-cleave *in vitro*. The effect of the small amount of *in vitro* self-cleavage of M1, and of M3 under other conditions, on the infectivity experiments was difficult to predict.

## 7-12 Inoculation of *Nicotiana clevlandii* with mutated and wild-type vLTSV-A cDNA and LTSV-N

*Nicotiana clevlandii* was chosen as the assay species; LTSV-N is reported to produce large chlorotic local lesions and occasional systemic chlorotic mottle when inoculated on this species (Forster and Jones, 1980), however no symptoms were visible during the course of these experiments (as has been found by others, A. Jeffries, pers. comm.).

Double-stranded DNA copies of the monomeric wild-type vLTSV-A sequence have been shown previously to be infectious when coinoculated with a suitable helper virus on host plants (Dunlop, 1986). The sequence of events following inoculation with wild-type vLTSV-A cDNA, leading to infection are predicted to be: ligation of the cDNA by a host ligase *in vivo* to form a circular cDNA (or multimeric cDNA), which is accepted as a template for an RNA polymerase (either host or viral). Transcription of the cDNA would produce either multimeric plus RNAs, multimeric minus RNAs, or both. *In vivo* cleavage (possibly self-cleavage) and ligation would form circular monomeric RNAs, which would then allow the virusoid to enter the normal rolling circle cycle.

*N. clevlandii* were inoculated with one of the four monomeric vLTSV-A cDNAs (M1, M2, M3 or wild-type) prepared by digestion of the pGem2 clones with SalI (7-3), and were coinoculated with LTSV-N as described in 7-4. After ten days, plants were harvested and virus particles purified (7-5). RNA was isolated from purified virions (7-7), to produce a mixture of viral RNA and virusoid RNAs.

## 7-13 Northern hybridization analysis of progeny plus vLTSV RNAs

Purified viral RNA was analysed by Northern hybridization analysis using a plus vLTSV specific RNA probe (7-8). As expected, no hybridization was observed when viral RNA of plants infected with virus only (not with virusoid) was probed (results not shown),

indicating that no contamination of vLTSV occurred. Purified viral RNAs from plants infected with LTSV-N and either mutated (M1, M2 or M3) or wild-type vLTSV-A sequences revealed the presence of oligomeric series of bands. Bands corresponding to eleven times the monomeric RNA unit length were visible in some experiments (Figure 7.4; estimation of size was based on RNA and DNA markers in early experiments). Therefore, the presence of the introduced mutations did not abolish the infectivity of any of the virusoid constructs. The pattern was similar to that obtained for vLTSV-N by Hutchins *et al.* (1985), however, in my work a greater proportion of the higher multimers was obtained, perhaps reflecting the different gel and hybridization systems used. It is not possible to quantitate the RNA levels of the mutated virusoids relative to the wild-type virusoid from these hybridization experiments, as no attempt was made to determine whether the filters were saturated with RNA.

Intriguingly, an additional series of bands was present in the viral RNA extracted following M1, M2 and M3 inoculation, but not following inoculation with the wild-type vLTSV-A cDNA. These bands migrated roughly half-way between the bands of the main multimeric series (Figure 7.4, lanes 1,2,3). Such bands have not been described previously for wild-type plus vLTSV, but are reminiscent of the X-bands reported for ASBV and vVTMoV (Hutchins *et al.*, 1985). The second band of the minus dimer doublet (see below) may also correspond to one band of a non-integral multimeric series. It has been suggested that the bands are a consequence of cleavage of the RNA at "cryptic sites", which may occur if cleavage at the correct site is inhibited for some reason (Branch *et al.*, 1985). Alternatively, they may arise due to inefficient cleavage at the first processing site (i.e., the extreme 5' site) of a rolling circle transcript. Each band of the minor series may represent an integral multimer, with additional sequences at the 5'-end, which correspond to the sequence between the *in vivo* transcriptional initiation start site and the first cleavage site. Neither of these possibilities, however, explains why the M1, M2 and M3 plus vLTSV RNAs, and not the wild-type plus vLTSV RNAs, have this series of bands, unless the introduced mutations result in slower cleavage of the plus sense RNAs.

**Figure 7.4** Northern hybridization analysis of plus vLTSV RNAs extracted from purified virus. RNAs were extracted from purified virus isolated from *Nicotiana clevlandii* infected with M1 (**lane 1**), M2 (**lane 2**), M3 (**lane 3**) or wild-type (**lane 4**) vLTSV-A cDNA inoculum and co-inoculated with LTSV-N, then analysed by Northern hybridization using a plus vLTSV specific $^{32}$P-labelled RNA probe. Bands corresponding to monomer, dimer and trimer are indicated. Autoradiography was for 4 h at -80°C with an intensifying screen.

**Figure 7.5** Northern hybridization analysis of minus vLTSV RNAs extracted from purified virus. RNAs were extracted from purified virus isolated from *Nicotiana clevlandii* infected with M1 (**lane 1**), M2 (**lane 2**), M3 (**lane 3**) or wild-type (**lane 4**) vLTSV-A cDNA inoculum and co-inoculated with LTSV-N, then analysed by Northern hybridization using a minus vLTSV specific $^{32}$P-labelled RNA probe. Bands corresponding to monomer and dimer are indicated. Autoradiography was for 7 days at -80°C with an intensifying screen. Note that the dimeric doublet bands in lanes 1, 3 and 4 were clearly visible on autoradiogram.

**FIGURE 7.4**

**PLUS**

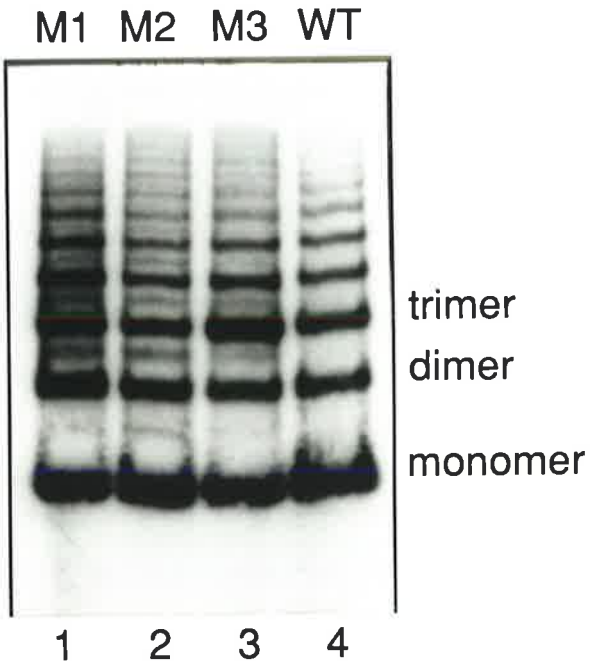
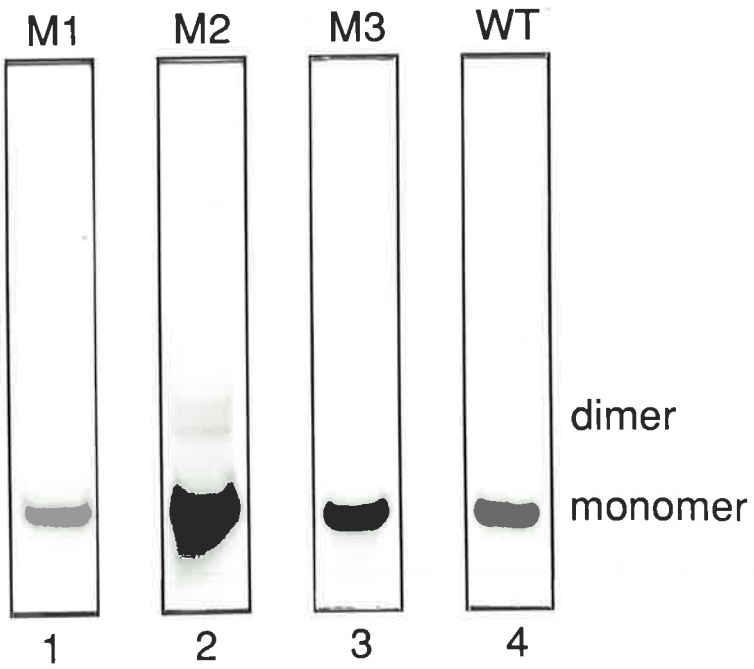



