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Olsthoorn, R.R.C.L.; Duin, J. van

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Evolutionary reconstruction of a hairpin deleted from the genome of an RNA virus

(RNA evolution/RNA structure/RNA recombination/translation/ribosome binding)

RENÉ C. L. OLSTHOORN* AND JAN VAN DUIN†

Department of Biochemistry, Leiden Institute of Chemistry, Gorlaeus Laboratories, University of Leiden, P.O. Box 9502, 2300 RA Leiden, The Netherlands

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ABSTRACT **The intercistronic region between the maturation and coat-protein genes of RNA phage MS2 contains important regulatory and structural information. The sequence participates in two adjacent stem-loop structures, one of which, the coat-initiator hairpin, controls coat-gene translation and is thus under strong selection pressure. We have removed 19 out of the 23 nucleotides constituting the intercistronic region, thereby destroying the capacity of the phage to build the two hairpins. The deletion lowered coat-protein yield more than 1000-fold, and the titer of the infectious clone carrying the deletion dropped 10 orders of magnitude as compared with the wild type. Two types of revertants were recovered. One had, in two steps, recruited 18 new nucleotides that served to rebuild the two hairpins and the lost Shine-Dalgarno sequence. The other type had deleted an additional six nucleotides, which allowed the reconstruction of the Shine-Dalgarno sequence and the initiator hairpin, albeit by sacrificing the remnants of the other stem-loop. The results visualize the immense genetic repertoire created by, what appears as, random RNA recombination. It would seem that in this genetic ensemble every possible new RNA combination is represented.**

The single-stranded RNA coliphages are of mRNA polarity and are about 4000-nucleotides long. The genome encodes four proteins, needed for RNA replication and for the construction and spreading of the virions (Fig. 1*A*). New plus strands are generated via a minus-strand intermediate by an error-prone replicase. A single-phage burst releases some 10,000 descendants per cell, of which only 5–10% appear infectious (1). A large number of the progeny phage carry single substitutions, but multiple base changes and recombinants are present as well (2–4). This collection of mutant genomes, the so-called quasispecies, allows the phage population to rapidly adapt to changes in its environment (5, 6).

Besides encoding the phage proteins the nucleotide sequence endows the RNA with the proper phenotype, i.e., it prescribes the proper secondary structure needed for translational control mechanisms, protein binding sites and other functions (7).

To understand the significance of this phenotype we have constructed an infectious MS2 cDNA clone and used this clone to introduce changes that compromise preselected structure elements.

One particularly delicate structure element is located around the start of the major coat protein (CP) gene. For group I phage MS2 this element is shown in Fig. 1*B*. The sequence folds into two hairpins, one of which has the CP start codon in the loop, while the lower part of the stem contains the Shine-Dalgarno (SD) sequence (boxed), which mediates ribosome binding. In a previous evolutionary experiment we could show that the thermodynamic stability of this hairpin is of great

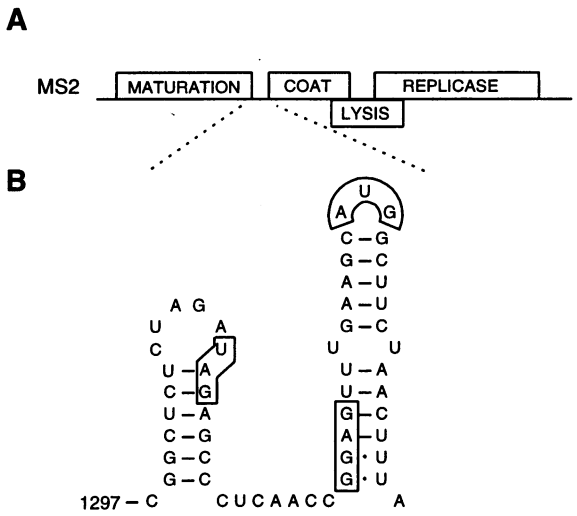

FIG. 1. (*A*) Genetic map of the MS2 RNA genome. (*B*) Secondary structure at the start of the CP gene. Boxed residues indicate, from 5′ to 3′, the stop codon of the maturation gene, the SD sequence, and the initiation codon of the CP gene.

importance to the fitness of the phage. Changes from its wild-type ΔG° value immediately triggered the selection of suppressor mutations in which the original stability, but not the original sequence, was restored (8). The maturation-gene terminator-helix (Fig. 1*B*) is conserved in all RNA phages but neither its stem length nor its loop sequence seems subject to strong selection pressure (9).

