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Translational control by delayed RNA folding: Identification of the kinetic trap

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ABSTRACT

The maturation or A-protein gene of single-stranded RNA phage MS2 is preceded by a 130-nt long untranslated leader. When MS2 RNA folding is at equilibrium, the gene is untranslatable because the leader adopts a well-defined cloverleaf structure in which the Shine–Dalgarno (SD) sequence of the maturation gene is taken up in long-distance base pairing with an upstream complementary sequence (UCS). Synthesis of the A-protein takes place transiently while the RNA is synthesized from the minus strand. This requires that formation of the inhibitory cloverleaf is slow. In vitro, the folding delay was on the order of minutes. Here, we present evidence that this postponed folding is caused by the formation of a metastable intermediate. This intermediate is a small local hairpin that contains the UCS in its loop, thereby preventing or slowing down its pairing with the SD sequence. Mutants in which the small hairpin could not be formed made no detectable amounts of A-protein and were barely viable. Apparently, here the cloverleaf formed quicker than ribosomes could bind. On the other hand, mutants in which the small intermediary hairpin was stabilized produced more A-protein than wild type and were viable. One hardly growing mutant that could not form the metastable hairpin and did not make detectable amounts of A-protein was evolved. The emerging pseudo-revertant had selected two second site repressor mutations that allowed reconstruction of a variant of the metastable intermediate. The pseudo-revertant had also regained the capacity to produce the A-protein.

Keywords: kinetic trap; metastable state; MS2; phage; RNA folding; translation

INTRODUCTION

One of the aspects that make RNA coliphages interesting biological systems is the way they coordinate and control the translation of their genome into protein. Lacking both transcription and subgenomic messengers, regulation exploits folding and unfolding of the RNA, sometimes in combination with repressor proteins.

In Figure 1A we show the genetic map of RNA phage MS2. In full-length RNA, only the coat gene is directly accessible to ribosomes. The other three are closed by RNA secondary structure. Translation of the lysis and replicase genes is coupled to reading the coat sequence as described elsewhere (Berkhout et al., 1987; Adhin & van Duin, 1990; van Himbergen et al., 1993). In contrast, expression of the maturation gene is not in any way coupled to that of the coat. Maturation (A) protein is present at one copy per virion and is required for attachment of the phage to the *Escherichia coli* sex pili (Paranchych & Frost, 1988).

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We would like to understand the mechanism that controls its synthesis. As a first step Groeneveld et al. (1995) determined the secondary structure of the 5′ UTR of MS2 (Fig. 1C). First, there is the 5′ terminal hairpin common to all phages. The remainder of the region folds into a cloverleaf structure in which the Shine–Dalgarno (SD) sequence (AGGAGGU) is sequestered in a long-distance interaction (LDI) with an upstream complementary sequence (UCS). It was shown that this pairing acts indeed as a negative control element because mismatches in the LDI such as CC3435AA and deletion of the UCS caused an ~10-fold increase in A-protein synthesis. There were, however, some unexpected observations. The increase in expression did not correspond with the prediction based on the stability loss of the LDI (de Smit & van Duin, 1990). More importantly, stabilization of the LDI by the U•G to C-G change (U32C) did not exhibit any of the expected decrease in translation. These data suggested that translation originated mainly or fully from a nonequilibrium structure in which the LDI had not yet been formed. Such delayed folding is often caused by trapping in a metastable structure (Clodi et al., 1999; Nagel et al., 1999). This possibility was supported by an experiment

