Long-range translational coupling in single-stranded RNA bacteriophages: an evolutionary analysis

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ABSTRACT

In coliphage MS2 RNA a long-distance interaction (LDI) between an internal segment of the upstream coat gene and the start region of the replicase gene prevents initiation of replicase synthesis in the absence of coat gene translation. Elongating ribosomes break up the repressor LDI and thus activate the hidden initiation site. Expression studies on partial MS2 cDNA clones identified base pairing between 1427–1433 and 1738–1744, the so-called Min Jou (MJ) interaction, as the molecular basis for the long-range coupling mechanism. Here, we examine the biological significance of this interaction for the control of replicase gene translation. The LDI was disrupted by mutations in the 3′-side and the evolutionary adaptation was monitored upon phage passaging. Two categories of pseudorevertants emerged. The first type had restored the MJ interaction but not necessarily the native sequence. The pseudorevertants of the second type acquired a compensatory substitution some 80 nt downstream of the MJ interaction that stabilizes an adjacent LDI. In one examined case we confirmed that the second site mutations had restored coat–replicase translational coupling. Our results show the importance of translational control for fitness of the phage. They also reveal that the structure that buries the replicase start extends to structure elements bordering the MJ interaction.

INTRODUCTION

Translation initiation is usually the rate limiting step in setting the level of gene expression in prokaryotes (1) and, as such, it represents the focal point of many regulatory mechanisms (2). Secondary structures in messenger RNA can effectively restrict the accessibility of the initiation site (3). In the single-stranded RNA bacteriophage MS2 genomic RNA folding sequesters the start of the replicase cistron and the transit of ribosomes through the proximal part of the upstream coat gene is needed for its activation (4). Previously, it was proposed that in the unperturbed state of the viral RNA the replicase initiation site is hidden by a long-range base pairing to the internal 1409–1433 coat gene segment (5). Ribosomes translating the coat message temporarily unfold the LDI, which allows other ribosomes to bind at the replicase start. A refinement of this model came with the finding that deletions reaching down to 1419 still showed full coupling, while those extending to 1432 had fully lost it (6).

Further analysis of the sequence and structure of MS2 RNA showed only a possibility for 7 bp between nt 1427–1433 and 1738–1744. This was called the Min Jou (MJ) interaction (7). Subsequent expression studies on partial MS2 cDNA clones provided distinct evidence for the role of this LDI in replicase repression (8). Disruption of the MJ pairing by substitutions on either side led to increased replicase synthesis in the absence of coat translation, whereas repair of the mismatches by compensatory base substitutions had the opposite effect.

Just recently, the structure model of MS2 RNA around the replicase start site has been further refined (9; Fig. 1a). The main difference with the previous model (7) is that the MJ interaction is now flanked by another long-distance pairing called the VD interaction, involving nt 1419–1426 on one side and 1810–1817 on the other. One argument in proposing this structure was the distinct resemblance to Qβ RNA folding in the homologous region (10,11).

In this study we analyse the significance of coat–replicase coupling in live phage by taking advantage of the ease with which RNA phages adapt to mutational perturbations. If translational control is important, phage mutants in which the pairing is destroyed are expected to evolve suppressor mutations restoring the coupling. Our results support the existence of both the MJ and the VD interactions.

MATERIALS AND METHODS

Bacterial strains and plasmids

Plasmid pMS2000 contains full-length MS2 cDNA under control of the thermoinducible P$_{lac}$ promoter of phage λ (12) and bacteria transformed with pMS2000 produce viable phage. Vector plasmid pD carries the linker fragment TCTAGAaggcccCTTAAG (lower case letters refer to non-phage sequence) in place of the phage cDNA XbaI–BfrI fragment (1303–1901). Cells transformed with this vector do not produce phage. Random PCR-directed mutagenesis was carried out on the phage XbaI–BamHI (1303–2057) cDNA fragment subcloned into vector pTZ19 and the resulting plasmid was designated pTN1. In plasmids used for measurements of replicase gene expression the MS2 cDNA fragments are located behind the P$_{lac}$ promoter and the lacZ gene is fused at the BamHI site to the replicase gene (8).

