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Rescue of the RNA phage genome from RNase III cleavage

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ABSTRACT

The secondary structure of the RNA from the single-stranded RNA bacteriophages, like MS2 and Q_β, has evolved to serve a variety of functions such as controlling gene expression, exposing binding sites for the replicase and capsid proteins, allowing strand separation and so forth. On the other hand, all of these foldings have to perform in bacterial cells in which various RNA splitting enzymes are present. We therefore examined whether phage RNA structure is under selective pressure by host RNases. Here we show this to be true for RNase III. A fully double-stranded hairpin of 17 bp, which is an RNase III target, was inserted into a non-coding region of the MS2 RNA genome. In an RNase III$^-$ host these phages survived but in wild-type bacteria they did not. Here the stem underwent Darwinian evolution to a structure that was no longer a substrate for RNase III. This was achieved in three different ways: (i) the perfect stem was maintained but shortened by removing all or most of the insert; (ii) the stem acquired suppressor mutations that replaced Watson–Crick base pairs by mismatches; (iii) the stem acquired small deletions or insertions that created bulges. These insertions consist of short stretches of non-templated A or U residues. Their origin is ascribed to polyadenylation at the site of the RNase III cut (in the + or – strand) either by *Escherichia coli* poly(A) polymerase or by idling MS2 replicase.

INTRODUCTION

The single-stranded (ss)RNA phages, like MS2, are plus strand viruses containing a genome of ~3500 nt (Fig. 1). Apart from encoding the four proteins necessary for phage multiplication and spreading, the sequence also prescribes a certain RNA secondary structure that substantially contributes to the reproductive success of the molecule. For example, genotypically neutral mutations that affect critical aspects of RNA folding can lead to a dramatic loss in phage titer (1,2). Here we ask whether phage RNA structures, in general, represent a compromise since folding may also be subject to selection pressure exerted by host RNases. We showed, for instance, that large single-stranded regions are selected against, probably because they are sensitive to RNases (3). In this paper we focus on the question whether RNA phage structure is adapted to RNase III and, if so, how RNase III targets in the genome are inactivated.

RNase III has been found in eubacteria and eukarya (4). In bacteria the enzyme is encoded by the *rnc* locus and is involved in processing of rRNA from the precursor 30S transcript (5), but it also regulates expression of several operons, including its own (6–8). RNase III cleavage sites have been determined for mRNA species of the DNA phages T3, T7 and λ (9) but RNA from the ssRNA phages was reported as resistant to cleavage (10).

Though it is not precisely defined what the exclusive features of an RNase III site are, it has been well documented that fully double-stranded (ds)RNA structures of sufficient length are cut on one or both sides of the stem (11,12). A 3′ overhang of 2 nt results when scission occurs on both sides. Here we introduce such long dsRNA structures in an intercistronic region of the MS2 genome. These mutant phages grow normally in an *rnc*$^-$ host, but not in the wild-type bacterium. There the RNA falls prey to RNase III, but is rescued by the replication machinery as an RNase III-resistant structure.

MATERIALS AND METHODS

Bacterial strains

Plasmids containing the full-length MS2 cDNA and derivatives thereof were grown in *Escherichia coli* F$^-$ strain M5219 at 28°C as previously described (3). Phage were amplified in *E.coli* F$^+$ strain KA797 or RCL1 at 37°C. RCL1, which is an *rnc105* derivative of KA797, was obtained by transduction with a P1 lysate generously provided by P.Regnier. Transductants were screened for tetracycline resistance and assayed by SDS–PAGE for overproduction of PNPase (90 kDa), which is regulated by RNase III (13). The *rnc105* mutant has a Gly→Asp substitution that inactivates the protein.

Plasmids

Plasmids pMS.A, pMS.C, pMS.D, pMS.R and pMS.S are derivatives of the infectious cDNA clone pMS2000 (14), which contain a 34 bp insert at the *Xba*I site (position 1303 of the MS2 genome). The construction of pMS.A, pMS.C and pMS.D has been described before (3). Inserts R and S are made in the same way. pMS.F and pMS.H contain oligonucleotides C (5′-CTAGATAGAACT**GCTAGC**TAACTAGCTGTTCTTT)

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followed by D (5′-CTAGAAAGAACAGCTAGTTA**GCTAG-C**AGTTCTAT) in the coding strand, while in pMS.H the order is reversed (DC). Phage generated by cells harboring pMS.R, pMS.S, pMS.F or pMS.H are denoted R.n, S.n, F.n or H.n respectively, where n = 0 specifies the starting mutants. Their evolutionary offspring are indicated by R.1, R.2, etc. This numbering is arbitrary and does not necessarily imply an evolutionary route. The single letter code is sometimes used for simplicity to indicate the starting mutant.