**FIGURE 7.5**

**MINUS**



## 7-14 Northern hybridization analysis of progeny minus vLTSV RNAs

It is believed that minus viroid RNAs are present at about 10 to 50 fold lower levels than the plus RNAs *in vivo* (Branch *et al.*, 1988), however, no figures are available for the relative levels of plus and minus vLTSV RNAs. Difficulties in detecting minus species of viroids and virusoids in the presence of excess plus RNAs have been reported by a number of researchers (Branch *et al.*, 1981, Bruening *et al.*, 1982, Branch and Robertson, 1984, Hutchins *et al.*, 1985, Branch *et al.*, 1988). Problems, due to the cross-hybridization of the minus probe ($^{32}$P-labelled plus RNA transcripts) to plus RNA bound to the filter (possible because of the self-complementary nature of the viroids and virusoids), can largely be overcome by the use of full-length RNA (rather than DNA) probes, and high temperatures of hybridization and washing (Hutchins *et al.*, 1985). Other complications stem from the fact that more than 50 % of the nucleic acid covalently attached to the nitrocellulose filter by baking, is released during prehybridization of the filter (Hutchins *et al.*, 1985). This released RNA is then available to hybridize to filter bound RNA, causing a number of artifacts (as discussed in Hutchins *et al.*, 1985 and Branch and Robertson, 1984), or to bind to the probe in solution, hence diluting the effective concentration of the probe. Hutchins *et al.* (1985) found that these problems could be overcome by washing the filter in water at 90°C for 10 min prior to the prehybridization step, this removes most of the loosely attached nucleic acid from the filter.

Attempts at analysis of the minus vLTSV species by Northern hybridization using a minus vLTSV specific probe (7-8) produced results similar to the artifactual results reported by Branch *et al.* (1981), Bruening *et al.* (1982), Branch and Robertson (1984) and Hutchins *et al.* (1985) (results not shown). Hybridization to the entire track occurred with either regions of no hybridization ("windows"), or additional faint bands, at the position of monomer, dimer and trimer (results not shown). Therefore, despite the filter washing treatment, it appeared that artifactual results still occurred, as was found by Jaspars *et al.* (1985).

More stringent conditions were employed to eliminate possible artifactual results. To remove as much loosely bound RNA as possible, washing the filters at 90°C was increased to 15 minutes. The prehybridization buffer was replaced with fresh solution after 12 h, incubated for a further 6 - 12 h, and then the filter was rinsed with fresh solution prior to

hybridization. To eliminate any cross-hybridization of probes, or any "sandwich" hybridization (see Hutchins *et al.*, 1985), hybridization and washing of filters was carried out at 70°C, rather than 65°C. In addition, one quarter of the amount of viral RNA was loaded on the gel, and twice as much $^{32}$P-labelled probe was used during hybridization. This was to decrease the amount of unlabelled plus RNA present on, and potentially released from, the filter relative to the $^{32}$P-labelled plus RNA (i.e., the minus probe). As a further precaution, each track of the filter was prehybridized, hybridized and washed separately from other tracks to overcome any possible interference of RNA from one track to another (e.g., Jaspars *et al.*, 1985).

The results of the experiments done under these more stringent conditions were quite different from the earlier attempts. Surprisingly, the minus vLTSV specific probe resulted in the same pattern and intensity of hybridization for RNA derived from all three mutated (M1, M2 and M3) vLTSV-A and the wild-type vLTSV-A inoculated plants (Figure 7.5). Predominantly monomeric species, with a lesser amount of dimeric were evident (Figure 7.5). A doublet of bands at the dimeric position was visible, as previously reported for the wild-type RNA (Hutchins *et al.*, 1985). The minus hybridization pattern differed noticably from the plus pattern by the absence of the non-integral multimeric bands (X-bands), and the presence of the dimeric doublet. Hence, it was considered unlikely that the minus pattern was due to artifactual detection of the plus species.

No hybridization of the minus probe to high molecular weight material of the purified viral RNA (extracted from virions) is evident in Figure 7.5. Possibly, the high molecular weight minus vLTSV RNAs were not detected because they were not packaged into virion particles, however, this seems unlikely, as the high molecular weight minus vVTMoV and vSCMoV RNAs are packaged into their respective helper virus capsids (Hutchins *et al.*, 1985, Davies *et al.*, 1990). Nevertheless, two further infectivity experiments were attempted in order to isolate total RNA from infected plants, unfortunately, both resulted in an extemely low level of infection, unsuitable for Northern hybridization analysis of minus species. It appears that the stock of virus prepared at the start of the work had deteriorated. Due to time constraints, it was not possible to return to the dried leaf material stocks of virus and prepare a new working stock of virus.

**Table 7.1** Summary of sequencing data

| Inoculum | No. of Progeny Clones Sequenced | % of Clones Revertant | Total No. of Base Changes (1) (2) | Base Change Frequency (3) | Base Change Frequency Relative to Wild-Type |
|---|---|---|---|---|---|
| M1 | Mutant 12 | 20 % | 14 | $4.2 \times 10^{-3}$ | 5.25 |
| | Revertant 3 | | 2 | $2.4 \times 10^{-3}$ | 3 |
| M2 | Mutant 12 | 8 % | 8 | $2.4 \times 10^{-3}$ | 3 |
| | Revertant 1 | | 0 | 0 | - |
| M3 | Mutant 12 | 14 % | 10 | $3.0 \times 10^{-3}$ | 3.75 |
| | Pseudo-Revertant 2 | | 0 | 0 | - |
| WT | 9 | | 2 | $0.8 \times 10^{-3}$ | 1 |

(1) Base changes in this column do not include the reversion of the original mutation.

(2) M2 base changes involving two or more linked nucleotides in the same clone are counted as one base change.

(3) Base change frequency is the number of base changes per base sequenced. Of the 324 nt of vLTSV only 280 were sequenced as the primers covered 44 nt.

In summary, suprisingly the results from the Northern hybridization analysis suggested that monomeric (and dimeric) minus vLTSV RNAs were produced, despite the presence in the cDNA inocula of mutations that disrupted *in vitro* self-cleavage of the minus vLTSV-A RNA transcripts. A possible explanation was that the mutated sequences had reverted to the wild-type vLTSV-A sequence. Therefore, it was important to sequence the progeny RNAs, to determine whether the original mutations were present in the progeny vLTSV RNAs.