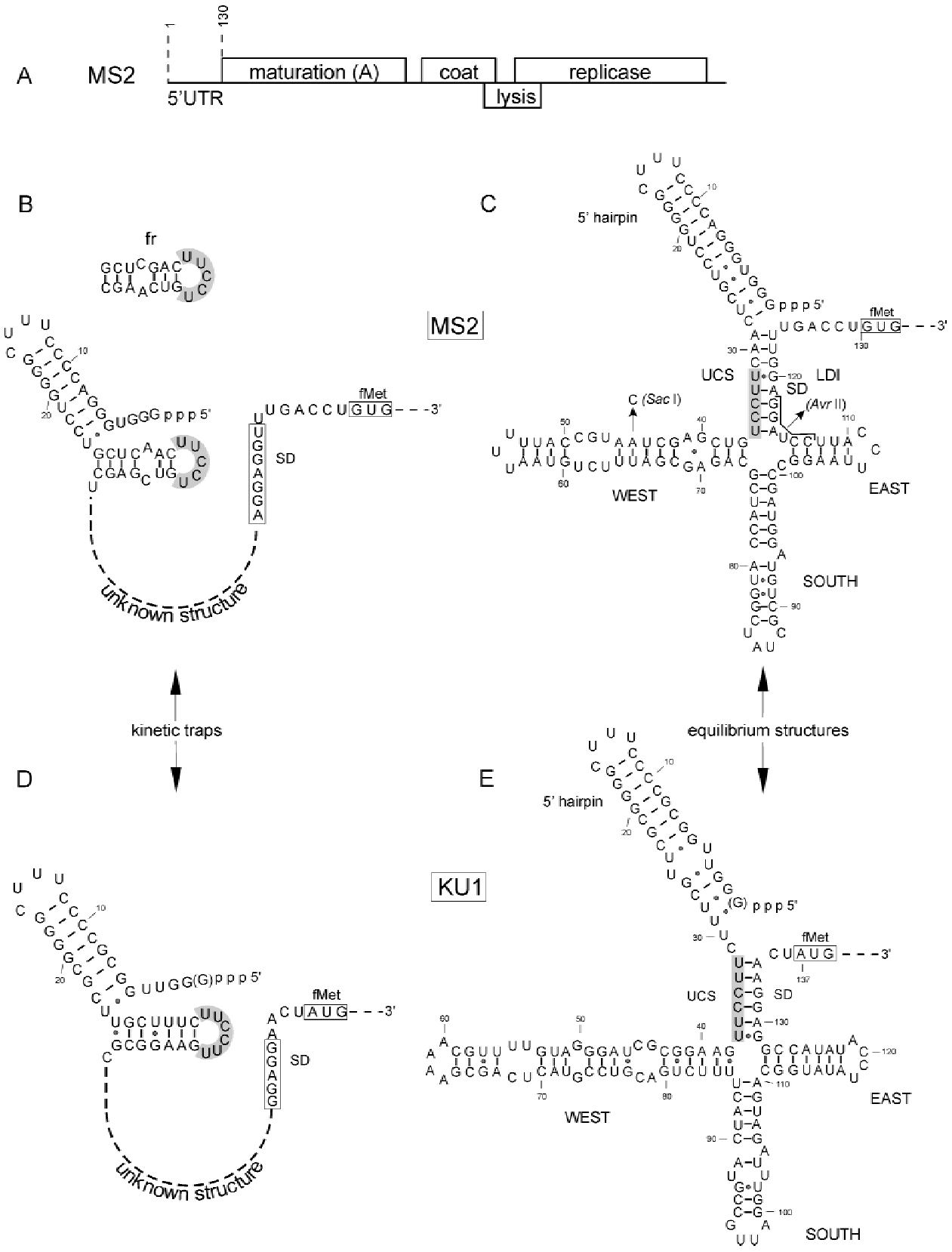

FIGURE 1. **A**: Genetic map of RNA coliphage MS2. **B**: Metastable folding intermediate for the 5′ UTR of MS2 and the related phage fr as proposed by us. **C**: Equilibrium structure for the 5′ UTR. UCS: upstream complementary sequence; SD: Shine–Dalgarno; LDI: long-distance interaction. For convenience a *Sac*I was introduced (A45C). The mutation did not measurable affect phage titer. **D**: Proposed metastable intermediate for phage KU1. **E**: Equilibrium structure for KU1. One G at the terminus is in brackets. Its presence could not unequivocally be determined by RNA sequencing but is inferred by analogy with all other sequenced coliphages. Structures C and E are taken from Groeneveld et al. (1995).

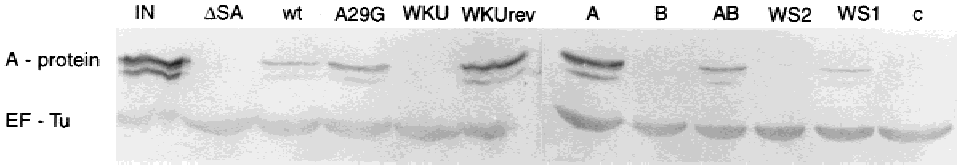

FIGURE 2. Western blots of several selected mutants showing the amounts of A-protein made. A short staining with antibodies against EF-Tu is carried out to verify that similar amounts of cell extract were applied in all slots. C: control not encoding the A-protein. IN: mutant carrying an insertion of 100 nt in the South arm showing a 10-fold increased A-protein synthesis (Groeneveld et al., 1995). In ΔSA, the combined East, South, and West arms have been replaced by a single 5 nt loop (see the Introduction). Splitting of the A-protein band is an electrophoresis artefact.

in which the combined East, South, and West arms of the cloverleaf were replaced by a single 5-nt loop bridging the LDI. In this truncated construct (ΔSA), metastable intermediates are not likely to exist and indeed expression of the A-protein was 10-fold reduced as compared to wild type (Fig. 2) (Groeneveld et al., 1995).

A next step to support the kinetic model was set by Poot et al. (1997). Here, RNA from wild type and the ΔSA mutant was prepared by in vitro transcription. The transcripts were heat denatured, snap cooled in liquid N_2, and their refolding at 37 °C measured by the loss of their ability to bind ribosomes. These experiments showed that ΔSA folded instantly, whereas for wild type, it took several minutes before all the RNA had reached its final closed structure. Furthermore it was demonstrated that this slow folding is a biological necessity. A man-made mutant, with a 100-nt insertion in the South arm (mutant IN) showed a 10-fold increased A-protein synthesis (Fig. 2). In the in vitro refolding assay, the mutant RNA remained permanently accessible to ribosomes. When this mutant was evolved, most of the insert was deleted and the revertant had regained the slow refolding behavior typical for wild type (Poot et al., 1997).

We measured a half-life of cloverleaf (Fig. 1C) folding in vitro of several minutes. tRNA, which has a similar size and two-dimensional structure, folds in the millisecond range (Crothers et al., 1974), and even though this molecule may have evolved to fold quickly (Higgs, 1993, 2000), it is clear that our cloverleaf is extraordinarily slow in reaching the lowest free energy state. As mentioned, it is generally assumed that long folding times can only be achieved if the RNA gets trapped in an alternative structure from which it cannot easily escape (reviewed by Thirumalai & Woodson, 2000). Here, we identify this kinetic trap for the A-protein start using genetic, biochemical, and evolutionary approaches.

RESULTS

The strategy

The model to be tested is that the folding of the inhibitory cloverleaf is postponed by a kinetic trap allowing temporary synthesis of the A-protein. If so, it must be possible to make phage mutants that have lost the potential to form the intermediate. These are predicted to show a negligible A-protein yield and therefore a low titer. Passaging such mutants will produce high fitness pseudo-revertants containing suppressor mutations that yield wild-type levels of A-protein. Analysis of the sequences and phenotypes of mutants and revertants should allow us to approximately localize and eventually identify the kinetic trap if it exists.

We have at our disposal an infectious MS2 cDNA clone. To obtain phages, it is sufficient to transform *E. coli* cells with the plasmid containing the complete MS2 cDNA sequence (Olsthoorn et al., 1994). The supernatant of overnight cultures of transformed cells yields about 10^{11} pfu/mL for the wild-type sequence. Depending on the mutations introduced, this number goes down, sometimes to the point where no survivors can be found. In such a case, we can enhance the sensitivity of the procedure by artificial bacteriolysis (see Materials and Methods).

Practically, we use a two-plasmid system to produce phage mutants. One plasmid carries the MS2 sequence 1 to 741 with the mutations. The second plasmid, compatible with the first one, contains the wild-type sequence 113 to 3′ end. Using wild-type sequences this plasmid combination yields 10^8–10^9 pfu/mL. In the two-plasmid system phage generation results from genetic recombination at the RNA and/or the DNA level.

Mutants are also examined for their ability to produce maturation protein. To do so the relevant partial MS2 cDNA clones carrying nt 1 to 2057 are brought under the transcriptional control of the thermo-inducible P_L promoter from phage λ and the yields measured by western blots.

Sequences in the arms of the cloverleaf contribute to A-protein synthesis

We first wanted to exclude the possibility that slow folding of the LDI is a general property of every cloverleaf structure whereas the millisecond folding of tRNA might be the exception. Therefore as a first experiment to gain confidence that there was a secret to be found in