Phage evolution

Phage obtained after overnight growth of M5219 cells containing a mutant MS2 cDNA clone (cycle 1) were plated on lawns of KA797 cells (cycle 2) as described (2). Subsequently, plaques were amplified overnight at 37°C in liquid cultures of KA797 in 2.5 ml LC broth. This is defined as cycle 3. Thereafter, phages were either further passaged or purified by PEG precipitation as described before (2).

Phages from *rnc*$^{-}$ cells were obtained as follows. Infectious plasmids (~50 ng) were directly transformed into RCL1 cells (F^{+}) by means of electroporation (12.5 kV/cm). After incubating the transformed cells for 1 h at 37°C, 100 µl fresh RCL1 cells and 2 ml LC containing 50 µg/ml tetracycline were added and the total mixture kept with shaking overnight at 37°C (cycle 1). Appropriate dilutions were then plated either on lawns of RCL1 or KA797 cells (cycle 2). Individual plaques were picked and amplified in liquid cultures of RCL1 or KA797 cells (cycle 3).

RNA isolation, RT-PCR and sequence analysis

Phage RNA was purified by phenol/chloroform extraction and precipitation with ethanol. The pellet was dissolved in H_2O and this solution was used for reverse transcription in a total volume of 20 µl using primer DUI59 as described before (2). The resulting cDNA was amplified by PCR according to standard procedures as recommended by the supplier (Eurogentec), using primers DUI59 (complementary to nt 1409–1422) and DUI715 (homologous to nt 1182–1205). Samples were analyzed on 2% agarose gels. PCR fragments were sequenced with DUI59 after separation of the strands with Dyna beads (Dynal). Revertants from pMS.F and pMS.H were sequenced at the RNA level.

Determination of RNase III cleavage sites

A sample of 1 µg phage RNA was briefly pretreated at 70°C to equilibrate the structure. Subsequently the sample was incubated with 0.5 U RNase III for 10 min at the indicated temperature in buffer containing 10 mM Tris–HCl, pH 7.5, 100 mM KCl, 10 mM $MgCl_2$, 1 mM β-mercaptoethanol and 5% glycerol. After extracting the RNA with phenol, extension of primer DUI59 was used to determine the cleavage sites. (RNase III was prepared in the laboratory of D.Court and made available to us by P.Regnier.)

RESULTS

The system

Some time ago we prepared an infectious MS2 cDNA clone. Bacteria harboring this sequence in a plasmid produce overnight ~10^{11} infectious phage/ml culture. These phage are released into the medium. To control the problem of reinfection we generally use bacteria that lack the fertility (F) factor and can therefore not be attacked by these phage from without.

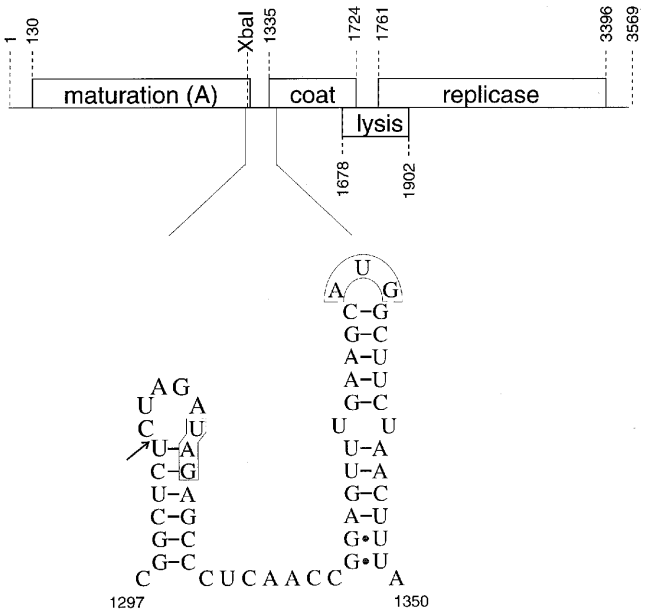

Figure 1. Genetic map of RNA phage MS2 and secondary structure in the maturation–coat intercistronic region. The *Xba*I sequence used to insert various structure elements is indicated by an arrow. Stop and start codons are boxed. The structure is supported by comparative analysis and probing (35).

Synonymous codon substitutions in the MS2 cDNA that affect important RNA structure elements lead to a decrease in phage yield by several orders of magnitude, but, usually, passaging of such mutants rapidly results in selection of second site suppressor mutations that restore the structure element and raise the titer to a level almost indistinguishable from wild-type (1,2,15–17).

Background on the insertion site

Viruses are known for their economic use of genetic material, as witnessed by the presence of overlapping genes in their genomes. MS2 is no exception and, as can be seen in the genetic map of Figure 1, there are only three small non-coding regions. Position 1303, close to the maturation–coat spacing, was found to tolerate small insertions. The local structure of the wild-type RNA is shown in Figure 1 and the *Xba*I site just ahead of the maturation stop codon can be conveniently used to insert extra material (3). Actually, some of these inserts extend the C-terminus of the maturation protein somewhat, but there was never an indication that this affected phage growth.