## 7-15 The majority of the M1, M2 and M3 progeny cDNA clones contained the original mutations

Full-length cDNA clones in M13mp18 were prepared (7-9) from the progeny vLTSV RNAs resulting from inoculation with M1, M2, M3 and wild-type vLTSV-A cDNA. The cDNA clones were sequenced (2-22) and the sequence of all cDNA clones was compared to that of vLTSV-A (Keese *et al.*, 1983). The majority of the 15 M1, 13 M2 and 14 M3 progeny cDNA clones sequenced contained the original introduced mutation, but in each case there were a small number of cDNA clones in which the original mutation had either reverted to the wild-type vLTSV-A sequence, or pseudo-reverted (see Table 7.1). The presence of the original introduced mutations in the majority of the progeny cDNA clones indicates that the vLTSV from the M1, M2 and M3 inoculated plants was not a result of contamination with the wild-type vLTSV-A cDNA inoculum. In addition, the sequences of all progeny cDNA clones, including the nine derived from wild-type vLTSV-A inoculation, were predominantly the vLTSV-A sequence (vLTSV-A and vLTSV-N differ by eight nucleotides (Keese *et al.*, 1983); see Figure 7.6), indicating that the progeny virusoids were derived from the vLTSV-A inoculum, and were not due to trace amounts of vLTSV-N in the apparantly virusoid free LTSV-N.

As the progeny cDNA clones were generated from the progeny plus vLTSV-A RNA, it can be concluded that the majority of the progeny plus RNAs contained the original mutations. It seems likely, therefore, that the minus RNAs (predominantly monomeric and dimeric, as demonstrated by Northern analysis) also contained the mutations. To demonstrate unequivocally, that the minus monomeric RNAs did contain the original

mutations, it would be necessary to generate cDNA clones using primers specific for the minus sequence from purified monomeric RNA; this was not done here.

In summary, it appears that the progeny virusoid RNA extracted from each set of inoculated plants were derived from the cDNA inocula, and were not due to contamination. Therefore, the results suggest that minus RNAs containing the original mutations (which disrupted *in vitro* self-cleavage) were capable of cleavage to monomeric forms *in vivo*. A small proportion of progeny virusoids from the M1, M2 and M3 inoculated plants had reverted, presumably, indicating that the wild-type vLTSV-A sequence has a greater replicative ability than the mutated sequences; this is explored further in the Discussion.

## 7-16 The progeny cDNA clones contained base changes compared to the cDNA inoculum

Sequencing of the progeny cDNA clones revealed many base changes in the progeny M1, M2 and M3 virusoid sequences compared to the sequence of the vLTSV-A cDNA inoculum used. The number of individual base changes was lower in the progeny cDNA clones generated from wild-type vLTSV-A inoculated plants than from M1, M2 or M3 inoculated plants (summarised in Table 7.1). The base changes present in all progeny cDNA clones are detailed in Table 7.2 and their positions in the vLTSV-A sequence are shown in Figure 7.6.

As discussed above, Northern hybridization analysis revealed that the progeny M1, M2 and M3 vLTSV RNAs contained monomeric minus RNAs (Figure 7.5), presumably indicating that multimeric minus RNAs containing the M1, M2 or M3 mutations were capable of cleavage to monomeric forms. As hammerhead self-cleavage was believed to be involved in this process (Forster and Symons, 1987a), the observation that there were base changes in at least 50 % of the M1, M2 and M3 progeny cDNA clones (Tables 7.1, 7.2), suggested that some or all of the base changes may have resulted in the rescue of the *in vivo* self-cleavage activity of the mutated vLTSV RNAs. Therefore, several of the base changes within the conserved and non-conserved residues of the minus hammerhead sequence (Figure 7.8) were examined to determine whether they could rescue *in vitro* self-cleavage activity of the mutated minus RNA transcripts.

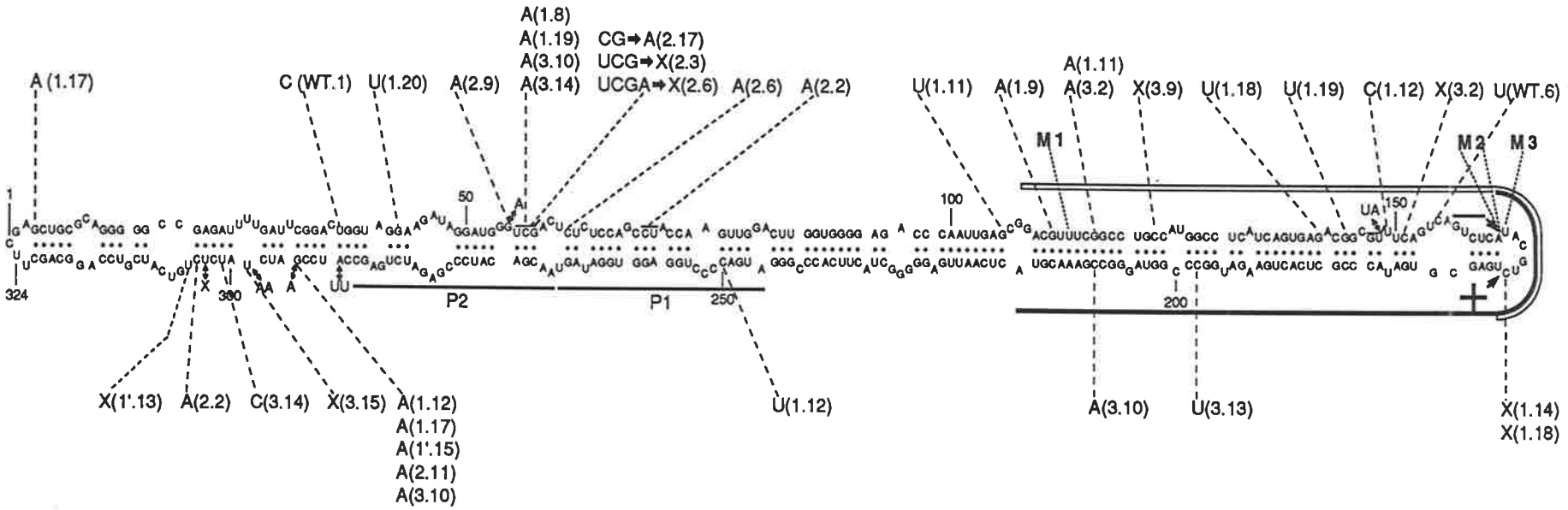



**Figure 7.6** The wild-type vLTSV-A sequence drawn as the proposed rod-like structure (Keese et al., 1983), with the sites of the introduced mutations (M1, M2, M3) and the base changes present in the progeny cDNA clones indicated. The differences between the vLTSV-A and vLTSV-N sequences are indicated by the double-headed arrows. The sites of plus and minus self-cleavage are indicated by the single-headed arrows on the right-hand side of the molecule. Position of hybridization of primers used for cDNA synthesis and PCR are indicated (P1, P2). The sequences comprising the plus and minus hammerhead structures are indicated by the solid and outlined boxes, respectively, on the right-hand side of the molecule. The type of base substitutions are indicated, and deletions are indicated by an X. The clone reference number adjacent to each base change refers to the progeny cDNA clone in which the base change was present (1' indicates an M1 revertant clone). Reversions of the original mutations are not shown.