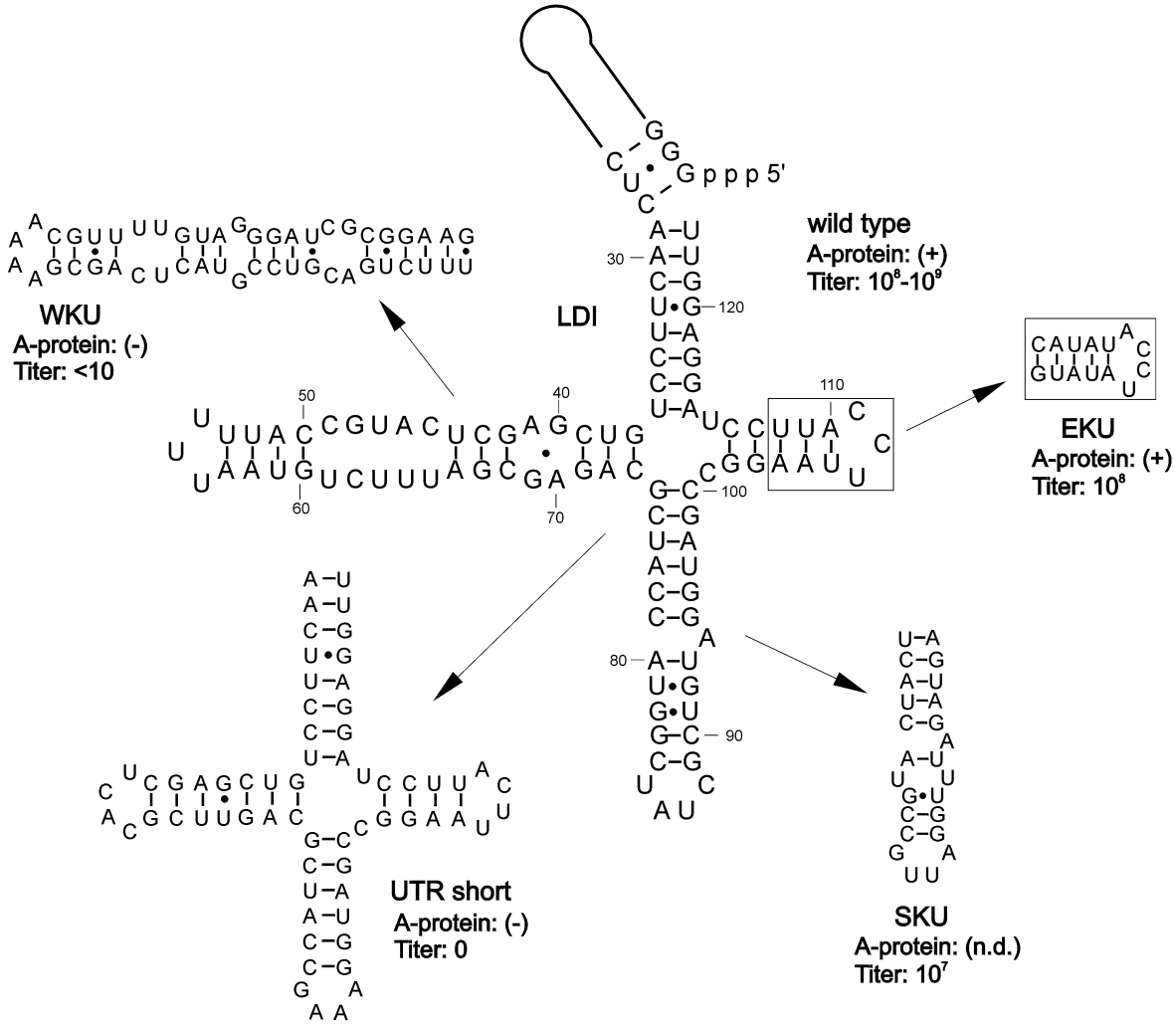

FIGURE 3. Four mutants used in this study. In "UTR short," only the LDI has not been changed. In WKU and SKU, the complete West or South arms have been replaced by the KU1 homolog. In EKU, only the boxed part of the East arm was exchanged because of the cloning site used (*Avr*II (113–118)).

the arms of the cloverleaf, we prepared mutant "UTR short" (Fig. 3). In this mutant, the LDI and the bottom parts of the three arms are unaltered but the top parts of the West and South hairpins have been shortened and simplified (for the East arm only one C residue was taken away). This construct produced no phages and no maturation protein. The absence of phages can be explained in many ways, for instance, a replication defect, but the absence of translation from the expression plasmid even though the ribosome binding site remained unaltered, suggests that in UTR short, the features that cause slow folding are lacking.

Arm-by-arm exchange

It is reasonable to assume that the putative kinetic trap must involve sequences of more than one arm. Therefore a swift procedure to approximately localize the intermediate seemed to be to exchange arm by arm for a different sequence. To minimize eventual complications that might arise from a completely new sequence, the most prudent approach seemed to take the arms from the related phage KU1. This phage was sequenced and studied by Groeneveld et al. (1995). KU1 is in the same genus as MS2 but belongs to a different species (species II). It has the same genetic map as MS2 (species I) but sequence identity is very low except when conserved protein motifs are encoded. Other members of species II are GA and JP34 (Inokuchi et al., 1986; Adhin et al., 1989). We could not take arms from other species I phages such as R17, f2, and fr because their sequence is virtually identical to MS2.

The 5′ UTR of KU1 forms a cloverleaf much like that of MS2 but its sequence is very different (Fig. 1E). We showed previously that the complete 5′ UTR of KU1 can functionally replace that of MS2 to produce high-titer hybrids (Groeneveld, 1997). Thus, the KU1 arms should be better substitutes than artificial sequences.

First, the East arm was replaced by its KU1 homolog. This construct, EKU, is displayed in Figure 3 (for practical reasons only the boxed sequence was exchanged). The titer of EKU is only slightly lower than wild type and maturation protein is made in wild-type quantity (not shown). Considering that the East arm can be replaced by a different hairpin without affecting A-protein yield and titer, we tentatively conclude that the East arm does not contribute to slow folding.

In mutant SKU, the South arm is exchanged for that of KU1 (Fig. 3). This replacement resulted in only a one to two log decrease in titer. Six plaques were evolved for six infection cycles. Three showed a single suppressor mutation in the East arm, but the other three were unchanged. No evolutionary changes were found in the new South arm. Based on the high original titer of mutant SKU the South arm was not considered as contributing to slow folding.

These two constructs were made using the convenient *Sac*I (40) and *Avr*II (113) sites (Fig. 1C). The *Sac*I site in the West arm (40–45) had been artificially introduced by the A45C substitution. This mutation did not affect fitness and it only very slowly reverted to wild type. This *Sac*I site is not suitable for replacing the complete West arm by that of KU1. As a next best solution, we replaced the sequence downstream of the *Sac*I site by one that created a shorter and more regular hairpin (WS1, Fig. 4). The newly introduced sequence is boxed. Considering both the size and the irregularities in the West arm such as the big internal loop and the G•A, A•G motif, we had expected strong effects from the changes. However,