Two of the inserts, whose fate was tested previously, are depicted in Figure 2 as A and C. It was then shown that type A inserts containing a large unstructured loop could not be stably maintained in the phage genome; deletions in the loop took place that reduced its size to between 4 and 7 nt. In contrast, equally sized structured insertions of type C were stable for many cycles (3).

Selection pressure of RNase III

At the time insert C was designed with two mismatches because we had never seen long uninterrupted stems in phage RNA (18–20). We reasoned that the absence of perfect stems might be

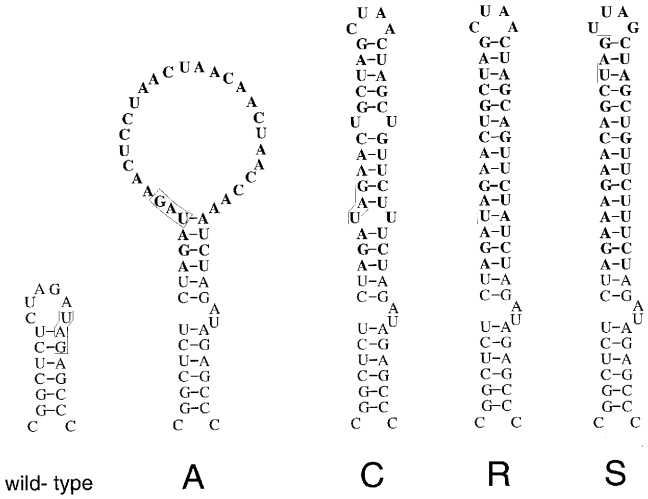

Figure 2. Four different structure elements inserted in MS2 cDNA at the *Xba*I site indicated in Figure 1. The stop codon of the maturation gene is boxed. When the insert is present in infectious MS2 cDNA the plasmid is called pMS.A, pMS.C, etc. Inserted nucleotides are in bold.

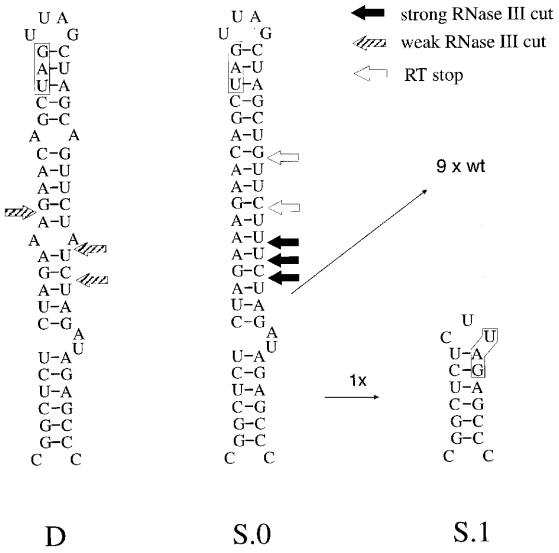

Figure 3. Probable structure of inserts S and D and their sensitivity to RNase III. Structure-induced stops of reverse transcriptase are shown as open arrows. On the right we indicate the progeny phages of insert S after cycle 3. Wild-type is found nine times and S.1 once.

related to the activity of RNase III in *E.coli*. To test this idea we prepared inserts R and S in which the two mismatches have been replaced by Watson–Crick pairs. R and S differ in the loop sequence and 2 bp. F⁻ cells were then transformed with plasmids carrying the inserts in MS2 cDNA.

Replacement of the mismatches by base pairs has dramatic effects; the titer of the respective plasmids pMS.R and pMS.S drops 1000 times as compared with pMS.C. Furthermore, whereas insert C is stably integrated into the MS2 RNA genome, insertions R and S are not. Ten plaques derived from the MS2 cDNA construct with insert S were sequenced. Nine had suffered a deletion that yielded wild-type and one turned out to be one triplet shorter than wild-type (S.1 in Fig. 3). (Mutant D will be discussed later.)

The fate of insert R was studied more extensively. We analyzed 50 plaques by RT-PCR. Of these, 41 produced the wild-type size PCR fragment. For 13 samples identity with wild-type was then confirmed by sequence analysis. The nine plaques that maintained a larger than wild-type size RT-PCR fragment were also sequenced. All of these had suffered insertions or a combination of insertions and deletions that destroyed the regularity of the stem and thus its potential to be an RNase III target. The likely structures of these nine revertants are shown in Figure 4 as R.1–R.9. The inserted nucleotides are shown in black boxes and the deletions are marked with a Δ.

To identify RNase III as the cause of the instability of inserts R or S we constructed an *E.coli* strain that was *rnc*⁻. This strain was transformed with pMS.R and pMS.S and the resulting phage were examined by RT-PCR and sequence analysis. The complete inserts R and S were still present in the 10 plaques analyzed, strongly arguing that their removal or modification in a wild-type *rnc* strain was due to negative selection pressure by RNase III. (There was no difference in titer between wild-type and R.0 and S.0 phage when grown in *rnc*⁻ hosts.)