**Table 7.2** Base changes in progeny cDNA clones

| Clone Reference | Base Changes (1) |
|---|---|
| **M1 NON-REVERTANT CLONES** | |
| M1.8 | C57→A |
| M1.9 | G112→A |
| M1.11 | G106→U, G117→A |
| M1.12 | U149→C, C250→U (2), G295→A |
| M1.14 | C168 deleted (3) |
| M1.17 | G4→A, G295→A |
| M1.18 | G141→U, C168 deleted (3) |
| M1.19 | C57→A, G144→U |
| M1.20 | G42→U |
| **MI REVERTANT CLONES** | |
| M1.6 | U114 inserted (reversion) |
| M1.13 | U114 inserted (reversion), U305 deleted |
| M1.15 | U114 inserted (reversion), G295→A |
| **M2 NON-REVERTANT CLONES** | |
| M2.2 | C71U72→A, C304→A |
| M2.3 | U56C57G58 deleted |
| M2.6 | C62→A, U56C57G58A59 deleted |
| M2.9 | G55→A |
| M2.11 | G295→A |
| M2.17 | C57G58→A |
| **M2 REVERTANT CLONE** | |
| M2.10 | G162/163 deleted (reversion) (4) |
| **M3 NON-REVERTANT CLONES** | |
| M3.2 | G117→A, C151 deleted |
| M3.9 | C123 deleted |
| M3.10 | C57→A, C209→A, G295→A |
| M3.13 | C198→U |
| M3.14 | C57→A, U301→C |
| M3.15 | U299 deleted |
| **M3 PSEUDO-REVERTANT CLONES** | |
| M3.3 | G163 inserted |
| M3.4 | A163 inserted |
| **WILD-TYPE CLONES** | |
| WT.1 | C36→U |
| WT.6 | C155→U |

(1) Base changes compared to the mutated or wild-type vLTSV-A cDNA inoculum.

(2) Base change due to error in the primer (refer to text).

(3) C168 deletion may be due to reverse transcriptase error (refer to text).

(4) M2 mutation was an insertion of a G residue (in the plus sense sequence) between nucleotides 162 and 163.

M13 single-stranded DNA from M13mp18 progeny cDNA clones (M1.9, M1.11, M1.14, M1.18 and M3.2) was used in the generation of cDNA clones in the plasmid vector pGem2 (described in 7-9). Appropriate pGem2 cDNA clones were digested with HindIII and EcoRI and transcribed with SP6 or T7 RNA polymerase to generate minus and plus RNA transcripts, respectively. Transcription patterns are shown in Figure 7.7 (lanes 1-10), alongside those of a wild-type vLTSV-A cDNA clone (Figure 7.7, lanes 13,14). The sites of the base changes in the progeny minus (and plus) hammerhead sequence compared to the wild-type vLTSV-A sequence, are indicated, and the self-cleavage results are summarised in Figure 7.8. The minus RNA transcripts from the five cDNA clones containing base changes in the minus hammerhead sequence (in addition to the original mutations) did not self-cleave during *in vitro* transcription (Figure 7.7). Hence, none of the base changes (Figure 7.8) rescued the *in vitro* self-cleavage activity of the minus RNA. Three base changes in either the stem I and II of the minus hammerhead sequence, present in other cDNA clones (M1.12, M1.19 and M3.9; Figure 7.8), were not tested for their effect on the *in vitro* self-cleavage of the minus RNA.

Base changes within the plus hammerhead structure (present in progeny cDNA clones M1.14, M1.18 and M3.10) were examined for their effect on the *in vitro* self-cleavage of plus vLTSV-A RNA transcripts. Interestingly, the progeny cDNA clones (M1.14, M1.18) both contained the same base change in the plus hammerhead sequence, resulting in the loss of *in vitro* self-cleavage of the plus RNA (Figure 7.7, lanes 5,7, Figure 7.8); this is further considered in the discussion. The base change in progeny cDNA clone M3.10 results in a mismatch in stem II of the plus hammerhead structure (Figure 7.8); similar mismatches also occur in the plus RNA of sBYDV (Miller *et al.*, 1991) and in a sequence variant of the self-cleaving newt RNA (Epstein and Pabon-Pena, 1991). Plus RNA transcripts derived from this clone self-cleaved as efficiently as wild-type vLTSV-A RNA transcripts (Figure 7.7, lane 11).

In summary, base changes from the vLTSV-A sequence were detected in progeny cDNA clones and the frequencies of base changes were higher in the M1, M2 and M3 progeny cDNA clones, than they were in the progeny cDNA clones generated from wild-type vLTSV-A inoculated plants. The presence of those base changes tested did not result in the rescue of the *in vitro* self-cleavage activity of the mutated minus vLTSV-A RNA

**Figure 7.7** Plus and minus vLTSV RNAs generated during transcription from full-length progeny DNA clones in pGem2, containing base changes in either the plus or the minus hammerhead structures. **Lane 1.** Transcription of the M1.9 progeny cDNA clone, linearised with EcoRI, and transcribed with T7 RNA polymerase, to generate the plus RNA transcript. **Lane 2.** As in lane 1, except that the clone was linearised with HindIII, and transcribed with SP6 RNA polymerase, to generate the minus RNA transcript. **Lanes 3 and 4.** As for lanes 1 and 2, except that the cDNA clone was the M1.11 progeny cDNA clone. **Lanes 5 and 6.** As for lanes 1 and 2, except that the cDNA clone was the M1.14 progeny cDNA clone. **Lanes 7 and 8.** As for lanes 1 and 2, except that the cDNA clone was the M1.18 progeny cDNA clone. **Lanes 9 and 10.** As for lanes 1 and 2, except that the cDNA clone was the M3.2 progeny cDNA clone. **Lanes 11 and 12.** As for lanes 1 and 2, except that the cDNA clone was the M3.10 progeny cDNA clone. **Lanes 13 and 14.** As for lanes 1 and 2, except that the cDNA clone was a wild-type vLTSV-A progeny cDNA clone (containing no base changes). **Lane 15.** Single-stranded $^{32}$P-labelled HpaII pUC19 DNA markers (2-27), the sizes of which are given on the right hand side of the figure. The full-length plus RNA transcripts (FL) are 384 nt, and if self-cleavage occurred, a 270 nt 5'-self-cleavage fragment (5'F(+)) and a 114 nt 3'-self-cleavage fragment (3'F(+)) were produced. The full-length minus RNA transcripts (FL) are 383 nt, and if self-cleavage occurred, a 130 nt 5'-self-cleavage fragment (5'F(-)) and a 253 nt 3'-self-cleavage fragment (3'F(-)) were produced. Refer to Figure 7.8 for the base changes in the plus or minus hammerhead structures of each RNA transcript, compared to the wild-type vLTSV-A sequence.

