Requirements for Template Activity of RNA in RNA Replication

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Abstract

RNA is generally believed to have preceded DNA as carrier of genetic information. In present organisms, RNA replication is restricted to viral RNA. We investigated the requirements for replication of RNA by phage Qβ replicase. RNA replication is highly selective in accepting RNA species as template; it uses single-stranded templates, synthesizing a complementary replica followed by recycling of template and enzyme. The RNA template and replica are not merely substrate and product, but participate actively in the replication process. Sequence comparison of RNA species that are replicated does not reveal a consensus sequence except of the 5′ terminal GGG and the 3′-terminal CCCA. However, the secondary structures of extremely short replicating RNA sequences show a common secondary structure: the 5′ terminus is structured, while the 3′ terminus—where replication starts—is not. It is a severe constraint that this structure must be present in both complementary strands; this is only possible by the participation of G:U base pairs. Mutations altering the secondary structure abolish the template activity. Artificially designed short RNA sequences having this structural property were synthesized and shown to replicate; during amplification the structure was further imporved by selection of advantageous mutants. There is evidence that this structure aids two important features of RNA replication: strand separation during replication and minimizing double strand formation between replica and template strands.

Introduction

When discussing possibilities of artificial life, one has the considerable difficulty to list all criteria a system has to meet to qualify as living. A subset of these conditions already suffices to warrant Darwinian evolution: i) Metabolism, i.e., the system must be far from equilibrium; ii) Autocatalysis, an information carrier must reproduce its information with high fidelity; iii) Mutation, the reproduction fidelity must have a certain limit. There are systems which meet this criteria, the earliest (Mills *et al.*, 1967) and best-understood being RNA replication by an RNA replicase. RNA replication is not used in the expression of normal cells, but is quite commonly used among RNA viruses to amplify there genomes. The RNA replicase of the coliphage $Q\beta$ is particularly stable, can be easily purified to homogeneity and replicates besides $Q\beta$ RNA itself also several short-chained RNA species that are easy to investigate.

Some features of RNA replication are particularly interesting for considering primitive replicating systems and shall be discussed in this paper in more detail: (i) Even though Watson-Crick base-pairing is most likely the basis of replication, replica and template are liberated from the enzyme as single strands (Weissmann *et al.*, 1967; Dobkin *et al.*, 1979, Biebricher *et al.*, 1982). No other proteins than the replicase are involved in the reaction, however, and no nucleoside triphosphates are consumed except the ones that are incorporated into RNA. (ii) The replicase is highly discriminating in accepting RNA as template (Haruna *et al.*, 1963; Biebricher 1983). Therefore, the RNA template is sharing the catalytic properties with the enzyme. It does this by folding its chain into a defined secondary and tertiary structure which is recognized by the replicase and takes part in catalysing the many steps of the replication mechanism.

Quantitative description of evolution parameters

As has been shown qualitatively by Spiegelman and collaborators (Mills *et al.*, 1967; Kramer *et al.*, 1974), the selectivity of the RNA in directing its replication by replicase can be easily used for evolution experiments *in vitro*. This system was used in our studies to derive quantitative evolution parameters from physical chemical properties of the involved RNA species.

The RNA replication mechanism of $Q\beta$ replicase is now well understood (Spiegelman *et al.*, 1965; Weissmann *et al.*, 1967; Mills *et al.*, 1978; Dobkin *et al.*, 1979; Biebricher *et al.*, 1981b, 1982, 1983a,b, 1984, 1985, 1991). The replicase binds in a first step to the RNA template. The binding strength, however, is not strongly correlated to the efficiency of replication: Several RNA unable to replicate, e.g., rRNA, bind replicase quite strongly (August *et al.*, 1968; Silverman, 1973), while some short-chained replicated RNA species bind rather weakly (Prives & Silverman, 1972; Biebricher & Luce, 1993). Replication is initiated by geminal association of two GTP molecules at the 3′ terminus of the template followed by phosphodiester formation. Chain elongation is by synthesizing a replica strand that is antiparallel and complementary to the template from the appropriate nucleoside triphosphate substrates. Replication termination involves replica liberation and

eventually the slow dissociation of the resulting inactive template-replicase complex into its components.