RNase III sensitivity of the dsRNA inserts

More direct evidence for RNase III pressure was obtained by *in vitro* experiments. Phage S.0, carrying the full insert S, was obtained from *rnc*⁻ cells and the RNA isolated and probed with RNase III. Cuts were detected by inhibition of primer extension. The results are shown in Figure 5. In the absence of RNase III at 37°C two strong stops can be seen at positions indicated with open arrows (Figs 3 and 5). As these stops do not occur when the long stem is disrupted (see below), we interpret these bands as stops induced by the high stability of the stem, preventing progression of reverse transcriptase. In the presence of RNase III at 37°C we see three bands at further downstream positions marking the enzyme cleavage sites, as shown by solid arrows in Figures 3 and 5.

The enzyme may also cut on the 5′-side of the helix, but this could not be detected. Perhaps the cuts on the 3′-side reached completion. We tried to use less enzyme and a lower temperature (0°C). However, in this case no cuts at all are observed and we only detect the structure stops (Fig. 5, lane 6).

Revertants from insert R are RNase III resistant

Above we showed that perfectly base paired inserts are indeed cleaved by RNase III. This then leads to the question whether the revertants have acquired RNase III resistance. To test this we isolated RNA from revertants R.8 and R.9 and from the parental phage R.0. This last RNA was, as before, obtained from *rnc*⁻ cells. All three RNAs were probed with RNase III and the results are shown in Figure 6. The sensitivity pattern obtained from parental R.0 is virtually identical to that of S.0 (Fig. 5). This is no surprise, since the difference between R.0 and S.0 is no more than two A-U→U-A base pair changes and an inverted loop sequence.