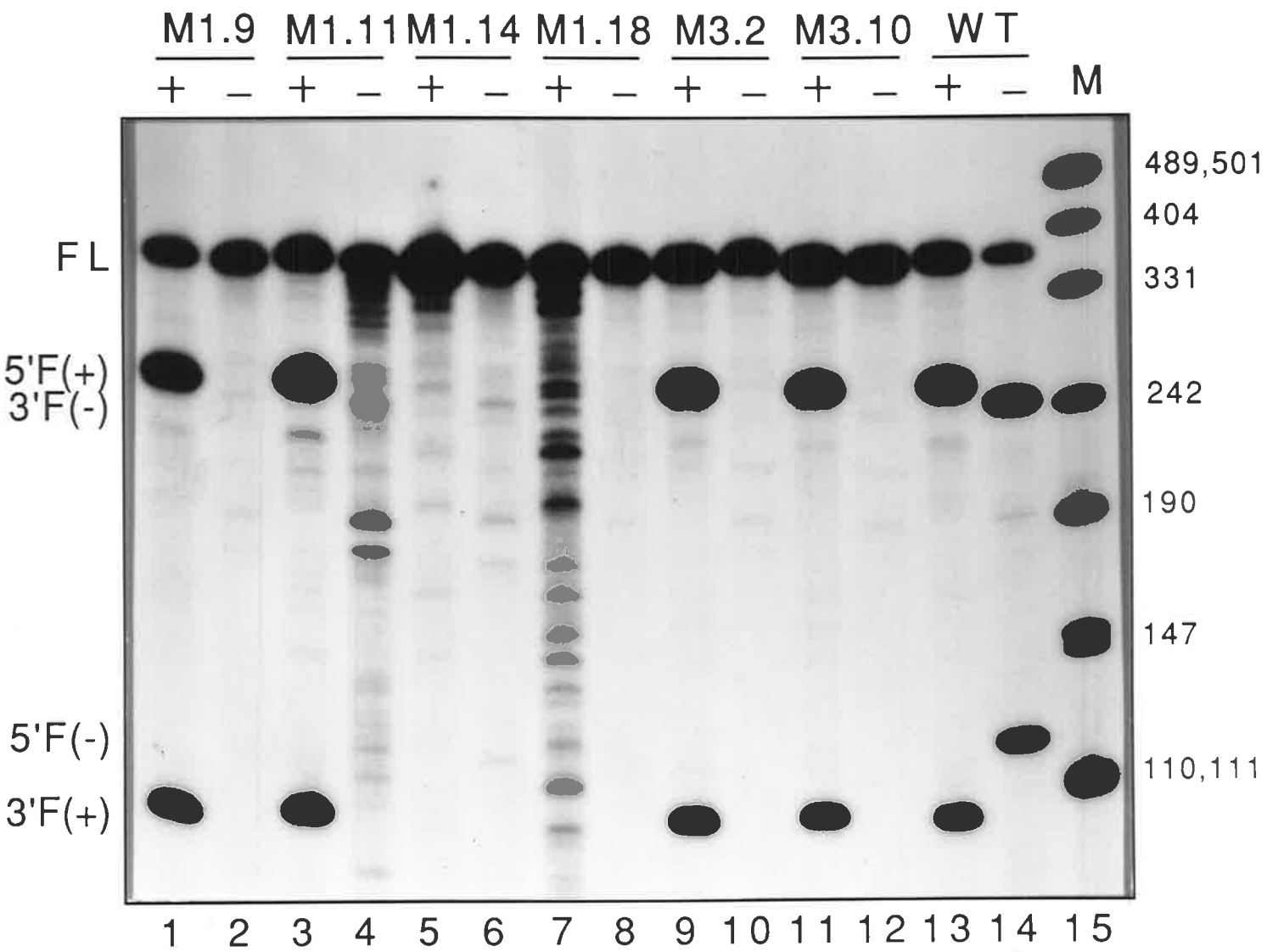
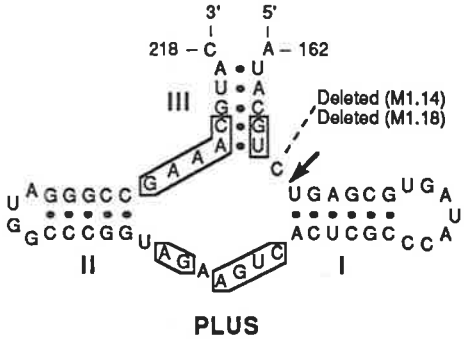
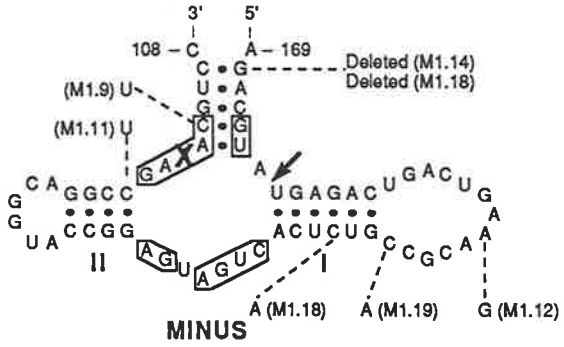

M1.9
M1.11
M1.14
M1.18
M3.2
M3.10
WT
+ − + − + − + − + − + − + − M
FL
5'F(+)
3'F(-)
5'F(-)
3'F(+)
489,501
404
331
242
190
147
110,111
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

**Figure 7.8** Plus and minus hammerhead structures indicating the sites of the base changes present in the progeny cDNA clones generated from plants inoculated with mutated (M1 and M3) or wild-type vLTSV-A cDNA. (Reversions of the original mutations are not indicated.) The *in vitro* self-cleavage ability of the plus and minus RNA transcripts generated from some of the progeny clones are indicated in the tables beneath. Nucleotides conserved between most naturally occurring hammerhead structures are boxed. Arrows indicate the sites of self-cleavage in the wild-type vLTSV-A molecule. Stems are numbered according to the convention of Forster and Symons (1987a), and vLTSV-A numbering is as according to Keese *et al.* (1983). The types of base substitution or deletion are indicated. The clone reference number adjacent to each base change refers to the progeny cDNA clone in which the base change was present. The X in the minus hammerhead structures of M1 and M3 indicates the site of the introduced mutations (Figure 7.1). No base changes were present in the plus or minus hammerhead structures of the M2 progeny clones, nor in the plus hammerhead structure of the progeny cDNA clones generated from wild-type vLTSV-A inoculated plants.