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Figure 1. (a) Genetic map of RNA coliphage MS2 and RNA secondary structure model at the replicase initiation site (9). The start codon of the replicase and the stop codon of the coat gene are marked by a box. Local stem-loop structures are indicated as CT, R33 and R32. MJ designates the MJ interaction region. The names of the various phages reflect their origin. For instance, MJ2.2, MJ2.5 and MJ2.7 are pseudorevertants of mutant MJ2. The initial mutations are in grey, while suppressor mutations are marked by black boxes. The left column shows revertants with a reconstructed MJ interaction. (b) Comparison of wild-type, mutant and pseudorevertant phage RNA sequences and structures of the MJ interaction region. The names of the various phages reflect their origin. For instance, MJ2.2, MJ2.5 and MJ2.7 are pseudorevertants of mutant MJ2. The initial mutations are in grey, while suppressor mutations are marked by black boxes. The left column shows revertants with a reconstructed MJ interaction. The right column compiles the descendants which did not restore the original wild-type sequence.

Clones containing the phage λ P1 promoter were grown in Escherichia coli K12 strain M5219 (M72 trpA<sub>attb</sub>, lacZ<sub>attb</sub> Sm<sup>−</sup> (λbio<sub>SS2</sub>, c<sub>LS7</sub>ΔH1)), which encodes the thermosensitive λ repressor (c<sub>LS7</sub>) and the transcriptional antitermination factor N (14). Escherichia coli AB259 F<sup>+</sup> (Hfr3000, Thi<sup>+</sup>, Suc<sup>−</sup>) were used for amplification of phages. Escherichia coli JM109 [F<sup>−</sup> trdΔ36 proAB lacP4(lac-Z) M15-endedA1] was the host strain for plasmid pTN1. Bacteria were grown on LB broth containing 10 g/l bacitracin, 5 g/l yeast extract and 10 g/l NaCl and included an antibiotic if required.

Construction of vector plasmid pD

To construct plasmid pD 3 pmol phosphorylated oligonucleotides CTAGGttaccgcC and TTAAGggtaccgttaacT were combined, incubated at 70°C for 5 min and kept at room temperature for an additional 15 min to produce a double-stranded (ds)DNA linker with sticky XbaI and BfiI ends. The linker was then ligated to XbaI/BfiI-digested plasmid pMS2000 DNA.

Mutagenesis and cloning

The oligonucleotides used in this study, donated by Dr E.Stankevich, were synthesized on a Gene Assembler and purified on C18-Pep RPC HR 5/5 columns (Pharmacia). Complementary primers p52 and p53 were used to randomize the region of interest on MS2 cDNA by two-step PCR. Oligo p52 was synthesized as CGCCGG- nnnnnAACTAGGATTACCAGT (identical to the 1732–1764 sequence of the MS2 genome) and oligo p53 as CGACATGGGTAAATCTCAGTGTTnnnnnCCGGG (complementary to the 1734–1766 sequence of the MS2 genome), where n stands for any nucleotide. In the first step two overlapping mutant MS2 cDNA fragments were created using primer pairs p52 and p109 (complementary to the 2042–2062 sequence of the MS2 genome) and p53 and reverse primer CAGGAAACAGCTATT- p109 (complementary to the 2042–2062 sequence of the MS2 genome), where n stands for any nucleotide. In the first step two overlapping mutant MS2 cDNA fragments were created using primer pairs p52 and p109 (complementary to the 2042–2062 sequence of the MS2 genome) and p53 and reverse primer CAGGAAACAGCTATT-GAC (M13pUC reverse sequencing primer; Fermentas). Reaction mixtures (100 µl) contained 5 µg/ml pTN1 plasmid DNA, 0.5 µM each primer pair, 200 µM dNTPs, 0.1 mg/ml BSA, 2.5 mM MgCl<sub>2</sub>, 10 µl 10× PCR buffer (100 mM Tris–HCl, pH 8.8, 500 mM KCl, 0.8% Nonidet P40) and 2 U Taq DNA polymerase (Fermentas). The reaction was carried out by 25 cycles: denaturation at 95°C for 45 s, annealing at 40°C for 45 s and elongation at 72°C for 90 s. The desired DNA fragments were then purified electrophoretically in a 6% polyacrylamide gel. In the second step, the two overlapping MS2 cDNA fragments from the first PCR were combined and PCR with flanking p109 and the reverse primer was carried out as above, except that the p52 and p53 primers as well as the pTN1 plasmid were omitted from the reaction mixture.