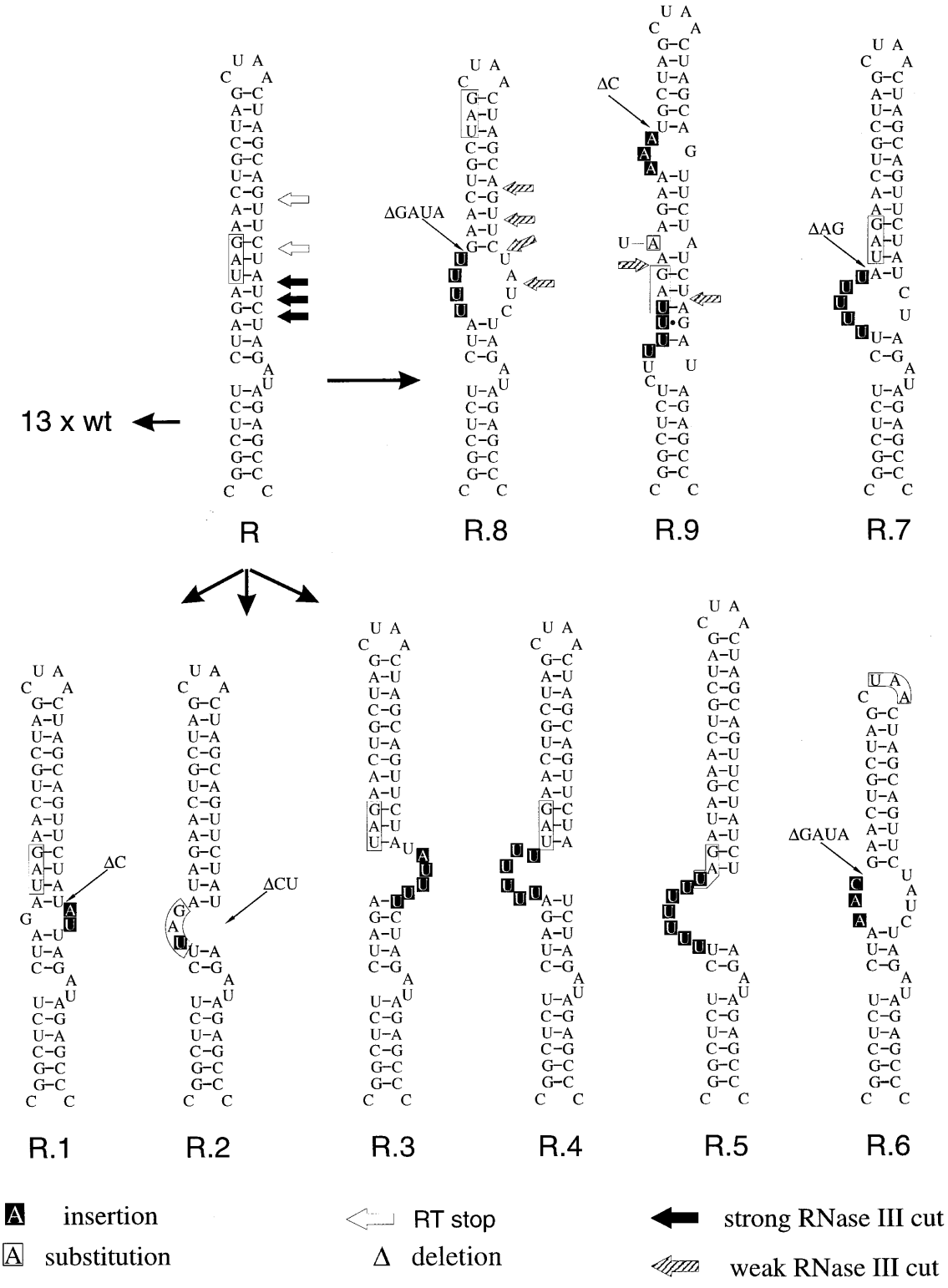

Figure 4. Phage generated by MS2 cDNA carrying insert R after cycle 3. The original insert is indicated as R. Numbers R.1, R.2, etc. are arbitrary. Untemplated residues are in black, substitutions in white boxes. Deletions are indicated. Open arrows are structure-induced stops of the reverse transcriptase. The in-frame stop codon is boxed.

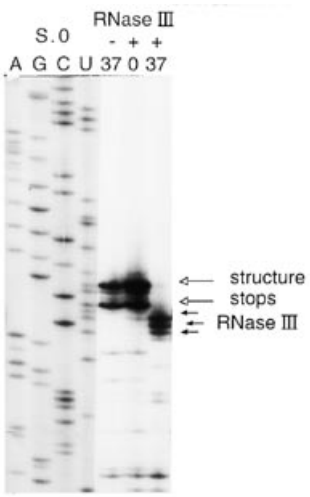

Figure 5. RNase III cleavage sites in mutant S.0 as revealed by primer extension inhibition. The sequence lanes are obtained from the corresponding PCR fragment. In lane 6 one tenth of the amount of enzyme was used at 0°C. The right three lanes are of a shorter exposure to better visualize the individual bands.

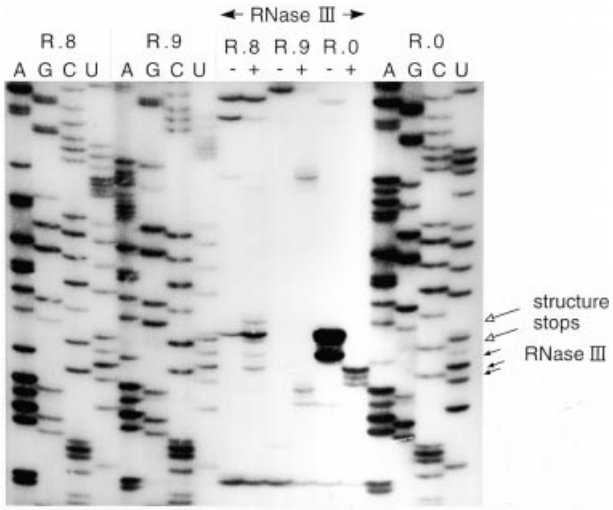

Figure 6. RNase III cleavage sites in phage mutant R.0 and its revertants R.8 and R.9. Sequence lanes of the original mutant and the two revertants are displayed. Only the stronger RNase III cuts in R.8 and R.9 have been indicated.

In contrast, in revertants R.8 and R.9 we notice that RNase III cuts are absent or strongly reduced as compared with R.0 (Fig. 6). This can be understood as the stem has now evolved bulges or internal loops, features that, in general, confer RNase III resistance in stem structures (see for instance 21).

We also notice that the reverse transcriptase stops present in R.0 and S.0 have diminished or disappeared in the revertant structures. This makes sense, since the stems in the revertants have been considerably weakened.

Revertants from phage R.0

We showed above that inserts R and S are stably maintained in the phage genome in *rnc*⁻ cells. This fact allows us to study the evolution of the insert when integrated in the phage RNA genome. In the previous experiments the insert was only encoded in the MS2 cDNA.

Accordingly, *rnc*⁺ cells were infected with mutant phage R.0. As expected, the titer on *rnc*⁺ is three to four orders of magnitude down as compared with *rnc*⁻. Ten plaques were picked, amplified and sequenced. The revertants fall into three classes. In one class, represented by R.10–R.13, mismatches in the stem are created, presumably leading to decreased RNase III sensitivity (Fig. 7). These revertants look very much like phage carrying insert C or D (Figs 2 and 3), which were found to be stable in *rnc*⁺ cells while showing only weak RNase III cuts (indicated in Fig. 3).

The second kind of revertant is characterized by small deletions that result in a bulge in the stem (R.14 and R.15). For R.14 the deletion might be facilitated by the UAGA repeat, but for R.15 this sort of explanation does not hold.

The third class has suffered a large deletion leading to a reduction in hairpin size (R16 and wild-type). Formation of wild-type structure may be favored by a 10 nt repeat around the *Xba*I sites.