The concentration profiles of RNA show several replication phases:

i) An exponential growth phase where replicase is in excess over template. Both replica and template bind to replicase and start a new cycle. Replica and template are released from the enzyme at different times (Dobkin *et al.*, 1979; Biebricher *et al.*, 1981b) resulting in a Fibonaccian mechanism (Biebricher *et al.*, 1991; Eigen *et al.*, 1991).

ii) A linear growth phase where replicase is saturated with templates. The slow step of template release from the enzyme (which recycles the enzyme) becomes rate-determining (Biebricher *et al.*, 1981b, 1983).

iii) A stationary growth phase where synthesis of single-stranded RNA by replication is balanced by the loss of template strands due to double-strand formation. In this phase, the single-stranded RNA concentration reaches a steady state, and only the concentration of double strands increases (Biebricher *et al.*, 1984).

iv) A decay phase where the synthesis rate drops by reversible binding of double strands to enzyme and eventually by substrate depletion (Biebricher 1986).

The concentration profiles can be calculated by numerical integration of the pertaining differential equations and by compact mathematical equations specific for the growth phase.

Two different RNA species in the same incubation medium share the same environment and compete for resources. Their relative populations change by selection forces. In this system, selection rate values (defined as relative change of the relative population of a genotype) can be calculated when the pertaining rates in the replication mechanism are known. The selection behaviour changes dramatically with the growth phases (Biebricher *et al.*, 1985, 1991; Biebricher 1986):

i) In the exponential growth phase each species grows with its characteristic overall replication rate, which gives a quantitative measure for the selection rate value.

ii) In the linear growth phase species must compete for free enzyme. The selection rate value is not correlated to the overall replication rate, but is proportional to the rate of enzyme binding. The selection rate values are not constant, but change with time and increasing concentrations of the different species.

iii) In the stationary growth phase of one species there is also strong selection for minimizing the rate of double strand formation. Selection values can be negative when the rate of double strand formation exceeds the rate of synthesis of a species (Biebricher *et al.*, 1985; Biebricher 1986, 1987).

iv) Finally, a steady state is obtained where the concentration ratios of the different species one to another remains constant. Depending on the conditions—particularly the rate constants of forming hybrid double strands—stable coexistence of several species or eradication of all species except the fittest is observed. The selective values disappear in this phase (Biebricher *et al.*, 1985, 1991).

Again, the quite complicated concentration profiles of the different RNA species calculated by numerical integration agree with the measured profiles determined by experiments.

Mutation and the formation of a quasispecies distribution

The error rates of RNA replication are regularly several orders of magnitude higher than that of DNA replication, because RNA replication lacks a proof-reading mechanism (Domingo & Holland, 1987; Eigen & Biebricher, 1987). Thus, mutants are produced during replication and compete with each other as different species do. The error rate of $Q\beta$ replicase is about 3×10^{-4} (Batschelet *et al.*, 1976). After a sufficient replication time, a steady state mutant distribution—the quasispecies—is obtained where each mutant type achieves its steady state mutant frequency as a function of its formation rates by mutation as well as its selection rate values (Eigen & Schuster, 1977; Eigen & Biebricher, 1987; Biebricher *et al.*, 1991).

The mutant distribution of the RNA species MNV-11 was investigated. Sequence analysis requires about 10^8 copies of the nucleic acid; thus one has to amplify the single strands. In order to obtain a representative sampling of the mutant population, amplification should be much more accurate than RNA replication and lack a selective bias. This was achieved by cloning the cDNA from the RNA strands into DNA plasmids (Biebricher, 1987; Biebricher & Luce, 1992). The probability of producing a mutated copy is calculated to be less than 3 % per strand and replication round, and one would expect that the majority among those mutants has only one sequence position altered. However, when sampling a population of MNV-11, only 40 % wild type mutants and mutants with several base substitutions were found; base insertions and deletions were also observed. This result is readily explained if one assumes that the mutant distribution is largely determined by selection forces and neutral or nearly neutral mutants thus predominate in the population. This assumption was confirmed by determining the selection rate values of homogeneous mutant RNA populations obtained by transcription from the DNA clones; they were found to be close to the selection rate value of the wild type (Hilliger, 1989; Rohde, Daum & Biebricher, unpublished). The preponderance of multi-error-mutants suggests that adversary mutations can be compensated by further mutations; indeed it was observed that the secondary structure of the RNA was conserved. Regions where the primary sequence was conserved suggest that they play an important role in recognition. If recognition is indeed based on the recognition of a specific nucleotide sequence, then sequence comparison of different RNA species should yield a consensus sequence.