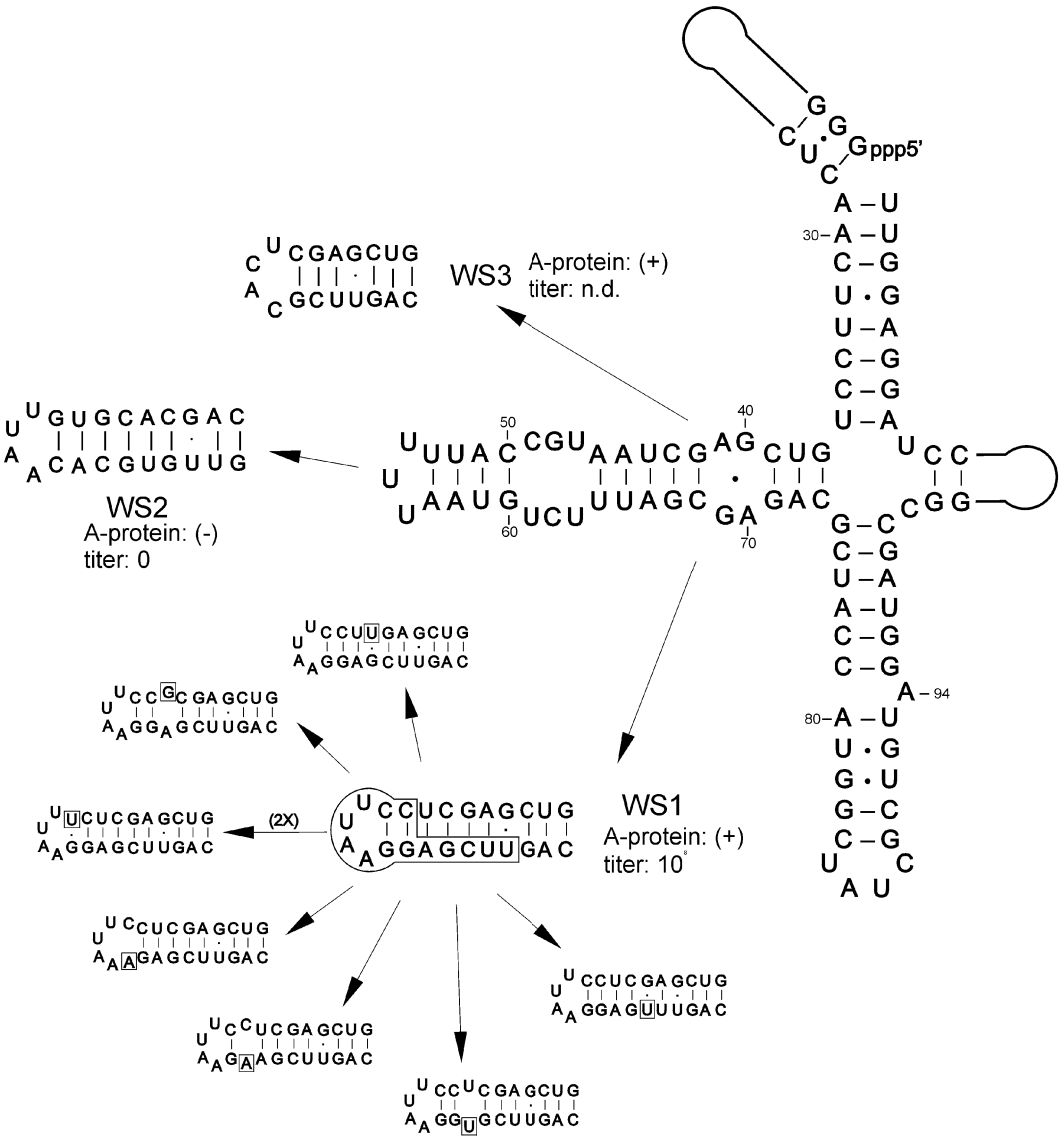

FIGURE 4. Structure of mutants WS1, WS2, and WS3. The complete West arm is exchanged for the sequence indicated. Suppressor mutations selected by WS1 are boxed. WS3 was taken from Groeneveld et al. (1995).

the titer of WS1 and the amount of maturation protein produced were only slightly lower than wild type (Fig. 2). We amplified eight plaques and determined their sequence after seven additional cycles. As shown in Figure 4, almost all of them had selected a different suppressor mutation suggesting that the pressure did not seem to act on the sequence but rather on the stability of the stem, which in WS1 is apparently too high for optimal performance.

Taken together, the mutants EKU, SKU, and WS1 suggested that all of the arms could be replaced by a different stem-loop without seriously affecting phage fitness or yield of maturation protein. Such a conclusion would contradict the presence of a functional metastable intermediate.

Single-nucleotide replacements

We therefore reconsidered the possibility that slow formation of the LDI is an inherent feature of four-way junctions and that tRNA is an exception perhaps due to the presence of the variable loop. However, insertion of AUA between the South and East arms to imitate such a variable loop had a negligible effect on both titer and A-protein yield.

Another possibility seemed that, by itself, the two-dimensional folding of the 5′ UTR is not sufficient to inhibit translation, but needs stabilization by some tertiary structure feature, for instance the pairwise coaxial stacking of the arms at the four-way junction (Thomson & Lilley, 1999). This stacking might then be the slow step. Accordingly, nucleotides at the junction were replaced (C73A; G74A; C101U,A,G), but no strong negative effects were observed. The most pronounced decrease, a three-log drop in titer, was associated with C73A. We also varied or deleted the bulged A94, which is conserved between MS2 and KU1. Here, a 100-fold drop in titer was obtained for the A94 deletion. Five out of six revertants from this mutant had selected the U93A suppressor mutation, suggesting that the A at this position is important. Nevertheless, the effects of ΔA94 and C73A on the titer were relatively mild and not what one would expect if these nucleotides were vital for an essential property of the RNA. Moreover, as none of these point mutations had a significant influence on A-protein synthesis, they probably affected aspects of the life cycle of the phage unrelated to regulation of the maturation protein.

Revisiting the West arm

Upon reanalyzing the revertants from WS1, we noticed that the suppressor mutations were clustered in the upper half of the stem. If weakening the stem would be their only purpose they might also have occurred closer to the junction. We then realized that by exploiting the *Sac*I site (40) for construction of our clones, the sequence in the 5′ part of the West arm (UCGAGCUG 5′) remains identical to wild type. Any intermediate comprising this sequence would have been missed by us. Accordingly, the last possibility to trace a simple kinetic intermediate was to substitute this sequence for a different one. We chose the one shown in Figure 4 as WS2. WS2 resembles WS1 except that many of its base pairs are reversed.

In sharp contrast to WS1, WS2 makes neither maturation protein, nor phages (Fig. 2). This suggests therefore that the wild-type sequence up to nt 44 is necessary for the production of phages and A-protein. In this respect we note that WS3 (Fig. 4), a shorter version of WS1 that still has the critical wild-type sequence, can make normal amounts of A-protein (Groeneveld et al., 1995). Thus, the West arm and in particular nt 37–45 may be involved in delayed cloverleaf folding.