Thus, in addition to similarities we also find two major differences between revertants obtained from mutant MS2 cDNA and mutant MS2 RNA. Revertants from MS2 RNA tend to develop mismatches, while revertants from MS2 cDNA mostly acquire inserts. A possible reason for these differences will be given in the Discussion.

Tolerance to longer inserts in *rnc*⁻ cells

We wondered how much more RNA could be stowed away in the genome and in the virion. Accordingly, double sized fragments containing uninterrupted stems of 34 rather than 17 bp were inserted in the *Xba*I site. The hairpins predicted to result from the inserts are shown in Figure 8 as F and H. Surprisingly, both hairpins remained stably incorporated in infectious MS2 phage for several cycles when propagated in *rnc*⁻ strains. However, when *rnc*⁺ cells were transformed with the plasmid the titer dropped 1000-fold. A small number of plaques was analyzed. The revertants had lost the top half or more of the hairpin, as indicated in Figure 8 by the dotted lines. One phage (H3) carried an extra A, not present in the insert.

The fact that 34 uninterrupted base pairs can be added to a pre-existing stem reveals an unexpected flexibility. After all, 34 bp in an RNA-A helix represent a length of ~90 Å, which is close to half the diameter of the virion.

DISCUSSION

The genome of the ssRNA phages assumes a specific secondary structure that maximizes the reproductive success of the molecule. It does so by optimizing strand separation (22,23), gene expression (24–26), replication (27,28) and probably many other processes. Here we have tried to understand to what extent these RNA structures are restrained by the fact that they must function in an environment where RNases operate.

Of all the nucleases characterized in *E.coli*, RNase III seems best suited to study its impact on shaping phage RNA structure. Defective mutants are available and *rnc*⁻ cells grow normally. In

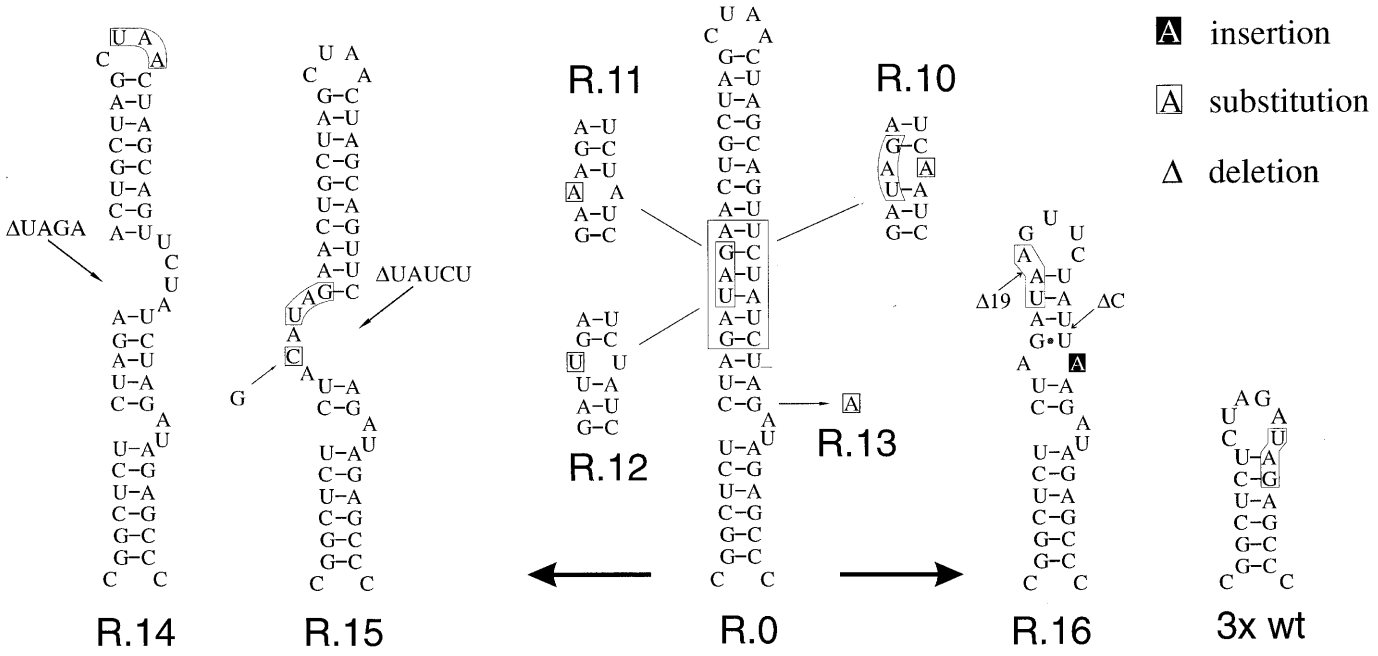

Figure 7. Revertants from phage R.0 upon passaging in *rnc*$^+$ hosts after cycle 3. Some revertants remained stable up to cycle 8, while others reverted back to wild-type.

addition, an RNase III substrate structure can be designed. Accordingly, various synthetic DNA fragments encoding a fully dsRNA hairpin were inserted in MS2 cDNA. *In vitro* experiments showed these structures to be RNase III targets.

