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In vitro recombination and terminal elongation of RNA by Qβ replicase

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SV-11 is a short-chain [115 nucleotides (nt)] RNA species that is replicated by Qβ replicase. It is reproducibly selected when MNV-11, another 87 nt RNA species, is extensively amplified by Qβ replicase at high ionic strength and long incubation times. Comparing the sequences of the two species reveals that SV-11 contains an inverse duplication of the high-melting domain of MNV-11. SV-11 is thus a recombinant between the plus and minus strands of MNV-11 resulting in a nearly palindromic sequence. During chain elongation in replication, the chain folds consecutively to a metastable secondary structure of the RNA, which can rearrange spontaneously to a more stable hairpin-form RNA. While the metastable form is an excellent template for Qβ replicase, the stable RNA is unable to serve as template. When initiation of a new chain is suppressed by replacing GTP in the replication mixture by ITP, Qβ replicase adds nucleotides to the 3′ terminus of RNA. The replicase uses parts of the RNA sequence, preferentially the 3′ terminal part for copying, thereby creating an interior duplication. This reaction is about five orders of magnitude slower than normal template-instructed synthesis. The reaction also adds nucleotides to the 3′ terminus of some RNA molecules that are unable to serve as templates for Qβ replicase.

Key words: copy choice mechanism/Qβ replicase/RNA recombination/RNA replication/self-replicating RNA

Introduction

Experimental observation of evolutionary adaptation has been possible using a system where certain RNA species are replicated by Qβ replicase (Mills *et al.*, 1967; Levisohn and Spiegelman, 1968, 1969; Saffhill *et al.*, 1970; Kramer *et al.*, 1974; Biebricher *et al.*, 1982; Biebricher, 1983). Evolution of RNA species is often accompanied by jumpwise changes of their molecular weight. In most experiments, the evolutionary changes were not reproducible due to the stochastic nature of such processes (Biebricher, 1983). However, when optimized self-replicating RNA species were used, reproducible selection of new variants could be observed, offering an opportunity to study the molecular processes *in vitro*. SV-11 (115 nt) is reproducibly selected from the optimized species MNV-11 (87 nt) under conditions of higher ionic strength and long incubation times (Biebricher *et al.*, 1982). These observations raise some puzzling questions. (i) Is SV-11 really an evolutionary descendant of MNV-11 or merely an impurity which gets selected under the new conditions? (ii) If it is a descendant, what is the origin of the added material in SV-11? (iii) Why does this process lead to reproducible products?

In this article, we supply experimental evidence that SV-11 is indeed a descendant of MNV-11 and that addition of new genetic material originates from copying parts of the sequence. Furthermore, we show that Qβ replicase can modify RNA molecules by attaching nucleotides to the 3′ terminus of the RNA in a non-random manner, using parts of the RNA molecule as a template. A possible mechanism for this RNA recombination is discussed.

Results

Selection of SV-11 from MNV-11

MNV-11 is an optimized self-replicating RNA species with a chain length of 87 nt. It can be amplified by Qβ replicase indefinitely without selection of another species; an evolutionary stable quasi-species distribution is formed (Biebricher, 1987). However, when MNV-11 was amplified with replicase for longer time periods in the presence of 50 mM $(NH_4)_2SO_4$, after a few serial transfers, a new species, SV-11, was reproducibly selected having a chain length of 115 nt (Biebricher *et al.*, 1982). When the experiment was repeated, the number of transfers required to select SV-11 varied, but the selection product was always SV-11. For the following reasons it is unlikely that SV-11 is merely an undetectable contaminant in the preparations of MNV-11 or replicase which is disadvantaged under conditions of low salt, but gets selected under conditions of high salt. (i) The sequences of both species are strongly related; even before their sequences were determined, the similarity of the fingerprints (Sanger *et al.*, 1965; Biebricher *et al.*, 1981) and the cross-hybridization of the two RNA species suggested a close kinship. (ii) If template is omitted, no self-replicating species can be found even after prolonged incubation in the presence of high salt (Biebricher *et al.*, 1981) ruling out the presence of a salt-resistant contaminant in the enzyme preparation or the incubation buffer; (iii) the species SV-11 can also be selected after recloning MNV-11 from a single RNA molecule; (iv) different salt-resistant species with unrelated properties are selected from RNA species other than MNV-11.

The reproducibility of the formed new species is in sharp contrast to the results of *de novo* synthesis (Biebricher *et al.*, 1981) and the optimization of inefficiently reproducing RNA species (Biebricher, 1983), which led to selection of different RNA species even when the experiments were repeated under identical conditions. The reproducibility in selection immediately suggests an instructed process; however, what is the source of the instruction?