A potential metastable hairpin involving this sequence is shown in Figure 1B. The loop of the hairpin contains the UCS, and the stem is formed at the expense of the adjacent 5′ hairpin and the beginning of the West arm just including the *Sac*I sequence present in WS1 and WS3 but not in WS2. This hairpin is potentially conserved in the related phages fr and KU1 (Fig. 1B,D). To further test its role, we made three new mutants A, B, and AB (Fig. 5). In mutants A and B the metastable hairpin cannot be formed because of several mismatches, but in AB, pairing is restored, albeit with other base pairs (Fig. 5 inset). Indeed, constructs A and B produce no plaques in our standard assay and only very few after artificial lysis. In contrast, mutant AB has a titer that is close to wild-type level (10^8 pfu/mL). This result strongly supports the notion that the metastable intermediate is essential for phage viability.

Mutant B is not expected to make maturation protein and it does not (Fig. 2). For the mutants A and AB, the interpretation is complicated by the fact that the mutations also destabilize the LDI. An A-U and a C-G pair have been replaced by G•U and U•G, respectively. The high level of A-protein synthesis in mutant A (Fig. 2) is probably due to leaky translation from the destabilized LDI. Mutant AB is expected to make similar amounts of maturation protein as A, but for unknown reasons it makes less. Nevertheless, the fact that mutant B does not make any maturation protein is strong evidence that the metastable hairpin is needed for translation of the maturation gene when the LDI is intact. The titer of the three mutants A, B, and AB shows that viability depends directly or indirectly on the potential to form the intermediate.

The sequence of mutant AB did not evolve for at least nine infection cycles, even though the B mutations destroyed complementarity at the bottom of the West arm. Usually, if a mutant has a serious defect, evolution to a better alternative takes a few infection cycles, mostly less than five. It seemed interesting to make a mutant in which the B sequence could again form base pairs with a new D sequence (ABD in Fig. 5).

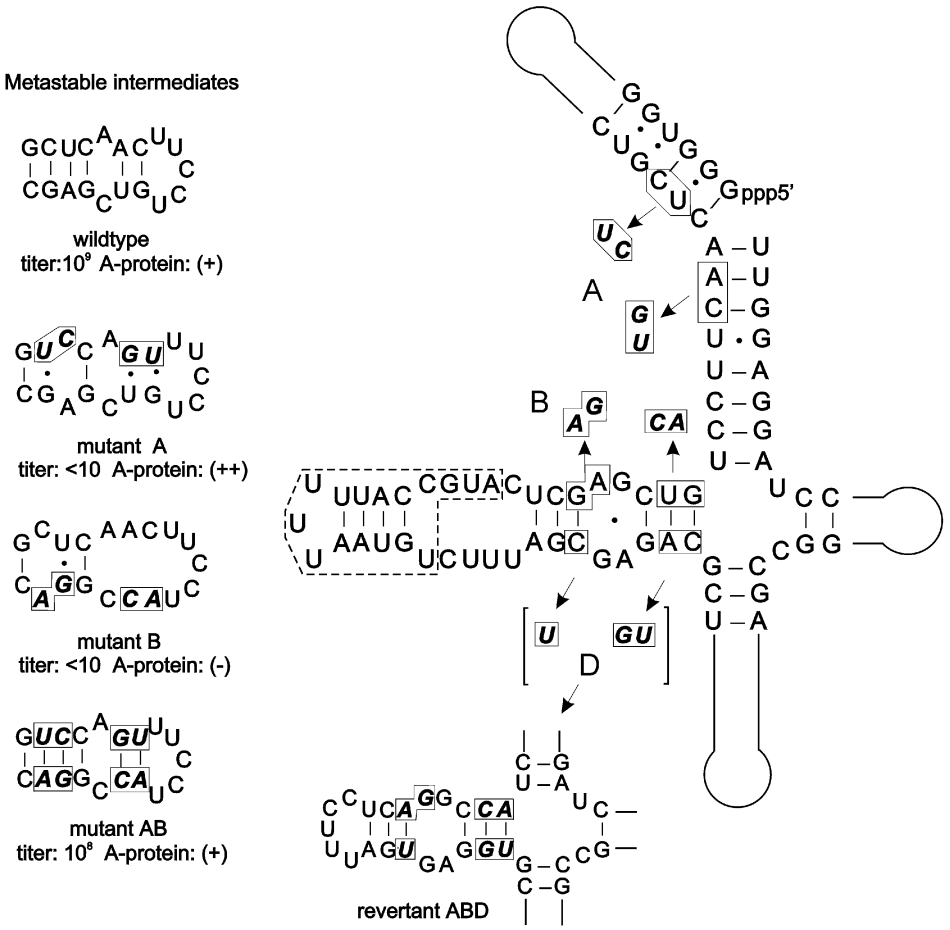

FIGURE 5. Nucleotide changes present in three mutants A, B, and D. On the left, the potential for formation of the metastable structure is shown. Mutations are in bold italics. The dashed box shows the sequence that is deleted upon evolution of mutant ABD. Revertants of mutants A and B carried reversions GU3031AC and AC3738GU, respectively, after nine cycles.

ABD made a normal amount of A-protein, but almost no plaques (<10/mL). The revertants that were obtained had deleted the upper half of the West arm (boxed in Fig. 5). Although we do not know what the driving force is for the deletion, we report the sequence of revertant ABD because it shows in a fully different way that only the bottom part of the West arm is essential. This revertant resembles WS1 in this respect.

Exchange of the West arm for its KU1 homolog

It now became of interest to replace the West arm by that of KU1 to create WKU (Figs. 3 and 6). As can be seen in Figure 1D, KU1 has the potential to form its own metastable structure but the hybrid phage WKU carrying only the West arm of KU1 has not because of the two A•A mismatches (Fig. 6, inset). Therefore, exchange of the West arm for that of KU1 is expected to have a strong effect on fitness and production of maturation protein. Indeed, the WKU hybrid does not produce any maturation protein (Fig. 2) and plaques could only be obtained after artificial lysis and an extra infection cycle in F^+ cells with this lysate (see Materials and Methods). Bulk evolution of the resulting lysate for six cycles yielded the interesting revertant in which two suppressor mutations were found, AA2930UU. As shown in Figure 6, these are precisely the substitutions that allow the revertant phage WKUrev to form a metastable structure closely resembling the ones predicted for MS2 and for KU1. These second site suppressor mutations, therefore, provide strong evidence that the proposed metastable structure is essential for phage survival by acting as the kinetic trap that allows the temporary expression of the maturation protein. In agreement, revertant WKUrev does produce maturation protein (Fig. 2). Although some of this translation may derive from the equilibrium structure, which is weakened by the two suppressor mutations, we note that it is very unlikely that the AA2930UU transversions were selected to get leaky translation. First, mutant A shows