Introducing the information for a 17 bp perfect stem in the infectious MS2 cDNA clone produced phage in possession of the full insert in an *rnc*$^-$ host. However, when the same plasmid was used to generate phage in an *rnc*$^+$ host the insert could no longer be found in the RNA genome. Nevertheless, if the same insert contained two mismatches it was stably maintained. These experiments show that RNase III exerts selection pressure on the structure of phage RNA.

Three ways to evolve RNase III resistance

Although the fully double-stranded hairpin cannot be maintained in *rnc*$^+$ cells, this does not mean that phage carrying this feature are doomed to die in this host. Revertants arise that have acquired resistance to the enzyme. This can occur in three different ways.

One major route is to reduce the length of the stem. Many times, but not always (see for instance S.1, F.2 and R.16), this leads to the wild-type. Another possibility is creation of bulges in the stem. This can be achieved either by small deletions or by insertions. The increased RNase III resistance of these structures was experimentally shown for R.8 and R.9 (Fig. 6).

Finally, escape from RNase III cleavage is attained by the creation of mismatches in the stem. This solution is illustrated by R.10–R.13. These revertants look very much like phage with insert C or D, which were found to be stable (3) and showed only weak RNase III sensitivity (Fig. 3).

Not all of our revertants belong to one class exclusively. Sometimes there is a mixture of the different resistance pathways. For instance, R.9 and R.16 have acquired a mismatch in addition to insertions and deletions respectively. Similarly, R.15 seems to have started with a C.C mismatch, created by a G→C substitution. Possibly, this conferred insufficient protection and the 5 nt deletion UAUCU appeared as a further improvement.

Origin of suppressor mutations

The conversion of an RNase III-sensitive to an RNase III-resistant structure is the apparent result of an evolutionary process that selects for the presence of imperfections in the long regular stem caused by deletions, insertions or substitutions.

The insertions are non-templated and consist of small rows of Us or As. It is known that Q_β replicase has a tendency to add A residues to the growing chain when the template strand is lost or otherwise absent (29). Thus, revertant R.3 (Fig. 4) could originate as follows. The MS2 replicase copying the plus strand will face a template cut by RNase III at one of the positions found by us. The idling replicase adds a few As before it manages to engage with the template again across the cut. In the plus strand the extra As now turn up as an oligo(U) bulge at the site of the RNase III cut. Probably, these events can also happen during copying of the cleaved minus strand, resulting in addition of As in the plus strand, as seen for R.6 and R.9. Similarly, insertions of Us on the 5′-side of the stem (e.g. R.5) could result from RNase III cleavage on the 5′-side of the plus strand.

Another interesting possibility to account for the insertions is to consider the action of host enzymes on RNase III-cleaved plus or minus strand phage RNA. It has been shown that *E.coli* poly(A) polymerase (PAP) can add A residues to 3′-ends created by RNase III or to the exonucleolytically resected products (30–32).

These two explanations, though tentative, are consistent with the fact that the insertions cluster at the measured or predicted RNase III cutting sites in the plus and minus strands. How the replicase crosses the cleavage site is enigmatic. Perhaps base