## M1



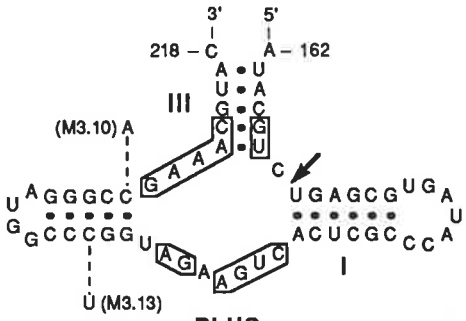

PLUS



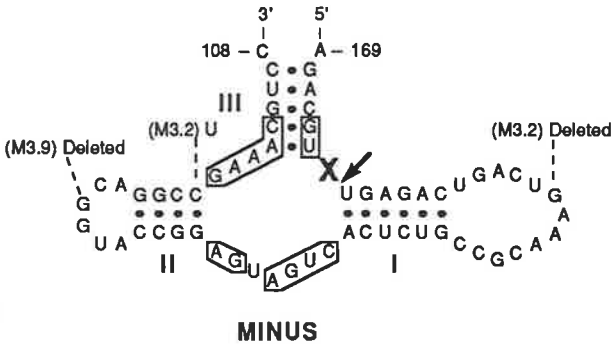

MINUS

| | PLUS | MINUS |
|---|---|---|
| M1.9 | Yes | No |
| M1.11 | Yes | No |
| M1.12 | Not Tested | |
| M1.14 | No | No |
| M1.18 | No | No |
| M1.19 | Not Tested | |

## M3



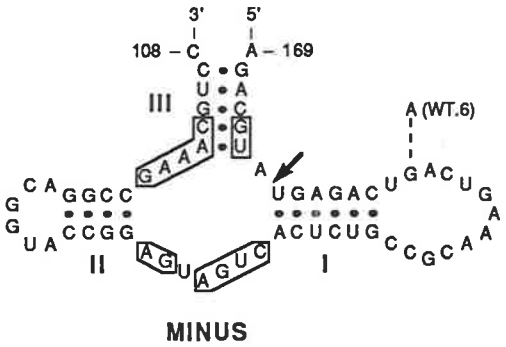

PLUS




MINUS

| | PLUS | MINUS |
|---|---|---|
| M3.2 | Yes | No |
| M3.9 | Not Tested | |
| M3.10 | Yes | No |
| M3.13 | Not Tested | |

## WILD-TYPE




MINUS

| | PLUS | MINUS |
|---|---|---|
| WT.6 | Not Tested | |

***IN VITRO***

**SELF-CLEAVAGE**

**ACTIVITY**

transcripts. It remains possible, however, that the presence of some or all of the base changes resulted in the rescue of the *in vivo* self-cleavage activity, or possibly some other activity, that was disrupted by the presence of the mutations. This, and other implications of the sequencing results are considered further in the discussion.

## Discussion

### 7-17 Monomeric minus RNAs are produced during replication of the mutated virusoids

In this Chapter, the possible involvement of the hammerhead mediated self-cleavage reaction in the formation of monomeric minus vLTSV RNAs *in vivo* was investigated. Plants were infected with mutated vLTSV-A sequences that were deficient in the minus *in vitro* self-cleavage activity, and the resulting infections analysed by Northern hybridization and sequencing of progeny cDNA clones. If the hammerhead mediated self-cleavage reaction is involved in the generation of monomer sized minus RNAs *in vivo*, then no monomer sized minus RNAs would be expected to arise during replication of the mutated virusoids. Rather, multimeric minus RNAs would be expected to accumulate, and would be detected by Northern hybridization analysis.

The mutated vLTSV-A cDNA inocula were infectious when coinoculated with LTSV-N onto *N. clevlandii*, as demonstrated by Northern analysis. As expected, Northern hybridization analysis the plus sense M1, M2, M3 and wild-type progeny RNAs revealed a multimeric series, with RNAs up to eleven times monomeric size (Figure 7.4). Hence, the mutations did not appear to affect the production of the plus RNA *in vivo*. Surprisingly, however, the minus RNA species from M1, M2, M3 and wild-type vLTSV-A inoculations appeared identical on Northern analysis, with both monomer and dimer bands evident (Figure 7.5). The sequencing results indicated that most of the encapsidated virusoid RNAs retained the introduced mutations (Table 7.1). Possible explanations for the appearance of monomeric minus RNAs in plants inoculated with the mutated vLTSV-A sequences are discussed below.

7-17-1 Base changes may have rescued self-cleavage activity *in vivo*

Sequencing of the M13 cDNA clones generated from the M1, M2 and M3 inoculated plants revealed many base differences from the sequence used for inoculation. Five base changes within the minus hammerhead sequence, present in five different progeny cDNA clones (Figure 7.8), did not rescue the *in vitro* self-cleavage activity of the mutated minus RNA transcripts (Figure 7.7). However, it remains possible that these and other base changes rescued the *in vivo* self-cleavage activity, thereby allowing the production of monomeric minus RNAs.

The M1, M2 and M3 progeny virusoid populations contained a proportion of either reversions or pseudo-reversions of the mutations introduced into the minus hammerhead sequence (Table 7.1). This selection for the wild-type vLTSV-A sequence, presumably indicated that the wild-type (or pseudo-wild-type) vLTSV-A sequence had a greater replicative ability than the M1, M2 and M3 sequences. Although not tested, it would be expected that minus RNA transcripts from the revertant and psuedo-revertant progeny cDNA clones would have regained *in vitro* self-cleavage activity. The reason for the selection of wild-type, or pseudo-wild-type sequences *in vivo* may be that the original mutations lowered the efficiency of a virusoid function, presumably the *in vivo* self-cleavage reaction. Reversion to wild-type would regain the full efficiency of this function.

Further infectivity experiments would be of interest to clarify this, for example, mixed infections of wild-type vLTSV-A and mutated (M1, M2 or M3) sequences, to assess their relative replicative abilities. It would also be interesting to infect plants with either M1, M2 or M3 virusoid sequences, and harvest the leaves at different intervals, e.g. after 3 days and after 8 weeks, to see whether there were any difference in sequence composition of the RNAs. If the wild-type vLTSV-A sequence were functionally superior, it might be expected that after 8 weeks, the majority of the population would have reverted to the wild-type or psuedo-wild-type sequence.

Of interest are the two pseudo-revertants of M3. Instead of the insertion of an A into the minus hammerhead sequence to reform the wild-type vLTSV-A sequence, either a C or a U was inserted. Only two other known naturally occurring RNAs, apart from minus vLTSV, have an A at that site: plus sBYDV (Miller *et al.*, 1991), and a sequence variant of minus ASBV (Rakowski and Symons, 1989), all other hammerhead RNAs have a C at this

site. All three isolates of vLTSV (-A, -N, and a Canadian isolate, -Ca) contain an A at this site, presumably, there must be some advantage to the virusoid to have an A, or else it might be expected to contain a C, like other hammerhead sequences. Possibly over time these pseudo-revertants would revert to true wild-type vLTSV-A sequence.

### 7-17-2 Involvement of host factors in *in vivo* self-cleavage ?

None of the mutated minus RNA transcripts generated from the pGem2 cDNA clones (from which the inoculum cDNA was derived) exhibited more than 1 - 2 % self-cleavage *in vitro*, under a range of different conditions (see section 7-11, Figure 7.4, and results not shown). Conditions *in vivo* are different from those tested *in vitro*, and it is possible that the intracellular *milieu* provides an ionic environment that allowed a greater efficiency of self-cleavage than occurred *in vitro*. An intriguing extension of this idea is that host proteins may have assisted in the *in vivo* cleavage reaction of the minus vLTSV RNAs; perhaps host RNA stabilising proteins (e.g., single-stranded binding proteins) overcame the predicted disruption of the *in vivo* "self-cleavage" due to the introduced mutations.