In our previous evolutionary studies, genomes carried at most five substitutions in the initiator hairpin. Recovery from the wrong ΔG° turned out to be very simple. Even in the worst case, where the phage titer of the mutant infectious cDNA clone had dropped by five orders of magnitude, millions of revertants would still turn up per milliliter of bacterial culture. (The wild-type infectious clone yields about 10^{11} viable phage per ml culture after overnight growth.)

In the present series of experiments we set out to hit the virus harder allowing us to fathom the magnitude of the genetic reservoir. Accordingly, a 19-nucleotide deletion was made in the intercistronic region between the maturation and CP genes. This deletion destroys both hairpins and also removes the SD signal. As a result CP yield dropped to 0.05% of the wild-type value.

In a standard survey no phages could be found in transformed cells, but by scrutinizing the insides of the bacteria we recovered two types of revertants that, by different means, had managed to reconstruct a surrogate initiator hairpin with the

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Abbreviation: SD, Shine-Dalgarno; CP, coat protein; pfu, plaque-forming unit(s).

*Present address: Plant Molecular Biology Center, Northern Illinois University, de Kalb, IL 60115.
†To whom reprint requests should be addressed.

proper stability and SD sequence. One had done so by sacrificing six more nucleotides, the other by recruiting 18 new nucleotides. Our experiments show the almost unlimited potential of random RNA recombination to generate new information. In addition, the results further confirm the crucial role of the initiator hairpin for phage viability.

MATERIALS AND METHODS

Bacterial Strains and Plasmids. Plasmids were grown in *Escherichia coli* K12 strain M5219, encoding the thermosensitive λ repressor (cI857) and the transcriptional antitermination factor N (10). Evolution experiments were performed in the *E. coli* F^+ strain GM-1 (11). All strains were grown in LC broth.

pMS2000 contains a complete cDNA copy of phage MS2 under transcriptional control of the thermoinducible promoter P_L from phage λ. This plasmid produces about 10^{11} plaque-forming units (pfu)/ml culture during overnight growth at 28°C even though the promoter is silent at this temperature.

pLUC19 is a derivative of pPLc28.33 (12) from which the *Pst*I sequence, located in the beta-lactamase gene, has been changed by exchange with the corresponding *Bsa*I–*Pvu*I fragment from pUC19. Subsequently, the polylinker of pUC19 was cloned between the *Eco*RI and *Hin*dIII sites directly downstream of the P_L promoter.

pCOAT184 is a pACYC184 derivative in which the complete MS2 coat gene (1221–1737) is cloned behind the constitutive *tet* promoter. It produces a low level of coat protein that is sufficient to repress synthesis of the toxic replicase (13). The presence of pCOAT184 is indispensable for infectious cDNA mutants that poorly express coat protein (8).

Construction of the Deletion Mutant. A 19-nucleotide deletion (nucleotides 1314–1332) in the intercistronic region between the A-protein and coat gene was found during oligonucleotide-directed mutagenesis on M13 mp11 carrying the *Xba*I(1303)–*Bam*HI(2057) fragment of MS2 cDNA (14) containing three codon wobble substitutions (U1340C, U1343A, and U1349C). The fragment carrying the deletion was cloned into vector pPLa236 (10). The *Xba*I(1303)–*Bst*XI(1551) fragment of the resulting plasmid (pMS198) was ligated into the corresponding sites of pMS2000. This cDNA clone, pMS2198, is unstable and can only be maintained in an M5219 strain carrying pCOAT184. A disadvantage of the presence of pCOAT184 is that recombination takes place leading to wild-type MS2 phage. Cells containing both pMS2198 and pCOAT184 give rise to about 10^6 wild-type phages per ml culture.