Mutants of the infectious clone were constructed by digesting the final PCR product with XbaI and BfiI restriction endonucleases and then ligating them to the corresponding restriction sites in the vector plasmid pD. The ligated DNA was used to transform E.coli M5219 cells. Transformed bacteria were seeded onto nitrocellulose filters (Millipore) on top of LB agar plates containing kanamycin (50 µg/ml) and grown for 16–20 h at 28°C. This was done to screen the phage-producing clones by replica plating. Plaque-forming colonies were identified by replica plating the nitrocellulose filters on a lawn of E.coli AB259 F<sup>+</sup> cells on 0.8% agar and they were then inoculated into 3 ml LB broth containing kanamycin (50 µg/ml) and grown overnight at 28°C. Dideoxynucleotide chain termination sequencing (14) of the target region of dsDNA was carried out with T7 DNA polymerase (Pharmacia) using primer p108 (identical to the 1628–1648 sequence of the MS2 genome).
Monitoring phage evolution and competition

Mutant phages were obtained after growth of the infectious phage cDNA clones overnight at 28°C. Diluted samples of the lysate containing $10^8$ plaque-forming units (p.f.u.) of the phage were then inoculated into 3 ml E. coli AB259 suspension (at an OD$_{670}$ of $0.6$) and the cultures were shaken overnight at 37°C. Diluted samples of the obtained lysate ($10^8$ p.f.u. phage) were used for the next infection and so on. The infections were continued for 20–40 growth cycles. If the RNA sequence after various passages showed genetic heterogeneity in the phage population, plating was used to separate individual genomes for subsequent sequence analysis.

To test the fitness of a mutant phage it was mixed with wild-type phage in a p.f.u. ratio of 1:1 and subsequently the mixture was allowed to complete two passages on F$^+$ bacteria. The phage RNA from the initial and the evolved samples was sequenced. The disappearance of the mutant sequence from the population is defined as a failure of the mutant to compete with the wild-type, whereas the preservation of mutant RNA in the evolved sample is defined as good competition.

Sequence analysis of phage RNA

Phage RNA was prepared from 1 ml lysate as described by Olsthoorn et al. (12) and dissolved in 20 µl water. Samples of 1–3 µl were taken for reverse transcription using primer p109 at 42°C for 30 min in 10 µl 50 mM Tris–HCl, pH 8.3, 4 mM KCl, 8 mM MgCl$_2$ and 10 mM DTT containing 1 U AMV reverse transcriptase (Pharmacia). The cDNA product from the reverse transcription reaction was PCR amplified with primers p109 and p180 (identical to the 1200–1217 region of MS2 genome) and then precipitated with 1 vol ethanol in the presence of 2.5 M ammonium acetate (15). The amplified cDNA (bases 1200–2062 of the MS2 genome) was directly sequenced with T7 DNA polymerase. Routinely, two oligonucleotides p108 and p105 (complementary to the 1572–1585 region of the MS2 genome) were used as primers for sequencing segments on either side of the MJ interaction, which included bases 1700–1900 and 1300–1500 on MS2 cDNA respectively. Other primers were also sometimes used to check the sequence of bases 1500–1700 in the phage genome.