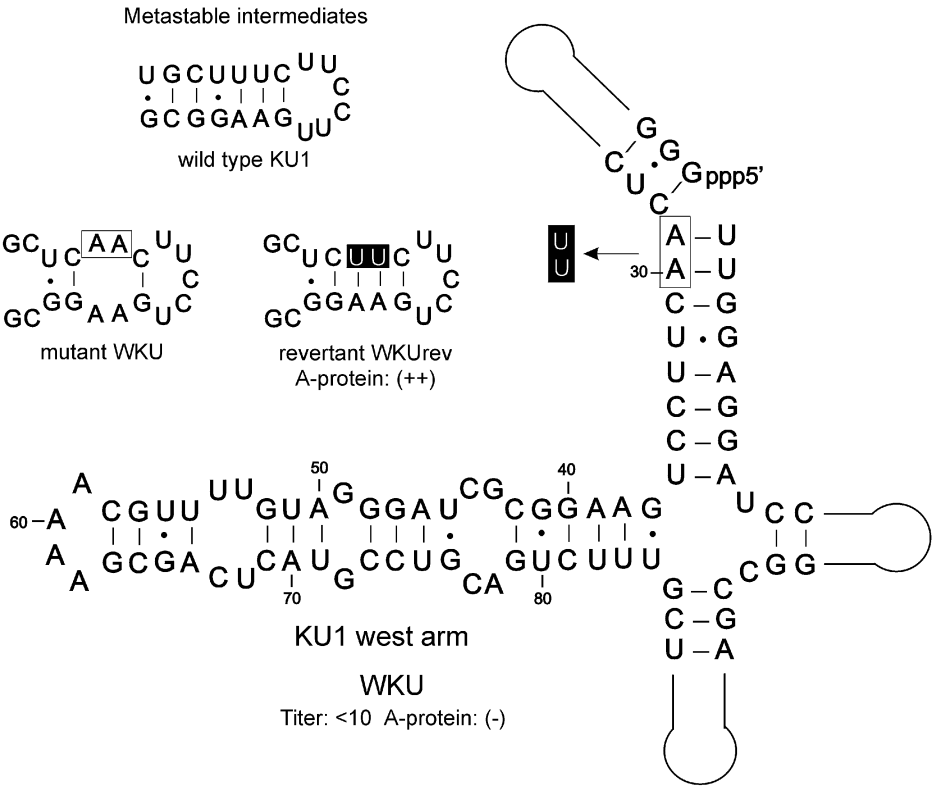

FIGURE 6. Structure of mutant WKU. Suppressor mutations are in black boxes. The inset shows the potential of mutant and revertant to form the metastable intermediate.

that leaky translation is not sufficient to make viable phages and second, leaky translation could have been induced by a variety of transitions in the UCS. Therefore, the only reasonable explanation for selecting the two A → U transversions at exactly these two positions is that the potential to form the metastable hairpin must be recovered.

DISCUSSION

The problem

Previously, we have reported evidence that translation of the MS2 A-protein gene takes place from a nonequilibrium structure of the RNA. Such a nonequilibrium structure is supposed to exist transiently on nascent strands when the RNA is being transcribed from the minus strand (Poot et al., 1997). The equilibrium structure is considered inactive in translation because the ribosome binding site, in particular the SD sequence, is buried in a LDI with an upstream sequence (Fig. 1C).

This LDI, and in fact the complete 5′ UTR structure, can form as soon as nt 123 has emerged from the replicative complex. At this stage, ribosomes cannot yet stably bind the growing RNA chain because the start codon is not there to allow initiator tRNA to clamp the ribosome and mRNA together (Hartz et al., 1991). Thus, in the absence of any folding delay, the A-protein gene would be untranslatable. Taking into account that a ribosome covers about 15 nt downstream of the start codon, a stable initiation complex can only be made when nt 145 appears. Assuming an average speed of 30 nt/s for coliphage replicase (Kondo, 1975), cloverleaf folding should at least be delayed by the 1 s it takes to produce the remaining part of the ribosome binding site.

Not a great deal is known about RNA folding times. tRNA was reported to fold in micro- to milliseconds (Crothers et al., 1974) but it took minutes for the heat-denatured cloverleaf of MS2 RNA to regain its equilibrium form, thus suggesting a special delaying mechanism (Poot et al., 1997). In many cases, such mechanisms turn out to involve a metastable intermediate that is easily formed but from which it is difficult to escape.

The new arguments

If such a kinetic trap would operate here, it is bound to exploit sequences beyond those of a single arm. Accordingly, the strategy was to replace one arm at a time with its KU1 homolog. If that arm donated nucleotides

for the kinetic trap, its replacement by another sequence would make the formation of the intermediate structure impossible. Exchange of the East arm or the South arm had only a marginal effect on A-protein synthesis or phage titer, but when exchanging the West-arm, the A-protein was not made and there was more than an eight-log drop in titer. This result implicated the West arm in the delayed cloverleaf folding. The revertant recovered from this WKU mutant carried the AA2930UU suppressor mutations and this indicated that the metastable intermediate responsible for slow folding was the small hairpin shown in Figures 1B and 6. This idea was strongly supported by comparing mutant WS1 and WS2. WS1 contained the sequence to form the metastable hairpin and this mutant made normal amounts of A-protein. Furthermore, it was almost as viable as wild type. WS2, on the other hand, having several base pairs reversed, could not form the intermediate and it produced neither A-protein nor phages. Further independent support for our model can be derived from mutants A, B, and AB. In A and B, destabilizing mutations are made in the ascending and descending, respectively, parts of the metastable hairpin (Fig. 5). As expected, these mutants produce almost no plaques. In contrast, in mutant AB base pairing is restored and this construct yields a wild-type titer.

The data presented therefore support a simple model in which a small intermediary hairpin carrying the UCS in its loop postpones cloverleaf folding and accounts for temporary translation.

Purpose of the transient translation

Our study seems the first example where a nonequilibrium structure represents the translationally active form while the lowest energy state is inactive. The biological purpose of this mechanism is probably to facilitate the translation–replication switch that must take place in all plus-strand RNA viruses. In the coliphages, the switch is achieved by competition between replicase and ribosome for a common binding site, that is, the coat gene start region (Kolakofsky & Weissmann, 1971). If replicase wins, this clears ribosomes from the three distal genes by translational polarity. This would still leave maturation covered with ribosomes. The translational inactivity of the equilibrium structure ensures that on full-size MS2 RNA, the A-protein gene is not accessible to ribosomes. As a result, replicase will not be dislodged by ribosomes during its travel to the 5′ end.