Sequence comparison of different RNA species

A large number of different RNA species replicated by $Q\beta$ replicase are published (Mills *et al.*, 1973, 1975; Schaffner *et al.*, 1977; Priano *et al.*, 1987; Biebricher 1987; Munishkin *et al.*, 1988; Biebricher & Luce, 1992, 1993). Of particular interest are the sequences of the very short (35–45 nucleotides) 'minihelices' that are formed independently in template-free incorporations

(Biebricher *et al.*, 1981a, 1986; Biebricher 1987; Biebricher & Luce, 1993). Sequence comparison, however, reveals no consensus sequence except for the invariant termini: the sequences of both complementary strands begin with pppGG(G) and end with (C)CCA, whereby the 3′-terminal A is attached by the replicase to the synthesized replica without a complementary nucleotide at the template. Even the natural templates, the viral Qβ RNA and its complement, bind to replicase by different mechanisms (Barrera *et al.*, 1993). Additional information regarding requirements for replication is obtained from experiments inserting foreign RNA into the sequence of replicating RNA species (Axelrod *et al.*, 1991; Wu *et al.*, 1992; Biebricher unpublished). Apparently quite large pieces of RNA can inserted as long the ends are preserved and the RNA insert meets some structural criteria (Axelrod *et al.*, 1991).

Furthermore, there is at least one replicating RNA species that can be folded into more than one secondary structure, only one of which is an active template (Biebricher *et al.*, 1983; Biebricher & Luce, 1992). Therefore, as in many other examples of RNA recognition (Giegé *et al.*, 1993), what is recognised is not the primary sequence, but rather certain chemical side groups in a defined sterical arrangement. How can one analyse the structural requirements? No tertiary structure has been determined yet and data about the secondary structures are also limited. It is still not possible to calculate tertiary structures from the nucleotide sequence of an RNA, despite considerable progress in modelling (Westhof & Michel, 1992). Good algorithms are available for calculating secondary structures, (Zuker & Stiegler, 1981; McCaskill 1990), even though often several structures with little energy differences are found; biochemical tools must decide among these.

The calculated secondary structures – particularly of the very short sequences where only one stem can be formed – show striking similarities. While the 5′ termini are usually involved in double-helical structures, the 3′ termini are unstructured. This latter feature has been noticed previously (Schaffner *et al.*, 1977). Since the complementary strands are antiparallel, one would expect that a double-helical 5′ end in one strand would correspond to a 3′-terminal double helix in the complementary strand. However, both complementary strands show 5′ terminal double helices, caused by the contributions of G:U base pairs at strategic positions (Biebricher & Luce, 1993).

Artificial RNA templates

The shortness of some replicating sequences suggests a synthetic approach to test whether the 5′ terminal structure is required: An RNA species found previously to replicate was synthesized by transcription from synthetic oligodeoxynucleotides by T7 RNA polymerase (Milligan & Uhlenbeck, 1989). As expected, the synthetic RNA was accepted by Qβ replicase. After 20 replication rounds, the progeny RNA was sequenced and found to be a quasispecies distribution around the starting sequence. This was expected, because the sequence had already reached the local optimum in the fitness landscape. A mutant of this sequence was synthesized where 4 point mutations stabilized the 5′ double helix in one strand, but favoured in the

complementary strand a terminal 3′ terminal double helix (Fig. 1). In agreement with the above hypothesis, this RNA could no longer be amplified by Qβ replicase and had thus also no chance to revert. This suggests that the structural condition is necessary.