Detection of replicase and lysis gene expression

Escherichia coli M5219 cells that harbour the specified plasmids were grown to logarithmic phase and then induced by shifting the cultures to 42°C. To measure replicase expression, cultures were put on ice after 30 min induction and the β-galactosidase activity was determined in 0.1 ml samples according to standard procedures (16). To monitor lysis gene expression the cell density at A$_{670}$ was recorded (17).

RESULTS

The experimental system

A complete cDNA copy of the MS2 genome is present on a plasmid under transcriptional control of the thermoad inducible promoter P$_t$ of phage λ (12). The plasmid is maintained in E.coli M5219 F$^+$ host cells, which produce phage spontaneously. Adverse mutations lower the titer of the phage. However, upon passing mutations can quickly restore high viability by base changes resulting from the low copying fidelity of phage RNA replicase (18). These (pseudo) revertants may predominate through natural selection if compensation for the inflicted defect occurs.

The mutants

To disrupt the MJ interaction in the phage RNA, substitutions were introduced in the infectious MS2 cDNA clone in the 3′-side of the LDI (Fig. 1a and Table 1). The 1738–1744 sequence codes for non-essential amino acids of the lysis protein (19) and was therefore preferred over the coat segment of the long-distance base pairing as the target for base changes. The mutations lowered phage fitness, as revealed by the decreased titers of the infectious MS2 cDNA clones (Table 1) and by their failure to compete with the wild-type during co-infections (not shown). From ~20 mutants sequenced, five (MJ2, MJ5, MJ19, MJ22 and MJ3) were taken for evolutionary analysis. The choice was based on our wish to have few as well as multiple substitutions present. MJ2 and MJ5, containing only two and three base changes respectively, would provide the phage with a reasonable chance to somehow re-establish the original regulatory LDI. Conversely, the multiple substitutions in MJ3, MJ19 and MJ22 are more likely to fully rearrange the structure and they render restoration of a MJ variant unlikely (17). This would force the phage to find an alternative to retain replicase expression under control. From the many mutants the specific choice of MJ5, MJ19 and MJ22 was made because these showed a low initial titer, which usually speeds up evolution. MJ3 was a random choice. The course of the evolutionary adaptation was followed for many growth cycles, as described in Materials and Methods.

Reconstruction of an MJ interaction

Several descendants of mutants MJ2 and MJ5 had reformed the MJ interaction (Fig. 1b, left). In one of them this led to wild-type phage (revertant MJ2.5) and this result is inconclusive. A more informative revertant is MJ2.7, which shows that the secondary structure of the phage RNA but not the sequence is important. Here, three base changes re-established the LDI, while only one of them is a true reversion. The G1737U substitution probably resulted in an extra U-A base pair for the interaction, albeit at the expense of the coat terminator (CT) hairpin.

Two revertants (MJ5.4 and MJ5.6) arose from mutant MJ5. Descendant MJ5.6 evolved a nearly native LDI but still preserved the U.G pair, where in the wild-type it is C-G. On the other hand, the C1732U second site substitution in the phage turned the mismatch of the CT hairpin into an A-U base pair. The CT hairpin possibly stabilizes the MJ interaction by coaxial stacking (8) and thus strengthening of this helix may help to suppress replicase translation. Perhaps, closing the mismatch is sufficient to compensate for the new U.G pair in the MJ interaction. Less straightforward is revertant MJ5.4. Besides two reversions in the LDI, this phage also acquired an A1746C substitution in the replicase initiator hairpin R33, a change that we cannot yet explain.

Progeny phages without an MJ interaction

The progeny of MJ19, MJ3 and MJ22, as well as one descendant of MJ2, did not restore the MJ interaction (right column of Fig. 1b), but their evolutionary adaptations strikingly resembled each other in that most of them had the U1817C base change some 50 nt downstream of the replicase start (MJ2.2, MJ19.2, MJ3.1 and
MJ22.3). Pseudorevertants MJ2.2 and MJ19.2 also had in common the A1746C substitution. This base change was also seen in MJ5.4, and we will take up this mutation in the Discussion.