To circumvent the need for pCOAT184 we reduced replicase expression by lowering the copy number of pMS2198. We have noticed that the copy number of the pPLa plasmids, of which pMS2198 is a derivative, can be down-regulated by the presence of the semicompatible pPLc vector. In most combinations of pPLc (Ap^R) and pPLa (Kn^R), the pPLc plasmid proves superior and the pPLa plasmid is hardly detectable in a standard DNA isolate (unpublished observation).

Accordingly, pMS2198 (Kn^R) was transformed to M5219 cells containing the empty pLUC19 vector, a derivative of pPLc28.33 (Ap^R). We obtained several Ap^RKn^R colonies, 12 of which were grown up in 2.5 ml of LC broth at 28°C. Ten microliters of supernatant from each overnight culture was plated on lawns of F^+ GM-1 cells. In these aliquots no pfus were present. Therefore, all 12 overnight cultures were pooled and precipitated with polyethylene glycol (PEG6000). The pellet, which includes cells and putative phages, was suspended in lysis buffer and treated with lysozyme according to standard procedures (15). After this treatment, small plaques (0.1 mm) were detected at about 0.3 pfu/ml culture, which is about 10 orders of magnitude lower than that of the wild-type infectious clone present in cells together with pLUC19.

Monitoring Phage Evolution. Ten plaques were picked and amplified in 2.5 ml LC containing 10^7 F^+ GM-1 cells. After overnight growth at 37°C, titers reached 10^{10} pfu/ml. Phage generation in M5219 is considered as cycle 1, growth as plaques as cycle 2, while the first growth in liquid culture is defined as cycle 3. Subsequent infection cycles start with the inoculation of about 10^7 GM-1 cells with 10^6–10^8 pfu. Phages present in 1 ml of supernatant were precipitated with PEG and the RNA in the region of interest was sequenced by primer extension as described before (8). The sequence of revertant 2.4 was also verified by double-stranded DNA sequence analysis of two independent reverse transcription–PCR fragments. To get this information RNA was isolated from two plaques obtained after plating of the lysate from cycle 21. cDNA was synthesized using primer DUI59 (5′-CCCCGTTAGCGAAG) and avian myeloblastosis virus reverse transcriptase (Promega). PCR (5 min at 95°C, [30 sec at 92°C, 1 min at 46°C, 45 sec at 72°C] × 40, 5′ 95°C) was carried out with *Taq* polymerase (Promega) and primers DUI59 and DUI715 (5′-CCCAAATCTCAGCCATGCATCGAGG).

Energy Parameters. ΔG° values were calculated using the energy parameters of Freier *et al.* (16). For the terminal G·U on C·A stack a value of −1.2 kcal/mol was derived by de Smit (17).

RESULTS

The System. The complete MS2 cDNA was cloned in a plasmid downstream of the P_L promoter of phage λ. The construct was maintained in strain M5219, which cannot be reinfected by MS2 because it does not produce F pili. Cultures harboring this plasmid generate phage spontaneously, even in the absence of induction of the promoter. The precise pathway leading from cDNA to tailored phage RNA is not known. Overnight cultures contain on average 10^{11} pfu/ml cell-free supernatant. The presence of adverse mutations in the MS2 cDNA reduces this titer. For instance, substitutions that reduce coat protein synthesis by stabilizing the initiator hairpin cause the titer to drop to 10^6 pfu/ml. The phages that are recovered from such cDNA mutants are revertants carrying destabilizing suppressor mutations that increase CP yield. Eventually, the revertants reach wild-type titer by additional substitutions that further adjust the stability of the helix to the wild-type value (8).

Recovery from a 19-Nucleotide Deletion. In the present analysis we have increased the pressure on the phage by deleting 19 nucleotides, including the SD sequence, from the intercistronic region between the maturation and CP genes (Fig. 2). The lower left panel of Fig. 3 shows the deletion in the secondary structure context of this region. As can be seen the missing nucleotides will severely weaken, if not destroy, both hairpins. Not surprisingly, synthesis of the coat protein in this mutant drops to 0.05% of the wild-type value as measured in partial cDNA clones (M. H. de Smit, unpublished data).