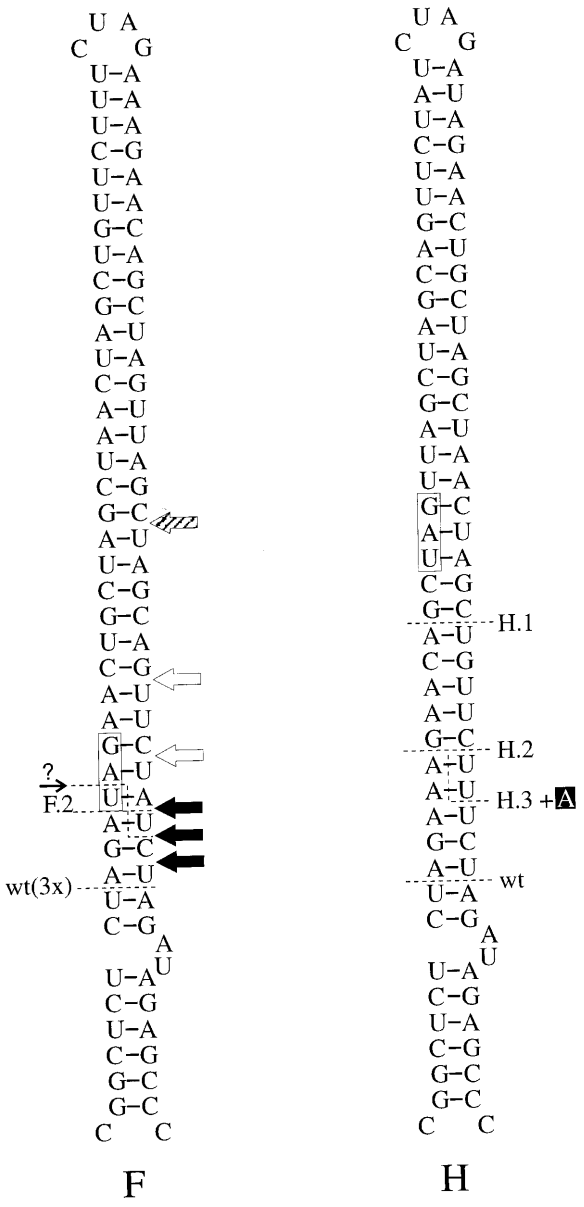

Figure 8. Phage generated by MS2 cDNA carrying inserts F or H after cycle 3. The dotted lines mark the part of the helix that was found removed in the various phage. Note that the path of the dotted line is not unambiguous for wild-type and for F.2. The thin arrow with a question mark above F.2 indicates a potential RNase III cut site. In combination with a 2 base staggered cut at the other site this could precisely lead to revertant F.2 by end-to-end ligation. Revertant H.3 carries four rather than the three encoded consecutive As.

pairing of the lower part of the stem keeps the two halves together, favoring a restart across the cleavage site (see below).

In many of the revertants the U/A insertions are accompanied by small deletions, the origin of which we do not know, but one could suggest that they result from imprecise replicase restarts across the site of the cut. Alternatively, these deletions are the result of 3′ exonucleolytic degradation by RNase II or PNPase at the site of cleavage (31).

Except for R.2, these scars on the RNA are found on only one side of the stem at a time, suggesting either that templates are cut on only one side by RNase III or that only those templates that are cut once can be rescued. We think the latter possibility is the more likely one, as non-templated Us are found on either side of the stem.

The large majority of our revertants are wild-type, arising by complete deletion of the insert. This event is probably the result of RNA recombination favored by the *Xba*I repeat sequence, but could, when MS2 cDNA is the infectious agent, also take place at the DNA level. On the other hand, deletions such as those occurring in R.15 and R.16 cannot easily be explained by any feature of the sequence and they may reflect the action of exonucleases on the cleaved RNA.

Finally, suppressor mutations are obtained by simple replication errors that create mismatches (R.10, R.11 and R.12). Here it is at first glance surprising that the mutations consist of the less likely transversions. We think transitions are not found because they can only lead to A.C mismatches or U.G pairs. U.G pairs probably do not sufficiently distort the helix to prevent RNase III cleavage. The A.C mismatches likewise will turn into U.G pairs in the minus strand.

Recently Chetverin *et al.* (33) reported evidence for RNA recombination by spontaneous covalent linkage between two base paired RNA half molecules. In their model the 3′-OH group of the 5′ half molecule attacks a phosphodiester bond in the 3′ half molecule, releasing the nucleotides 5′ of the splice site. In our system the two parts of the MS2 RNA genome are also held together by base pairs, which should favor the transesterification reaction. Furthermore, RNase III, as well as potential subsequent exonuclease, cleavage produces the required 3′-OH group. Many of our revertants could have been created by such a mechanism or by enzymatic joining of the half molecules (34). In particular, F.2 can be thought of as having arisen by end-to-end ligation after staggered RNase III cleavage (Fig. 8).

The infectious agent determines the revertant distribution

In the present study we have used two types of infectious agents. The insert, encoding the 17 bp perfect stem, was present either in infectious MS2 cDNA or in the intact phage RNA genome. The revertants obtained from either agent have in common that they all have developed RNase III resistance in *rnc*$^+$ cells, but they differ in the sort of solution reached. This, we believe, must reflect their different histories in the cell. One obvious dissimilarity is that the pathway leading from MS2 cDNA to live phage necessarily involves transcription of the DNA by *E.coli* RNA polymerase. It would seem that this stage in phage production is highly sensitive to RNase III. This we deduce from the finding that infection with MS2 cDNA with insert R does not lead to a solution with mismatches. Apparently, in these cells there is simply no intact MS2 RNA transcript present in which to introduce the mismatches. Instead, all of the revertants, except wild-type, bear the marks of RNase III cuts in the form of non-templated A or U residues.

Concluding remarks

Our results show that RNase III exerts selection pressure on the structure of the RNA phage genome. However, the phage adapts easily to this pressure. Upon introduction of an RNase III target there is an initial 1000-fold drop in titer, suggesting that during every replication round there is a 0.1% chance of accidently

creating a resistant phage. Adaptation to RNase III coincides with a return of the titer to a level indistinguishable from wild-type.

Although we have proposed several ways in which the insertions/deletions in the revertants could have arisen, the more attractive possibility seems that the initial RNase III cut earmarks the RNA for destruction by the combined actions of PAP and exonucleases (31). On the other hand, there is the replicase trying to save the RNA from extinction. The revertant sequences that are the outcome of these competing actions seem to offer a glimpse of the dynamics and intermediates involved in RNA degradation and replication.

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