Mutant phenotypes

In general, all mutants that can form the metastable hairpin produce A-protein and generate phages whereas mutants unable to form the hairpin make neither phages nor A-protein. Mutant A is an exception to this rule. It cannot form the intermediate, yet it makes A-protein. Most likely this is the result of leaky translation from the weakened LDI. As mentioned in the Introduction, mutations that destabilize the LDI increase translation. For the fact that mutant A makes maturation protein but virtually no phages one can think of several explanations.

It is conceivable that the mutations affect other steps in the phage life cycle such as replication or virion assembly. It is also possible that the unabated translation in mutant A precludes the translation–replication switch. We know from previous experiments that a maturation gene that is permanently open (mutant IN) results in a very low titer phage that evolves to regain the folding delay (Poot et al., 1997).

Another interesting and perhaps additional reason why mutant A does not make phages might be that the small metastable hairpin not only regulates translation but that it affects replication via its influence on the structure of the 3′ end of the minus strand. Due to the presence of several G•U pairs in the 5′ hairpin, it is likely that in the minus strand, the equivalent of the metastable hairpin in fact represents part of the equilibrium structure. As shown in Figure 7B, this leads to the exposure of the terminal C residues. In general, single-strandedness of these terminal C residues is needed for replication. However, the mutations present in mutant A disrupt the minus-strand version of the metastable hairpin and may thereby favor formation of the full-length 5′ hairpin (minus-strand version) in which the terminal Cs are not or not sufficiently exposed (Fig. 7C).

Such an indirect role of the metastable hairpin could explain why mutant WS2 is completely “dead.” If its only problem were the lack of A-protein synthesis, it would not be difficult to select one or two mismatches in the LDI to get leaky but unabated translation. However, to evolve the metastable hairpin from the WS2 sequence requires many simultaneous base changes and such an RNA is apparently not present in the quasispecies reservoir.

Consistent with a second role for the metastable hairpin is our finding that construct WS2 still fails to produce phages when the A-protein is provided *in trans*. (A more trivial explanation for our failure to complement mutant WS2 is that the maturation protein is needed *in cis*.)

In vitro and in vivo refolding

Because every virion contains only one copy of the maturation protein, the most efficient design would be that every newly synthesized plus strand would be translated only once. As calculated above, this would need a delay in folding of about 1 s after nt 123 has emerged from the replicase. In vitro, we measured several min-

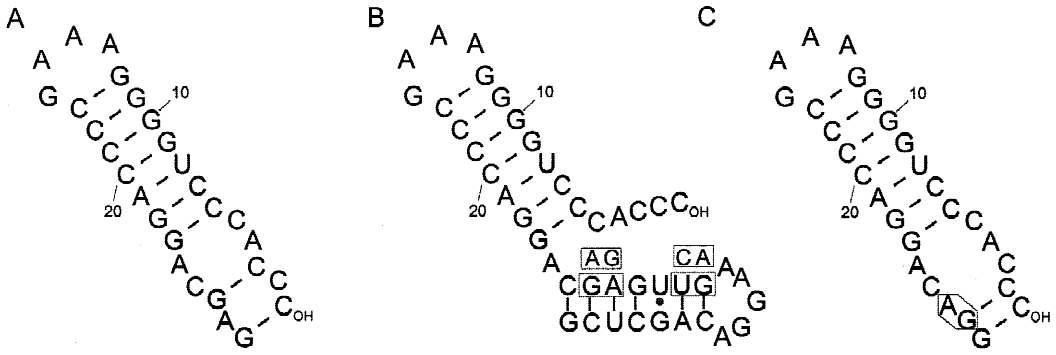

FIGURE 7. **A**: Minus-strand version of the 5′ terminal hairpin showing the destabilization of the lower part by the G•U → C A replacements. **B**: Proposed structure showing that the minus-strand equivalent of the metastable hairpin may permanently expose the terminal C residues. Boxed bases are the changes in mutant A (see Fig. 5). **C**: In mutant A, the minus-strand equivalent of the metastable hairpin is destabilized and this may favor the formation of the full-length 5′ hairpin equivalent in which the terminal Cs are again base paired.

utes of refolding time, and this would allow many translations and yield a large excess of A-protein over RNA. At present, we have no explanation for this apparent incongruity in the model. Possibly, folding in vivo is speeded up by chaperone proteins or the ionic conditions may be more favorable than those of our in vitro experiments (Poot et al., 1997).

The metastable hairpin

Several questions remain unsolved at this stage. For instance, is this alternative hairpin stabilized by interactions with elements in the other arms or by the fact that the initially chosen "wrong" pathway induces neighboring intermediates that must also be resolved to attain the equilibrium structure? These ideas arise because it seems difficult to imagine that this rather weak metastable structure can by itself postpone formation of the final structure. The experiments with the exchanged East arm suggest no major contribution from this hairpin because titers were not seriously affected. On the other hand, some results do indicate a contribution from the South hairpin. For instance, UTR short and WS3 have identical West arms and thus both contain the sequence to form the trap. Yet, only WS3 makes A-protein. WS3 has the wild-type South arm while UTR short has a truncated version of it. This does suggest some contribution to A-protein synthesis by this arm. The bulged A94 conserved between MS2 and KU1 may play a role in stabilizing the trap. Its removal from the MS2 (ΔA94) sequence caused a 100-fold drop in titer, and all revertants but one showed the U93A substitution. In UTR short, this A is not available while it is interacting with the G in the typical GNRA loop. Alternatively, the mere fact that UTR short has less nucleotides may speed up its folding while there are less alternative structures to engage in.

In general ribosomes face fully folded RNA even in nascent transcripts

It has often been suggested that the first ribosome to translate a nascent transcript would face not yet fully folded RNA. Our experiments do not support this notion. Rather, our results suggest that, generally speaking, RNA folds quicker than ribosomes can irreversibly bind. The examples are mutants UTR short, WS2, WKU, and B. None of these mutants produces any maturation protein, although the sequence at the translation initiation site is the same as wild type. This shows that the cloverleaf structure is formed within the time needed by ribosomes to bind irreversibly.

Mutations potentially extending the lifetime of the metastable structure

Although we cannot explain every single detail of the present study, we believe that the data strongly support the model that, during its synthesis, the 5′ UTR of MS2 RNA gets trapped in a metastable structure that allows a short burst of translation from the maturation-protein gene. It is easy to see how this alternative structure works. It captures most of the inhibitory nucleotides in the loop of a small hairpin, thus slowing down or precluding their pairing with the SD region. It is unknown along which pathway equilibrium is reached. Possibly, the loop of the intermediate hairpin forms a pseudoknot with the SD sequence, which then extends pair by pair to yield the final cloverleaf. Alternatively, the top part of the West arm, which can coexist with the trap, may extend itself at the expense of the intermediate. Some indirect evidence concerning this question exists. In a previous attempt to identify nucleotides involved in the kinetic trap, Groeneveld (1997) mutagenized the cloverleaf and mutants showing increased A-protein synthesis were sequenced. Most of these carried multiple mutations, but two changes were highly overrepre-

sented. One of them, U52C, falls outside the trap sequence. One simple explanation is that the mutation retards (or even prevents) formation of the top of the West arm and thus its downward extension to replace the metastable intermediate should also take longer.