To examine phage production the supernatant of an overnight culture harboring this MS2 cDNA deletion mutant was plated as usual on a lawn of F^+ cells (which can be infected by MS2). No plaques were found. In a second try we scrutinized 30 ml of the culture by lysing the bacteria. This lysate yielded 10 very small plaques. This number, 0.3 pfu/ml, represents a drop of about 10 orders of magnitude as compared with the yield that the wild-type construct gives under comparable circumstances (see *Materials and Methods*).

All plaques were picked and amplified overnight. By then their titers had reached 10^{10} pfu/ml, which is about 50× lower than the titer reached by wild-type phages. Phage RNA from the revertants was prepared and the region of interest sequenced by primer extension with reverse transcriptase.

Two types of revertants were recovered. One, Rev1.1, contains a further deletion of six bases that removes the

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Rev1.2          CGG......AGgUAGAG..................GCAUGGCCUCAAACUUCA
Rev1.1          CGG......AGAUAGAG..................GCAUGGCCUCAAACUUCA

starting mutant CGGCUCUCUAGAUAGAG..................GCAUGGCcUCAAACUUcA
wild type       CGGCUCUCUAGAUAGAGCCCUCAACCGGAGUUUGAAGCAUGGCUUCUAACUUUA
starting mutant CGGCUCUCUAGAUAGAG..................GCAUGGCcUCAAACUUcA

                ------------------>------------------>
Rev2.1          CGGCUCUCUAGAUAGAG.....GCAUGGAGAUAGAGGCAUGGCCUCAAACUUCA
Rev2.2          CGGCUCUCUAGAUAGAG.....cCAUGGAGAUAGAGGCAUGGCCUCAAACUUCA
Rev2.3          CGGCUCUCUAGAUAGAG.AGUGcCAUGGAGgUAGAGGCAUGGCCUCAAACUUCA
Rev2.4          CGaCUCUCUAGAUAGAG.AGUGgCAUGGAGgUAGAGGCAUGGCCUCAAACUUCA
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FIG. 2. Linear representation of the starting deletion mutant and the emerging revertants. SD sequences are shown in bold face type; stop codon of the maturation gene and initiation codon of the coat gene are shaded. Three codon wobble changes present in the starting mutant are shown in italics. Substitutions occurring during evolution are shown in lower case. Arrows indicate the duplicated sequence in Rev2.1.

penultimate two codons from the maturation gene (Fig. 2). In the other, Rev2.1, a 14-nucleotide insertion has taken place (Fig. 2). Remarkably, both changes recreate the wild-type SD sequence (GGAG) at the wild-type distance from the start codon (Fig. 2). But this is not the only achievement of the suppressor mutations. Both the deletion and the insertion allow the formation of a stem mimicking the initiator hairpin in shape and stability. In Rev1.1 this result is attained by sacrificing what is left from the terminator hairpin (Fig. 3). Upon further growth Rev1.1 rapidly acquired an A→G transition 8 nucleotides ahead of the start codon. In the discussion we try to interpret this mutation. Its effect is an extended SD sequence, a smaller internal loop and a higher stability of the initiator helix (Fig. 3). This revertant was not pursued any further.

The 14-nucleotide insertion in Rev2.1 not only reinstalls an initiator hairpin, it also rescues the terminator hairpin (Fig. 3). The further evolution of Rev2.1 followed two different pathways. In Rev3.1 the terminator hairpin was further stabilized by a G→U transversion, which almost brings back the wild-type structure by replacing the G·G mismatch with a G·U pair (Fig. 3). At the same time there is the A→G change at the edge of the SD sequence, which we also saw in the transition from Rev1.1 to Rev1.2. Evolution of Rev3.1 was stopped at this point.