Proteins are known to enhance the *in vitro* activity of some catalytic RNAs. Branch and Robertson (1991) have reported that addition of a nuclear extract from human liver cells accelerated the rate of *in vitro trans* cleavage of HDV RNA (which self-cleaves by the axe-head/pseudoknot structure). Many Group 1 introns, and the M1 RNA of RNase P are capable of mediating RNA cleavage and splicing reactions *in vitro*. However, the rates of these reactions are increased *in vitro* by the addition of the appropriate proteins (Kruger *et al.*, 1982, Guerrier-Takada *et al.*, 1983, McClain *et al.*, 1987, Reich *et al.*, 1988, Gampel *et al.*, 1989). In addition, the range of substrates accepted by RNase P *in vitro*, is greater in the presence of the protein (Guerrier-Takada and Altman, 1984, Guerrier-Takada *et al.*, 1984). Further, at least in a few cases, it has been demonstrated that Group 1 introns that are capable of self-splicing *in vitro*, require proteins for activity *in vivo* (Garriga and Lambowitz, 1984, Akins and Lambowitz, 1987, Gampel *et al.*, 1989).

Therefore, there are precedants for the enhancement of RNA mediated reactions by proteins, both *in vitro* and *in vivo*, and it remains possible that host proteins, or other factors, assisted in the "self-cleavage" activity of the mutated minus RNAs *in vivo*. If this is

the case, then it suggests that the *in vivo* hammerhead "self-cleavage" of wild-type vLTSV RNAs may also be assisted by host factors.

7-17-3 Is a symmetrical rolling circle mechanism involved in vLTSV-A replication ?

The hammerhead self-cleavage reaction was believed to be involved in the processing of multimeric plus and minus RNAs *in vivo*, as part of a symmetrical rolling circle mechanism. The results presented in this Chapter demonstrate that vLTSV-A containing mutations in the minus hammerhead sequence that disrupted *in vitro* self-cleavage of the minus RNA produced monomeric minus vLTSV RNAs *in vivo* (as detected by Northern analysis). One possible mechanism for the production of the monomeric and dimeric minus RNAs that would not be expected to be affected by the presence of the mutations, is that they are produced by the RNA polymerase "falling off" the circular plus RNA when it completes a revolution and encounters the double-stranded RNA formed by the 5'-end of the transcript. If monomeric minus RNAs were produced by this random method, it seems likely that monomeric minus RNAs would not be involved in replication, but rather may be "dead-end" products. The true template for plus strand synthesis may be multimeric minus RNA that was not detected by Northern hybridization of encapsidated virusoid RNA (Figure 7.5), but may have been detected by Northern analysis of total plant RNA extracts (not carried out in this Chapter; see section 7-14).

To demonstrate that mutated minus monomeric vLTSV-A RNAs are templates for the production of the plus RNA, it would be necessary to demonstrate that monomeric circular minus RNAs are part of the vLTSV replication complex. Double-stranded RNA (which may represent the virusoid replication complexes) can be purified from plant extracts using CF-11 chromatography (Branch and Robertson, 1984, Branch *et al.*, 1988). The presence of circular monomeric minus RNAs in this fraction could be detected by differential migration of linear and circular RNAs on polyacrylamide, urea gels, followed by Northern transfer to filters and hybridization with a minus vLTSV specific probe. These experiments would have to be undertaken before it could be concluded that the mutated minus monomeric RNAs are involved in vLTSV replication. By the same token, if high molecular weight minus RNA were detected in this fraction, it would suggest an asymmetrical rolling circle mechanism was involved.

## 7-18 Sequence analysis of progeny vLTSV populations

A total of 51 cDNA clones generated from encapsidated progeny virusoid RNA were sequenced. In addition to a small number of revertants, or pseudo-revertants (Table 7.1), the progeny M1, M2 and M3 cDNA clones contained many base changes (Table 7.2, Figure 7.6). The cDNA clones generated from the wild-type vLTSV-A inoculated plants contained fewer base changes than those from the M1, M2 and M3 inoculated plants (discussed below). The sequence heterogeneity of the virusoids is not surprising, in view of the quasispecies concept of RNA populations (discussed below), but what is intriguing is the difference in the numbers of base changes in the wild-type progeny cDNA clones, and those derived from M1, M2 and M3 infected plants.

### 7-18-1 Quasispecies concept of RNA populations

RNA (e.g., viral, viroid or virusoid) populations generally do not exist as a collection of RNA molecules with identical sequence, but rather as a distribution of sequence variants all related to a consensus sequence, differing from it by one or more base changes (Holland *et al.*, 1982, Domingo *et al.*, 1985). This is a consequence of the high error rates of RNA polymerases, which range from $10^{-3}$ to $10^{-4}$ (Domingo and Holland, 1988). The term quasispecies has been used to describe these heterogeneous populations (Holland *et al.*, 1982, Domingo *et al.*, 1985). A quasispecies equilibrium is established in RNA populations where base changes are introduced into the population (randomly by the RNA polymerase) and eliminated (by selection) at the same rate.

The sequences of vLTSV-A and vLTSV-N were originally determined by direct RNA sequencing, and were confirmed by dideoxy sequencing overlapping cDNA clones (Keese *et al.*, 1983). Direct RNA sequencing produces an average sequence of the whole population, i.e., the consensus sequence. As only one complete cDNA clone was sequenced for vLTSV-A, no variants from this sequence were detected. In the work presented in this Chapter, virions were purified from infected tissue ten days after inoculation. The RNA extracted from the purified virions reflects the RNA that has been packaged into virions up to that time; the cDNA clones that were sequenced represent a sample of this RNA. The base change frequencies observed (Table 7.1) are quite high relative to published mutation rates of RNA viruses, although the frequency of base changes

in progeny cDNA clones generated from wild-type vLTSV-A inoculated plants is within the normal range.

In some host plants LTSV is reported to cause local lesions approximately three days after inoculation (Forster and Jones, 1980), indicating that viral (and virusoid) RNA is packaged into virions within this time. Indeed, it is likely that viral RNA encapsidation begins soon after viral RNA replication begins (Matthews, 1991). The cDNA inoculum would have a limited existence within the cell (plasmid DNA was found to be largely degraded four hours after electroporation into barley protoplasts, Hughes *et al.*, 1977), however, it is possible that a proportion of the packaged plus virusoid RNAs was derived from RNA that was transcribed directly off the cDNA used for inoculation, and had not been through a cycle of replication.

### 7-18-2 The base changes in the progeny cDNA clones may confer a selective advantage

Interestingly, the progeny cDNA clones from M1, M2 and M3 inoculations had more base changes compared to the sequence used for inoculation than from wild-type vLTSV-A inoculations (Table 7.1). As the error rate of the RNA polymerase involved in *in vivo* replication would be expected to be constant, the same number of base changes would be introduced into each population.

If a base change occurs in the population, and has the same selective advantage as the original sequence (a "null" mutation) it would be expected to occur in the population at a level that reflects its rate of appearance. Hence, if the mutation rate was $10^{-3}$, any null base change would be expected to occur in 0.1 % of RNAs (Kurath and Palukaitis, 1990). Due to the small number of clones sequenced from each inoculum, it might be anticipated that any base changes detected (barring artifacts, and chance selection of a rare mutation) conferred a selective advantage. This suggests that M1, M2 or M3 sequences with additional base changes had selective advantages over the predominant sequences (the sequences used for infectivity). Presumably, the same base changes were either selectively disadvantageous, or neutral, to the wild-type vLTSV-A sequence. As discussed previously, the possibility exists that the extra mutations in the M1, M2 and M3 progeny were retained because they rescued

self-cleavage activity (or some other activity) that was disrupted *in vivo* by the original mutations.