The other mutation that led to increased A-protein production is A29G. This makes sense, because this mutation repairs the A C mismatch in the kinetic intermediate and would therefore extend its lifetime at the expense of the equilibrium structure. When measured separately, the A29G mutation indeed increased the A-protein yield (Fig. 2). This result is, however, not completely unambiguous because A29G may also destabilize the LDI slightly. Mutant A29G has a wild-type titer and, as expected from the high titer, its reversion to wild type takes place very slowly.

Kinetic intermediates in other biological systems

Kinetic intermediates with metastable secondary structures are widely used in nature as a way to facilitate and control biological processes. SV-11 RNA is about 200 nt long and is replicatable by Q_β replicase in its metastable form, but not in the ground state (Biebricher & Luce, 1993). Another well-studied example is that of plasmid R1-encoded *hok* mRNA. This RNA carries the information for a lethal protein, which is produced in cells that do not carry the antidote to this messenger, that is, antisense RNA. *Hok* mRNA goes through at least three different structures before it arrives at its translatable form (Gerdes et al., 1997). Here, the length of the transcript plays a role in the structure that is assumed (Nagel et al., 1999).

MATERIALS AND METHODS

Bacterial strains and plasmids

Wild-type and mutant MS2 cDNA plasmids were grown in strain JM109 (*F′ [traD36, proA$^+$ B$^+$, lacI^q, lacZΔM15] recA1, supE44, endA1, hsdR17, gyrA96, relA1 thi* Δ*(lac-proAB))* and M5219 (M72, *trpA$_{am}$*, *lacZ$_{am}$*, *Sm^r/*$\lambda\Delta$*bio$_{252}$*, *cI$_{857}$*, ΔH_1), the latter encoding the thermosensitive λ repressor (*cI$_{857}$*) and the transcriptional antitermination factor N (Remaut et al., 1981). *E. coli* F$^+$ KA797 (*F′ [lacI^Q*, *pro/ara],* Δ*lac-pro, thi*) was used as host to evolve mutant MS2 phages. The strains were grown on LC broth containing per liter 10 g bactotrypton, 5 g yeast extract, 8 g NaCl, 2 g $MgSO_4$, 140 mg thymine, and 1 mL 1 M Tris-HCl, pH 7.6.

Plasmid pUCMS2 is a derivative of plasmid pUC9 in which the *Pst*I-*Nde*I fragment was replaced by the MS2 sequence from position 1 (*Pst*I) to 741 (*Nde*I) taken from plasmid pUCMS21. This plasmid confers ampicilline resistance. Plasmids pMSΔBA and pMSΔSA (Groeneveld et al., 1995) carry the complete MS2 cDNA except for a deletion from the 5′ end to nt 113 and from nt 40 to nt 113, respectively. Plasmid pPLaMS22 contains an A-protein-coat protein fusion and is described in Groeneveld et al. (1995). Plasmids pMSΔBA, pMSΔSA, and pPLaMS22 confer kanamycin resistance.

Construction of mutants

The mutants were constructed by ligating complementary oligonucleotides in plasmid pUCMS2 cleaved with *Pst*I (preceding the MS2 5′ end) and *Avr*II (113) or *Sac*I (40) and *Avr*II (113) depending on the position of the mutation.

The oligonucleotides were hybridized by mixing them in a one to one molar ratio, heating to 70 °C, and slow cooling to room temperature. All clones were checked by nucleotide sequencing.

Phage generation, titer determination, and phage evolution

Phages were generated by the two-plasmid system. Cells harboring the plasmid pMSΔBA or pMSΔSA, carrying the complete MS2 sequence downstream from position 113, were transformed by heat shock, with plasmid pUCMS2 carrying the MS2 sequence from position 1 to 741. Three colonies were picked and grown overnight at 28 °C in the presence of antibiotics. Appropriate dilutions of the supernatant of each culture (cycle 1) were plated on a lawn of KA797 (F$^+$) cells and incubated overnight at 37 °C. Plaques (cycle 2) were counted and from each of the three cultures, two plaques were taken and amplified overnight on KA797 cells in liquid cultures (cycle 3). Phages were passaged for six more cycles by growing them overnight at 37 °C in 2-mL liquid cultures containing F$^+$ cells (cycles 4 to 9). After cycle 9, the sequence of the 5′ UTR (nt 1 to nt 130) was determined using RT-PCR. Revertants always had titers close to wild type.

Using the wild-type sequences, the two plasmids, pUCMS2 and pMSΔBA (or pMSΔSA), produced 10^8–10^9 pfu/mL overnight supernatant. When the mutations were severe, no plaques were obtained with this procedure. In this case, the cells of 10 mL of the overnight culture were pelleted and opened with lysozyme. After concentration by PEG6000, the solution was plated on KA797 cells. On several occasions, even this procedure failed to produce plaques. We considered that the plaques from low virulence phages might be too small to be visible. Accordingly, we introduced an extra cycle; the PEG6000 precipitate was used to infect a liquid culture of KA797. The supernatant of such an overnight culture was then analyzed for the presence of phages. If present, the lysate was evolved in bulk to obtain a single revertant sequence.

RT-PCR and sequence analysis

Phages from 1 μL of an overnight infected *E. coli* culture were dissolved in 10 μL H_2O and heated at 80 °C for 2 min. One microliter was used for RT-PCR in a total of 50 μL according to standard procedures recommended by the suppliers (Sigma-Aldrich and Eurogentec). The primers used were biotin-labeled BIO790 (complementary to nt 1–17) and unlabeled DUI427 (complementary to nt 716–741). PCR fragments were sequenced after separation and purification of the strands using Dynabeads (Dynal) with DUI360 (complementary to nt 146–167).

Expression studies

To measure the A-protein yield of mutants and revertants, the sequence of interest was cloned in the expression plasmid pPLaMS22 carrying the maturation gene fused in frame to the coat gene. Transcription of MS2 cDNA is under control of the thermosensitive P_L promoter from phage λ. *E. coli* M5219 cells harboring the plasmid were grown in 10 mL LC at 28 °C to an OD_{650} of 0.2 whereupon transcription was induced by heating to 42 °C. After 1 h, the whole culture was centrifuged and the pellet resuspended in Laemmli buffer. Half of the samples were used for visualization of the fusion protein by western blotting (de Smit & van Duin, 1990). For immunodetection, an antiserum against SDS-denatured MS2 coat protein was used.

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