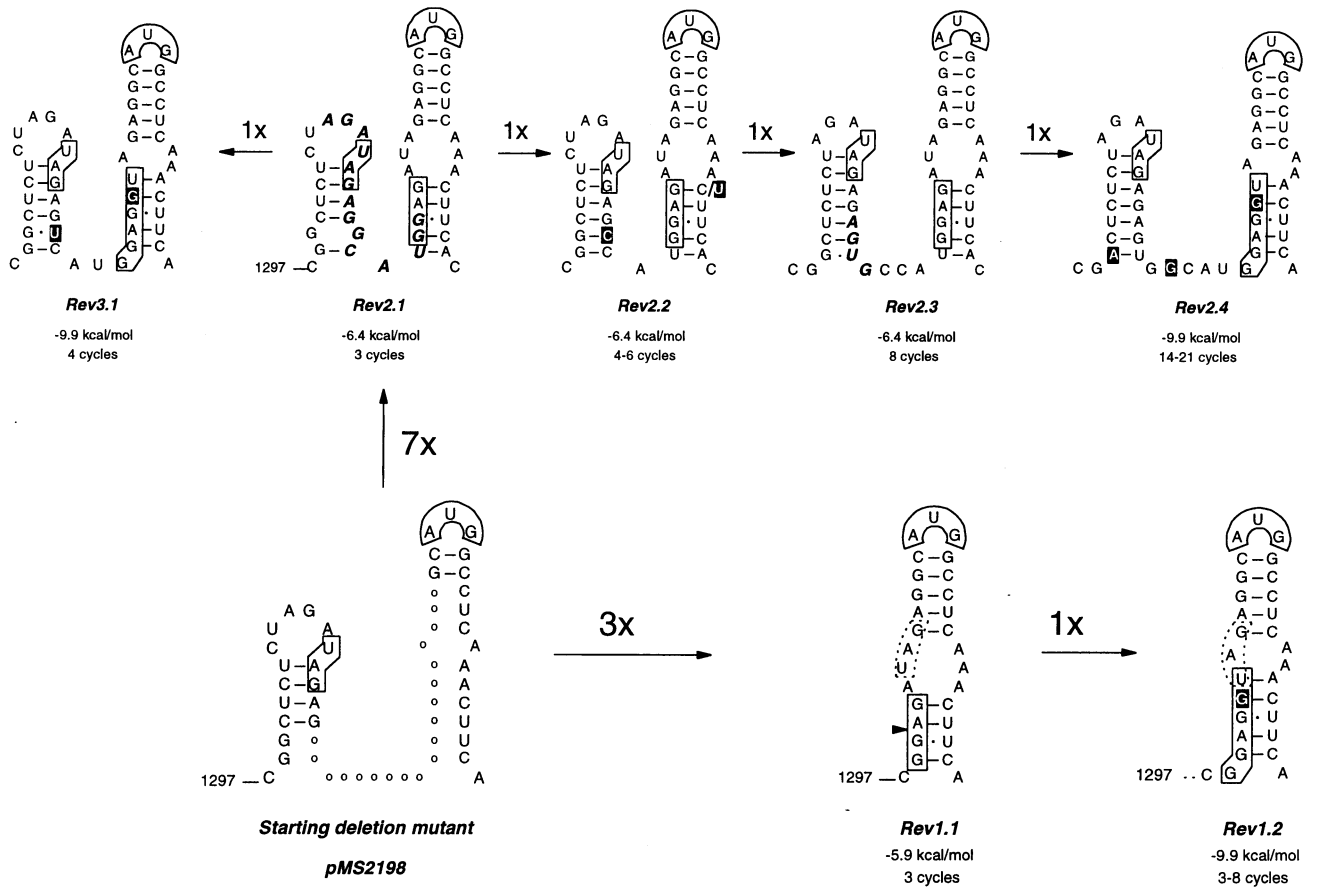

FIG. 3. Evolutionary pathways of the starting deletion mutant in the context of secondary structure. The deleted nucleotides in the starting mutant are indicated by open circles. Evolutionary routes are shown by arrows. The number specifies how many times a certain path was found. The arrowhead in Rev1.1 marks the position of the 6-nucleotide deletion. Insertions are shown in bold face type. Base substitutions occurring in the course of evolution are indicated by black squares. Rev2.2 is a mixture of two sequences. The stop codon in Rev1.1 and Rev1.2 is indicated by a dashed box. Helix stabilities were calculated as described in *Materials and Methods*. See Fig. 1 for further details. The secondary structures that we propose are largely supported by the pattern of suppressor mutations, in particular for the terminator hairpin. For the start hairpin such evidence is not available. However, in one of our revertant lines we have seen an A–U temporarily replacing the G–C pair at the fourth pair from the bottom (not shown).