Probably, all these second site mutations provide good compensation for absence of the MJ interaction.

Table 1. Properties of MJ mutants

<table>
<thead>
<tr>
<th>Clonea</th>
<th>RNA sequence 1738–1744b</th>
<th>Titer (p.f.u./ml)b</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>ccuucua</td>
<td>1 × 10^11</td>
</tr>
<tr>
<td>MJ6</td>
<td>ccUGAGAC (5)</td>
<td>4 × 10^10</td>
</tr>
<tr>
<td>MJ18</td>
<td>ccUAGGC (4)</td>
<td>4 × 10^10</td>
</tr>
<tr>
<td>MJ14</td>
<td>ccACGCGC (3)</td>
<td>4 × 10^10</td>
</tr>
<tr>
<td>MJ8</td>
<td>AUUGCCG (5)</td>
<td>3 × 10^10</td>
</tr>
<tr>
<td>MJ10</td>
<td>cUCuGcC (5)</td>
<td>3 × 10^10</td>
</tr>
<tr>
<td>MJ3</td>
<td>GeCCACGC (5)</td>
<td>3 × 10^10</td>
</tr>
<tr>
<td>MJ20</td>
<td>cUGAGGC (5)</td>
<td>3 × 10^10</td>
</tr>
<tr>
<td>MJ16</td>
<td>cUGCGGC (4)</td>
<td>3 × 10^10</td>
</tr>
<tr>
<td>MJ9</td>
<td>cUCGGUG (6)</td>
<td>3 × 10^10</td>
</tr>
<tr>
<td>MJ2</td>
<td>cUGACuA (2)</td>
<td>2 × 10^10</td>
</tr>
<tr>
<td>MJ15</td>
<td>GUuAusC (5)</td>
<td>2 × 10^10</td>
</tr>
<tr>
<td>MJ21</td>
<td>cACuGGC (4)</td>
<td>2 × 10^10</td>
</tr>
<tr>
<td>MJ4</td>
<td>uCCuugC (3)</td>
<td>2 × 10^10</td>
</tr>
<tr>
<td>MJ1</td>
<td>UCUGuA (4)</td>
<td>2 × 10^10</td>
</tr>
<tr>
<td>MJ7</td>
<td>UUCGCCG (6)</td>
<td>1 × 10^10</td>
</tr>
<tr>
<td>MJ17</td>
<td>AcGGACUG (6)</td>
<td>1 × 10^10</td>
</tr>
<tr>
<td>MJ12</td>
<td>cUGCGAua (5)</td>
<td>7 × 10^9</td>
</tr>
<tr>
<td>MJ22</td>
<td>cGGCCcC (5)</td>
<td>7 × 10^9</td>
</tr>
<tr>
<td>MJ19</td>
<td>uUUGGAa (6)</td>
<td>4 × 10^9</td>
</tr>
<tr>
<td>MJ11</td>
<td>cUGCCcC (4)</td>
<td>2 × 10^9</td>
</tr>
<tr>
<td>MJ5</td>
<td>UCGGcCA (3)</td>
<td>2 × 10^9</td>
</tr>
</tbody>
</table>

*The wild-type construct is pMS2000 (12). The mutants were created by PCR-directed mutagenesis using a primer with a randomized sequence corresponding to the 1738–1744 phage segment, except for MJ2.2, which was derived from the corresponding partial MS2 cDNA clone (8).

*The 1738–1744 RNA sequence of the mutants is aligned with that of the wild-type. Capital letters indicate mutations. Numbers in parentheses show the number of base changes as compared with the wild-type.

*Appropriate dilutions of supernatant from cDNA cultures grown overnight at 28°C were tested on lawns of E.coli AB259 F+ cells.