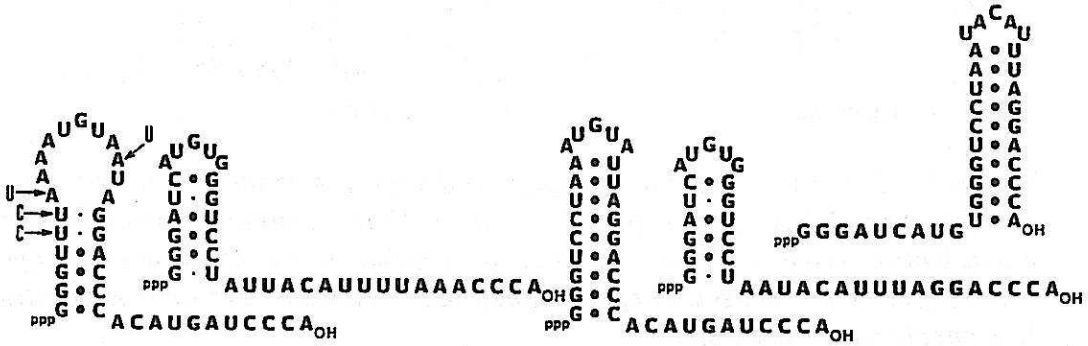

Figure 1: *Tentative structures of plus and minus strands of a short replicating RNA species (left) and of a mutant unable to replicate (right). The altered positions are indicated in the left part. Two structures are shown for the mutant minus strand, the rightmost being the most stable.*

A satisfying proof that the proposed condition is also sufficient for replication is nearly impossible to supply. A reasonable approach is to synthesize a few RNAs that have the required structural features, but were not found in previous experiments and test their ability to replicate. If they do replicate without exceptions, there is good evidence that the ability to replicate is correlated with the structural feature. So far, we have investigated only two examples, both of which are in good agreement with the hypothesis. Short RNA sequences with 5′ terminal helices in both complementary strands were synthesized and found to trigger synthesis by Qβ replicase, even though rather inefficiently. In devising an artificial template, it is quite unlikely to hit by chance a local fitness optimum in the sequence space. Thus the progeny RNA produced by replication was investigated by cloning and sequencing. A sequence drift with a few base exchanges was observed (Fig. 2). It was shown by another few rounds of replication followed by cloning and sequencing that this mutant RNA had now reached its local fitness maximum. The base exchanges or base insertions favoured by the sequence context did improve the structural feature we consider as being required for replication. In two other examples, optimization by mutation included duplication of sequence parts by recombination (Biebricher & Luce, 1992), accompanied by an increase in the nucleotide chain length of the RNA.

Minihelices are of course poor substitutes for optimized replicating RNA species that have longer chain lengths. Certainly many additional sequence features do contribute to improve the replication efficiency of RNA. In particular, the short sequences bind replicase only weakly and are thus inefficient templates; optimized RNA species with longer sequences probably contain some additional sequence parts specific for replicase binding (Nishihara *et al.*, 1983). Highly conserved interior stems are found in the RNA species MNV-11 which are probably responsible for replicase binding.

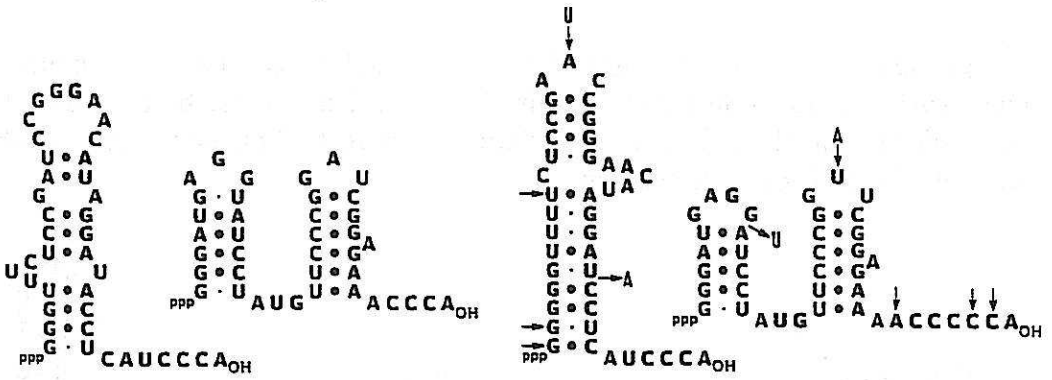

Figure 2: *Tentative structures of plus and minus strands of an artificially deviced short RNA species (left) and the optimized mutant formed during amplification (right). Arrows between two symbols designate base exchanges, arrows from internucleotide spaces mean base deletions and arrows pointing to symbols base insertions.*