The alternative evolutionary route of Rev2.1 also involved further repair of the terminator hairpin, this time to the wild-type structure (Fig. 3, Rev2.2). In a subsequent step there is an insertion of four bases (AGUG) that causes a slight rearrangement of the terminator hairpin and at the same time pushes the two helices away from each other in the direction of the original distance (Fig. 3, Rev2.3). Finally, some further adjustments take place, the major one being the familiar A→G change at the border of the SD sequence (see also Rev1.2 and Rev3.1). Applying the published stacking energies, this mutation is predicted to rearrange the internal loop, which in turn enlarges the interhelical distance to six nucleotides, one less than wild type. Meanwhile, it is remarkable that the three recorded revertants all end up with the same initiator helix.

DISCUSSION

We have put a heavy strain on RNA phage MS2 by deleting 19 nucleotides from the intercistronic region between the maturation and CP genes. This region contains the information for two stem-loop structures, one of which, the coat initiator hairpin, is subject to very strong selection pressure as its stability is related to CP yield. The deletion also removes the SD sequence required for ribosome binding.

At first glance the deletion seemed lethal, but upon scrutinizing the insides of the host bacteria we found a few phages, which had overcome the damage. In one kind (Rev1.1) a further deletion had taken place. In the other kind (Rev2.1) a 14-nucleotide insertion had occurred. Inspection of the new sequence revealed that it was in fact a duplication as indicated by the arrows in Fig. 2. Both mutations had essentially the same structural consequences; the wild-type SD sequence reappeared at the wild-type position, while at the same time an initiator hairpin emerged, only slightly different from the original one. The duplication also restored the terminator hairpin.

In Rev2.3 a further 4-nucleotide insertion, possibly a duplication of the neighboring AGAG sequence, restored the distance between the two hairpins. These major steps were accompanied by simple base substitutions that further adapted the new information to its structural and functional role. These refinements could sometimes, but not always, be understood (see below).

Evolution. The initial MS2 deletion mutant is not by itself a viable phage, but can become one during error-prone replication of MS2 cDNA transcripts. We distinguish two stages in this process. In the first one a very rare event takes place, without which no infectious phages exist. This step is the insertion or the deletion, leading to Rev2.1 and Rev1.1, respectively. Once this revertant has come into existence, the further perfection of the genome is relatively simple since we are now dealing with large numbers of progeny and the probability to find better adapted mutants or recombinants increases accordingly. In this respect it is relevant to note that we have never encountered a crippled revertant. No matter how serious the initial damage was, if a revertant emerged it always managed to optimize its genome such that a titer equal or very close to wild type was attained.

Since we cannot explain the observed insertions and deletion from special features of the surrounding sequence, we assume they arose randomly and that from the plethora of new combinations we have selected those that lead to a viable virus. As the titer of the mutant cDNA is 10 logs lower than the wild-type equivalent, the probability of the initial insertion or deletion to occur must be about 10^{-10}.

Recombination vs. Base Substitution. It is remarkable that we do not encounter simple base changes that might compensate for the initial deletion. For instance, the two transversions C1304 → G and U1305 → G, would create the same initiator helix as in Rev2.1 (Fig. 3). Possibly, the frequency of two simultaneous transversions is even lower than 10^{-10}. Misincorporation frequencies of 10^{-3} to 10^{-4} determined for Qβ replication apply to base transitions but little is known about transversions (18). If these would occur with a frequency of $<10^{-5}$ this could be one reason why we do not detect double transversions in our experiment.