It is relevant to note that population genetics of RNA species is poorly understood. Eigen and Biebricher (1988) have cautioned against interpreting results from quasispecies populations simplistically in terms of Darwinian selection. The high frequency of base changes in the M1, M2 and M3 populations may be a consequence of the introduced mutations (M1, M2 or M3) disturbing the distribution of sequences that would normally exist within a wild-type population.

In no case, following infectivity assays of mutated viroids (Ishikawa *et al.*, 1985, Owens *et al.*, 1986, Hammond and Owens, 1987, Owens, 1990, Owens *et al.*, 1991), or satellite RNAs (e.g., van Tol *et al.*, 1991, Sleat and Palukaitis, 1992), were a large number of progeny cDNA clones sequenced, as in this study. Therefore, it is not possible to compare the effect of introduced mutations on sequence heterogeneity in the populations from this study, with any other.

In summary, the accumulation of base changes in the M1, M2 and M3 populations, appears to be an interesting adaptive response to the presence of the introduced mutations. Whether this is due to the rescue *in vivo* of a disrupted function thereby increasing the fitness of the virusoid molecules, or the result of disturbing the quasispecies distribution, or some other reason, cannot be determined.

### 7-18-3 Distribution of base changes

The base changes in the progeny cDNA clones derived from the M1, M2, M3 and wild-type vLTSV-A inoculations appear to be localised mainly into three regions: the minus hammerhead domain, and the regions from residues 36 to 72, and 295 to 305 (Figure 7.6). Interestingly, these regions also contain the nucleotide differences between A and N isolates of vLTSV. The clustering of base changes into these regions may reflect that they are regions of vLTSV which tolerate base changes.

The distribution of the base changes within the vLTSV molecule differed in the M1, M2 and M3 progeny. For example, M1 progeny had many base changes in the minus hammerhead region, whereas M2 progeny had none in the minus hammerhead region; all the base changes in M2 progeny were in the left hand side of the vLTSV molecule. M3

progeny, in addition to containing base changes in the three regions indicated above, also contained base changes in the plus hammerhead sequence. Therefore, the location of the base changes within the progeny virusoid RNAs was dependent on which of the introduced mutations was present in the vLTSV-A inoculum.

Two bases changes (G294→A and C57→A), present in multiple clones, were similar in identity and location to single nucleotide changes between vLTSV-A and vLTSV-N isolates. The high frequency of occurrance of these base changes suggests that they confer a selective advantage. Possibly, the base changes allowed the virusoid molecules to replicate more efficiently within the N isolate of LTSV, or within *Nicotiana clevlandii*. (vLTSV-A that was sequenced originally was isolated from LTSV-A grown in *Chenopodium quinoa*, whereas vLTSV-N was isolated from LTSV-N grown in *N. clevlandii* (Keese *et al.*, 1983).) Examples of host plant selection for particular satellite RNA variants have been reported, e.g., by Kurath and Palukaitis (1990) and Moriones *et al.* (1991).

A Canadian vLTSV isolate (vLTSV-Ca) shares 80 % sequence similarity with LTSV-A and LTSV-N (Abouhaidar and Paliwal, 1988); the base differences between vLTSV-Ca and vLTSV-A are not localised into the same regions as the base changes described in this Chapter.

### 7-18-4 Three deletions in M2 progeny appear to be caused by deletion of the 5'-overhang of the cDNA inoculum

The inoculum used in these experiments, was double-stranded monomeric vLTSV-A cDNA excised with SalI from the pGem2 clones (Figure 7.2). This double-stranded cDNA had 5' overhangs of four nucleotides. Interestingly, three of the M2 mutations contained deletions of the corresponding bases in the virusoid RNA; M2.17 had a deletion of one base (as well as a base substitution in this region), M2.3 had a deletion of three bases, and M2.6 had the deletion of all four bases. Presumably, the single-stranded overhangs of the infecting cDNA were removed before the cDNA was transcribed *in vivo*. It is unclear why only M2 progeny RNAs contained these deletions, possibly the M2 cDNA was contaminated with a trace amount of exonuclease prior to inoculation.

7-18-5 Some base changes may be artifacts

It is possible that some base changes present in the progeny cDNA clones were caused by errors introduced by either the reverse transcriptase or Vent DNA polymerase during the generation of the cDNA clones. An upper limit for the frequency of these types of errors is the frequency of base changes in the progeny cDNA clones generated from wild-type vLTSV-A inoculated plants. Clearly, the majority of the base changes in the M1, M2 and M3 progeny cDNA clones were generated *in vivo*.

M1.14 and M1.18 contain deletions of the base 5' to the site of plus RNA self-cleavage (C168), this base change abolished *in vitro* self-cleavage of the plus RNA (Figure 7.7, lanes 5,7, Figure 7.8). This is the same base that was apparently deleted in certain cDNA clones of sBYDV (Miller *et al.*, 1991). The authors suggested that it may have been caused by the presence of a 2'-phosphate on the base at the self-cleavage site in the encapsidated RNA, as was reported by Kiberstis *et al.* (1985). The presence of this moiety may have caused the reverse transcriptase enzyme to skip over this base, and hence register as a deletion. Another possible explanation is that the cDNA was transcribed off a multimeric RNA that actually contained this deletion. Mutation of critical bases that abolish *in vivo* cleavage of multimeric RNAs may be one way in which the multimeric series is produced.

The substitution of C255 for a U residue in the region covered by the primers is also likely to be an artifact. This corresponds to a G to A conversion in primer 1: modification of guanosine to 2,6-diaminopurine (which mimics adenosine by having the capacity to base-pair with thymidine) is reported to be the most common modification during the automated synthesis of oligodeoxynucleotides (Eady and Davidson, 1987, and in Applied Biosystems Nucl. Acid Res. News No. 7, 1988).

## 7-19 Summary

In this work, three single base mutations were separately introduced into a vLTSV-A cDNA clone, resulting in the disruption of the *in vitro* self-cleavage of their minus RNA transcripts. When coinoculated onto *N. clevlandii* with LTSV-N these mutated vLTSV-A sequences appeared to replicate in a manner very similar to the wild-type vLTSV-A sequence. Monomeric minus RNAs could be detected by Northern analysis from plants

inoculated separately with the M1, M2 or M3 sequences. Hence the presence of the mutations did not appear to affect the production of the minus monomeric RNAs.

The presence of the reversions and psuedo-reversions appears to indicate that there was selection for the wild-type hammerhead sequence. This presumably indicates that molecules with the wild-type hammerhead sequence were able to replicate more effectively than the mutated sequences, suggesting that, rather than abolishing self-cleavage *in vivo*, the mutations reduced its efficiency. Two of the mutations resulted in a very low level of self-cleavage (less than 2 %) when the purified RNA transcripts were incubated *in vitro*. As discussed, it is possible that the intracellular environment (including host proteins) resulted in increased self-cleavage efficiency *in vivo* compared to that which occurred *in vitro*.

Sequencing of the progeny virusoid cDNA clones derived from the M1, M2, M3 and wild-type inoculated plants revealed that base changes were present at higher levels in the mutated progeny than those from wild-type vLTSV-A inoculations. This appears to be an interesting adaptive response on the part of the mutated vLTSV-A sequences.

## APPENDIX

## PUBLICATIONS

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