Replicase control in pseudorevertants is restored

To verify this question the 1365–2057 cDNA fragment of MJ19.2 and also the same region of the original mutant MJ19 were fused to the lacZ gene in an expression vector under transcriptional control of the Pr promoter (8). In these constructs the start codon of the coat gene is absent and we therefore measured the degree to which the replicase start was buried by structure. β-Galactosidase values for these clones (Table 2, first column) showed that the mutations in MJ19 indeed increased accessibility of the replicase start 3-fold. On the other hand, the mutations evolved in MJ19.2 decreased replicase synthesis ~6-fold and thus fully compensated for the inflicted activation of the replicase initiation site in mutant MJ19. In fact, MJ19.2 produced even less replicase than did the wild-type. These results demonstrate that evolution of pseudorevertants was directed towards suppression of replicase translation. The data, however, do not yet answer the essential question, i.e. whether the evolved mutations affected coat–replicase coupling. To resolve this point, the 1365–1628 sequence was deleted from the experimental constructs and replicase–β-galactosidase fusion protein in the resulting clones was measured (Table 2, second column). Because the removed region involves that part of the coat gene contributing to the MJ interaction, replicase expression in the shortened variants is no longer repressed by coat gene sequences and thereby goes up as compared with the corresponding parental clones. This increase gives a quantitative measure for coupling (coupling factor, Table 2).

In the wild-type, deletion of the coat cistron fragment raises replicase synthesis 9-fold (6). In MJ19 we expect an impaired long-range control due to disruption of the MJ interaction and this expectation is born out by the low coupling factor of three. The most important finding is that the suppressor mutations in MJ19.2 have restored the dependence of replicase gene translation on that of the coat. The coupling factor is back to eight. Thus, although the phage did not rebuild the MJ interaction, it did manage to restore coupling between the coat and replicase genes.

Table 2. Relative expression of the replicase gene

<table>
<thead>
<tr>
<th>RlacZ</th>
<th>MS2 cDNA fragment</th>
<th>Coupling factor</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1365–2057</td>
<td>1628–2057</td>
</tr>
<tr>
<td>Wild-type</td>
<td>1.0</td>
<td>9.0b</td>
</tr>
<tr>
<td>MJ19</td>
<td>3.1</td>
<td>9.2</td>
</tr>
<tr>
<td>MJ19.2</td>
<td>0.5</td>
<td>4.0</td>
</tr>
</tbody>
</table>

The efficiency of replicase gene translation is measured as activity of replicase–β-galactosidase fusion protein (RlacZ) using clones that contain either the 1365–2057 or 1628–2057 MS2 cDNA fragment fused to the lacZ gene at the BamHI site. The constructs lack the start of the phage coat cistron. In plasmids which begin at position 1365 of MS2 cDNA the coat gene segment that couples translation of replicase in wild-type phage is present (6). The RlacZ activity of the wild-type clone (8) is set at 1.0 (note that coupling is not absolute; 4). In constructs starting at position 1628 most of the coat gene is absent and replicase synthesis is uncoupled. Values are averaged from five experiments.

bCoupling is calculated as the ratio between the RlacZ activities of the corresponding clones starting at 1628 and 1365.

The question arises as to how replicase translation is regulated in MJ19.2. As mentioned above, A1746C is not understood, but U1817C makes sense in the context of the structural model shown in Figure 1a, as it stabilizes the VD interaction. It then falls into the same category as C1732U, found in MJ5.6. Both U1817C and C1732U stabilize flanking stems that can apparently strengthen the core interaction in an indirect way. Alternatively, it is clear that ribosome binding to the replicase start requires melting of hairpin R32. Perhaps, melting of R32 is somehow antagonized by the stronger VD interaction.