Another reason could be that the deletion has rendered the RNA more vulnerable to ribonucleases. This would lead to enhanced rates of recombination since the replicase would be forced to leave the broken template prematurely. Forced recombination by template breaks has been well documented for retroviruses (19–21). In this respect it may have significance that the 6-nucleotide deletion and the 14-nucleotide insertion can be thought to have originated from the same point, i.e. between U1305 and A1306 (Figs. 2 and 3). Pyrimidine-A junctions are known to be sensitive also to spontaneous cleavage.

The third and probably best explanation for the prevalence of recombinants over point mutations is that, although the above suggested double transversions would save the initiator hairpin, they would not restore the terminator helix. Rather, there would remain a long stretch of single-stranded RNA. Our previous work has shown strong selection against unstructured RNA (9), so it is conceivable that the terminator hairpin must either be fully restored (Rev2 and Rev3) or fully removed (Rev1). Clearly, this feat is more likely achieved by insertions or deletions than by base changes.

Structural Aspects. We have shown previously that the strength of the initiator hairpin is confined to a narrow range around −7.0 kcal/mol. Higher stabilities are selected against because they lead to reduced coat protein yield, but the selection pressure against more positive ΔG° values is not yet understood. In the present study, the first revertants that emerge, Rev1.1 and Rev2.1, have calculated stabilities around −6 kcal/mol. We have seen before (8) that this value is outside the permissible range and this is probably the reason why evolution continues until all revertants eventually end up having a hairpin with a substantially higher stability (−9.9 kcal/mol). This value seems even more off the optimum. However, we should take into account that the SD sequence in these revertants is extended from four to six bases and it is known that stronger ribosome binding sites can compensate for stronger hairpins (22, 23).

It is interesting to compare the structure of Rev2.4 with related phages and with previously obtained revertants (Fig. 4). The similarity between all these start regions is not the conservation of identical structural elements such as fixed bulges or constant hairpin-loop sequences. Such constant features are, in fact, not to be expected since one should bear in mind that the "active form" of the start helix, at least as far as translation is concerned, is the melted form. Thus, in the first place the strength of these helices should be similar and should, in combination with the extent of their SD complementarity, be weak enough to just be melted by a ribosome (22). Granted the uncertainties in thermodynamic parameters, by and large this condition appears to be met. Furthermore, the stability constraint can account for the presence and variable positions of bulges and internal loops, since we think they are merely there to restrain the ΔG°, which, given the length and composition of the stem, would otherwise exceed the allowable margin. Clearly, their position is not of prime importance.

A second common feature of interest is the almost constant distance between the start codon and the edge of the terminator helix. This distance, 19 ± 2 nucleotides, is the same as covered by a starting ribosome at its 5′ side. It is therefore conceivable that the terminator helix helps docking the ribosome by blocking its random lateral movement in the 5′ direction (26). If correct, this idea also identifies the pressure to enlarge the distance between the two helices as manifested