One can speculate why this structure may be important. During replication a single-stranded complementary replica strand is synthesized and then template and enzyme are recycled. What is occurring at the molecular level at the replication fork is still in the dark, but the presence of a complement suggests the transient formation of a double helix between replica and template. During chain elongation the replica must be peeled off the template by stepwise strand separation and the nascent replica strand must be protected from re-forming a double strand with the template. Stem structures within the replica (and probably also the template) could serve for this purpose and are particular important at the 5′ terminus of the replica where strand separation starts. There are several hints supporting this hypothesis: When strands can not separate, e.g., in the terminal elongation reaction producing hairpins (Biebricher & Luce, 1992), replication stops after incorporation of a few nucleotides. Furthermore, medium-length replicating RNA species are usually highly structured (Biebricher *et al.*, 1982), but formation of branched helices, e.g., cloverleaf structures, is not observed, since they could form only after liberation of a large single-stranded stretch. Inserting unstructured RNA stretches into the sequences of replicating RNA species diminishes the replication efficiency (Axelrod *et al.*, 1991).

Double strand formation

Replication of viral RNA produces its antisense RNA. Obviously the presence of the antisense RNA is not noxious, because formation of a double strand between sense and antisense RNA proceeds so slowly that the small proportion of the annihilated function can be tolerated. The sequence complexity of short-chain replicating RNA species, however, is much lower, and double strands form much more rapidly. The secondary structures of both complementary RNA strands inhibit double strand formation and replicating RNA species were found to melt cooperatively at abnormally high temperatures (Biebricher *et al.*, 1982). In our investigation of double strand formation (Biebricher *et al.*, 1984) we found that replicating RNA is protected by the replicase from double strand formation, but with some RNA

species double strand formation may occur already during replication (Priano *et al.*, 1987; Axelrod *et al.*, 1991). The kinetics of double strand formation of free plus and minus strands of the RNA species MNV-11 was investigated in our laboratory (Rohde, Daum & Biebricher, unpublished). In a first second-order reaction step, an adduct between the complementary strands is reversibly formed, where the intramoleular base-pairing is still largely preserved and the intermolecular double helix is restricted to a short stretch between exposed regions of the two strands. This adduct reacts in a cascade of irreversible first order reactions to the complete double helix between plus and minus strand. The exposed regions on the strands that make the first interstrand contact were localized by masking them with complementary oligonucleotides.

Optimized replicating RNA species are selected for minimizing their rate of double strand formation. In large RNA molecules, regions sensitive to double strand formation can be buried in the interior of the RNA bulk. Medium-sized replicating RNA species were found to contain particularly stable RNA stems (Biebricher & Luce, 1993; Tuerk *et al.*, 1988). Short-chained RNA species can minimize the rate of double strand formation by the described folding of the 5′ termini, because in this way the maximum number of bases is made unavailable for contact in either of the two strands; loops in one strand forming stems in the complementary strand.

Discussion

RNA is usually synthesized as single strands, be it by transcription or by replication. There are a few examples where DNA single strands are synthesized in replication, e.g. with some viruses or in bacterial conjugation, but single-stranded DNA must be always covered with proteins to prevent it from being converted into a double strand. At least in prokaryotes, there is no participation of melting proteins in synthesis of RNA strands. The secondary structure formation of the RNA probably suffices to prevent double strand formation. In RNA synthesis by transcription, the double-stranded DNA template is unwound before synthesis and is re-formed after transcription. In RNA replication, single strands are produced without a concurrent formation of a double strand; double strands are completely inactive to replicate (Biebricher *et al.*, 1982, 1984). It is unlikely that catalysing strand separation is restricted to RNA replicases, because the RNA polymerases of *Escherichia coli* (Biebricher & Orgel, 1973) and coliphage T7 (Konarska & Sharp, 1989) have been shown to behave as replicases if supplied with a specific template RNA. Therefore, the structures of template and replica RNA probably aid strand separation to a large extent. The extraordinary specificity of all RNA replicases suggests that much of the replication potential is provided by the RNA itself. However, we observed that RNA species replicated by $Q\beta$ replicase are not accepted by T7 RNA polymerase and vice versa (Biebricher, unpublished). Replication is thus a concerted action of the replicase and the template and replica RNA strands.

Primitive RNA replication could have been mediated by ribozymes (Doudna & Szostak, 1989; Doudna *et al.*, 1993). All known ribozymes are highly specific for their RNA substrates (Altman *et al.*, 1993; Cech *et al.*,

1993). It seems plausible that primitive RNA replication (i) was highly selective in accepting RNA templates and (ii) proceeded by strand separation and not by completion of a template-primer complex to a perfect double strand.

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