To verify that MJ19.2 did not contain substitutions outside the monitored 1300–1900 sequence, its 1303–1901 cDNA fragment was cloned back in the full-length MS2 cDNA copy. The titer of this infectious MJ19.2 clone had increased ~25 times as compared with the parental MJ19 clone. Also, phage produced from the MJ19.2 plasmid competed well with the wild-type during co-infections. These results show that the two suppressor mutations that re-establish translational control are also entirely responsible for the high viability of MJ19.2.

In wild-type RNA translation of the lysis gene is also coupled to coat gene reading (20). This coupling is enforced by a local stem-loop structure that occludes the lysis start (21). It is worthwhile noting that in both mutant MJ19 and revertant...
MJ19.2 coupling is still intact, i.e. in clones lacking the coat start signals lysis protein is not produced, whereas the protein is made when coat gene translation takes place (not shown).

**DISCUSSION**

Synthesis of replicase in the RNA coliphage MS2 is kept under dual control. One is repression by phage coat protein binding to the replicase initiator hairpin (22). The other is translational when coat gene translation takes place (not shown). Signals lysis protein is not produced, whereas the protein is made MJ19.2 coupling is still intact, i.e. in clones lacking the coat start.

3246 be effectuated by the long-distance MJ interaction (Fig. 1a). Here, we analyse the biological significance of this control. Mutations that disrupt the MJ interaction decrease the titer of the infectious clone up to 50 times. At the same time, such disruptions were shown to abolish the translational coupling. Importantly, in one studied case we could show that the coupling was re-instated by suppressor mutations. At the same time, the titer of such revertants was no longer distinguishable from the wild-type. We observed two types of revertants. In one type a different version of the original MJ interaction had evolved. In the other type revertants did not reform the MJ pairing, but had acquired substitutions mostly downstream of the 3′-side of the MJ interaction.

**Molecular basis for restoring the coupling**

A1772G and A1746C were found one and three times respectively.

How they restore coupling is not clear. Computer analysis (23) indicates that A1746C and the mutations accumulated in MJ22.2 set up different pairings between the replicase start and the coat gene, but we have no further support for such structures. The suppressor mutations U1817C and C1732U occur four times and once respectively. They make sense as they stabilize the structures flanking the MJ pairing. It is difficult to say whether they act by stabilizing the MJ LDI directly through, for instance, coaxial stacking (8) or whether their presence physically interferes with ribosomes diffusing to the replicase start. In this respect it is worth mentioning that the replicase initiator hairpin R33 by itself is too weak to shield the replicase start (24).

Whatever the mechanism, for the VD interaction we have independent evidence that it is involved in repressing replicase translation. We made two mutations, G1421A and C1424A, in the VD structure at wobble positions in the coat gene. They disrupt the LDI and stimulate replicase translation, measured as a lacZ fusion, ∼2-fold in a partial construct containing the 1365–2065 MS2 cDNA fragment. When these two mutations were introduced into an infectious clone, some of the revertants had acquired the U1817C suppressor mutation.

The results presented in this study show great similarity to a recent paper in which the importance of translational coupling between the coat and the lysis genes was analysed by the same evolutionary approach (17). From both studies it is clear that the coupling is not a fortuitous result of folding of the RNA. Rather, as in both studies coupling was restored by suppressor mutations, the mechanism must add to the fitness of the phage.

**Purpose of the translational coupling**

One problem in the life cycle of phage is that the genome must be able to switch from being a template for translation to being a template for replication. An RNA molecule can never be template for both processes at the same time, since they move in opposite directions. This problem is most likely avoided while ribosomes and replicase compete for a common site on the plus strand, i.e. the start of the coat protein cistron (25). Since lysis and replicase expression is coupled to coat gene translation, a replicase blocking the coat ribosome entry site effectively clears ribosomes from the three distal genes. The proximal maturation gene is not accessible to ribosomes in complete RNA, only on short non-equilibrated growing strands (26).

It is amazing to see how easy it is to re-invent these seemingly complex control mechanisms and how many different solutions there are. It is also remarkable that the perturbations caused by five to six substitutions can sometimes be neutralized by just two suppressor mutations.

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