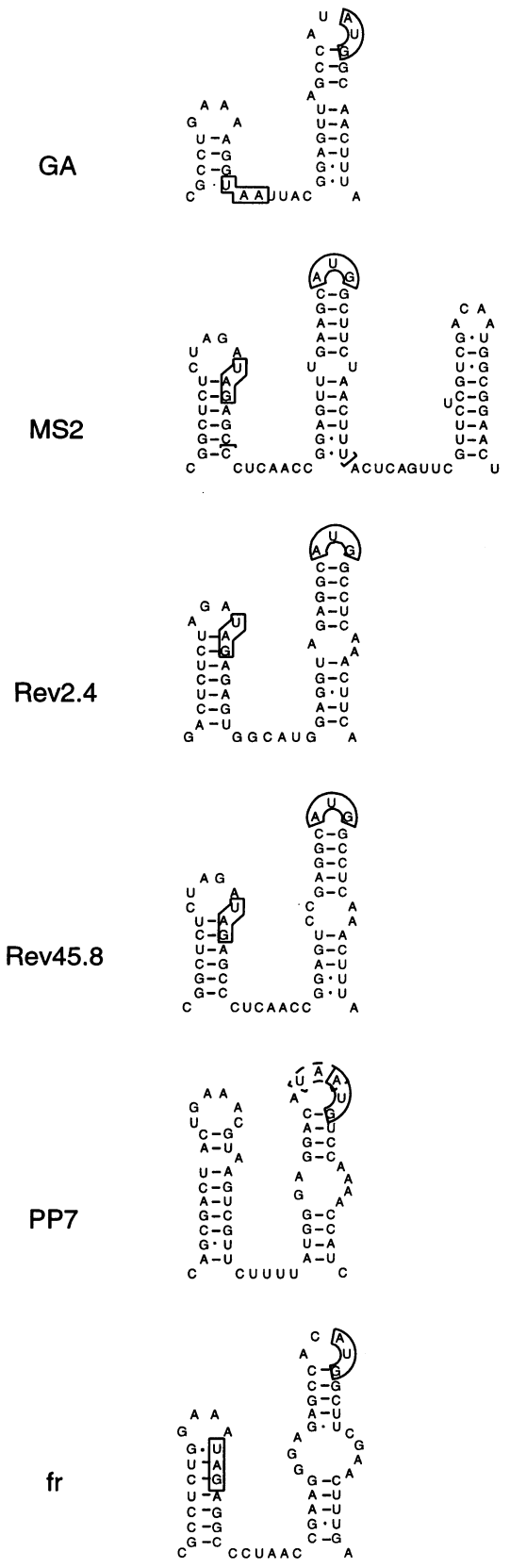

FIG. 4. Secondary structure at the start of the coat gene in several RNA phages and in two revertants. Stop and start codons are boxed. In PP7 the stop codon (dashed box) overlaps with the start codon. The stuctures of MS2, fr, and GA were taken from (24); PP7 from (25); Rev45.8 from (8). Only for MS2 the downstream hairpin is shown. Brackets in MS2 enclose the nucleotides protected by the initiating ribosome from nuclease attack.

by the four-nucleotide insertion that converts Rev2.2 into Rev2.3. If the terminator hairpin only serves as a barrier, this may explain its variable length (Fig. 4). Indeed, we could extend the terminator hairpin of MS2 by 15 base pairs without loss of fitness (9). In this experiment the distance between the start codon and the edge of the terminator helix remained the same.

It is worthwhile to point out that also at the 3′ side of the initiator hairpin a conserved small helix exists in all RNA phages, including Qβ and PP7 (25). Its 19 ± 2-nucleotide distance to the AUG codon is somewhat larger than what is covered by an initiating ribosome (shown only for MS2 in Fig. 4). In yeast mRNA the presence of a hairpin downstream of the start codon was reported to favor initiation by slowing down the scanning process (27).

Interestingly, this trio of small stem-loops is one of the few structural features conserved between distantly related phages and the link with translation is thus suggestive. We suppose the helices on either side present structural barriers that prevent the lateral escape of the ribosome from the start region, thereby increasing the probability to render the binding irreversible by codon-anticodon interaction.

Conclusion. The recovery from a large deletion demonstrates the rich potential of RNA phages to reanimate and subsequently optimize an almost dead specimen. The necessary tools include duplication, deletion, transversion, and transition, which are the kind of events that have been reported for other viruses as well (28). In other viral systems deletions appear to be repaired in a similar way: the gap is first filled in, usually by duplication of an adjacent sequence, and then the sequence is optimized by base mutations. For instance, in simian immunodeficiency virus a deletion of four codons from the *nef* gene was repaired by duplicating four adjacent codons. The new codons were adapted during further evolution to bring back the wild-type amino acids (29). In brome mosaic virus the removal of 17 nucleotides was partially restored by an 8-nucleotide duplication (30), whereas in poliovirus nucleotides of unknown origin were found at deletion sites (31). Such rare events can only be made visible in large populations of virus at sites under strong selection pressure. It is clear that the bacteriophage system with its high titers is extremely suitable to uncover the existence of low-probability events during replication.

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