Straightforward conclusions about the origin of SV-11 can be reached by sequence comparison of MNV-11 and SV-11. Figure 1 shows that the SV-11 sequence is homologous to the plus and the minus strand sequences of MNV-11;

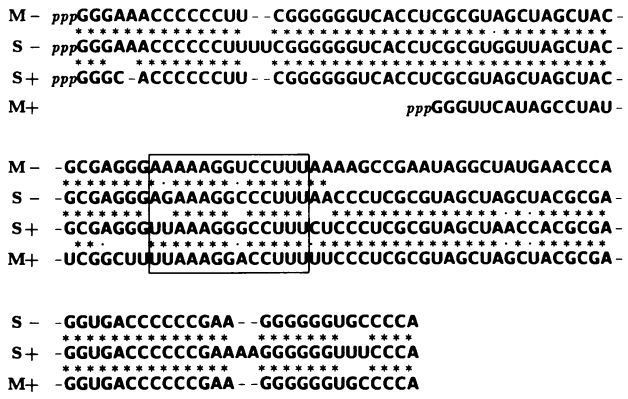

Fig. 1. Sequence homology between plus and minus strands of MNV-11 and SV-11. Aligned are the sequences, from top to bottom: MNV-11 minus strand, SV-11 plus strand, SV-11 minus strand, MNV-11 plus strand. Base homologies are marked with stars, base transitions with dots. The region where copy choice may have occurred is boxed; the most likely position is at the right edge of the box. Qβ replicase adds a 3′-terminal A residue to the replica strands (Weber and Weissmann, 1970).

apparently the SV-11 sequence is a recombinant between the plus and minus strands of MNV-11 and thus indeed an evolutionary descendant of MNV-11.

As little as one or a very few strands of MNV-11 or SV-11 could be readily detected within a large population of the other RNA species by amplifying the mixture under the appropriate selection conditions. MNV-11 has a higher overall replication rate than SV-11 and was thus selected in the exponential growth phase (Figure 2, top). It has been shown previously that in the linear growth phase RNA species are selected not for their overall growth rates, but rather for their rates of enzyme binding and for low rates of double strand formation (Biebricher *et al.*, 1985). Even the very rare recombination event producing SV-11 could be quantified by subjecting an MNV-11 population to several independent transfer experiments under conditions of 50 mM $(NH_4)_2SO_4$ and long incubation periods. The probability of formation of SV-11 from MNV-11 could then be estimated from recording the time of SV-11 emergence and extrapolating it to the production of the first strand of SV-11 using the measured competition kinetics in the linear growth phase (Figure 2, bottom). On average, one SV-11 strand was produced in 10^{13} replication rounds of MNV-11. The kinetic study confirmed that the MNV-11 population used for selection of SV-11 did not contain a single strand of SV-11. When the selection procedure was repeated, the number of transfers required for selection of SV-11 varied. In the absence of ammonium sulfate, SV-11 production from MNV-11 was not detected. This is not due to differences in the selection process, because SV-11 introduced into a MNV-11 population is selected in the linear growth phase at both high or low ionic strength; it is thus rather the frequency of recombination which is affected by the ionic strength.

Selection experiments under the selection conditions of SV-11, but using other self-replicating RNA as starting material, led to the different adapted self-replicating RNA species SV-5 and SV-7 whose fingerprints and other properties have been found to be unrelated to those of SV-11 (Biebricher *et al.*, 1982). They can be amplified indefinitely by serial transfers without selection of SV-11. Both were cloned and sequenced. Their sequences (Figure 3) indeed

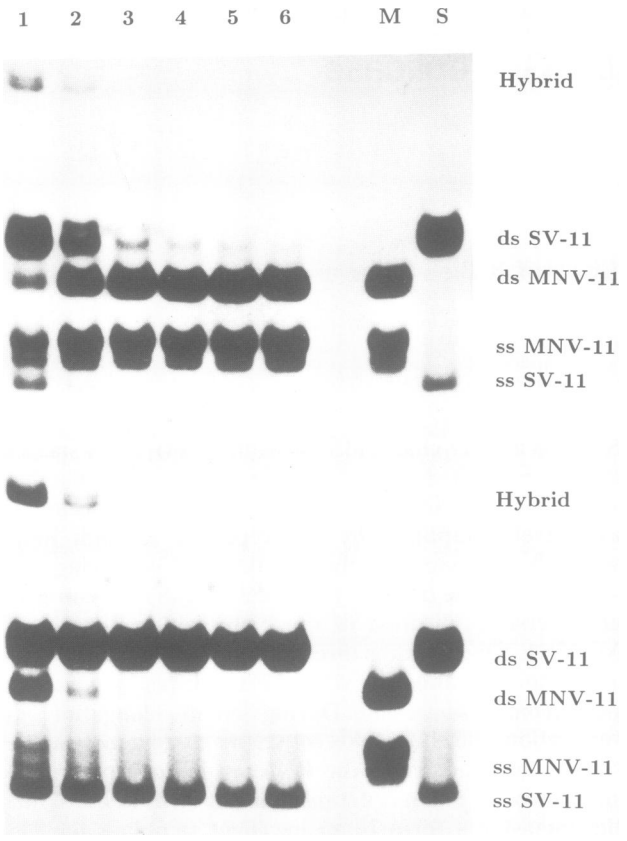

Fig. 2. Competition between MNV-11 and SV-11. Upper electropherogram: competition in the exponential growth phase. 20 pM MNV-11 and 80 pm SV-11 were incubated in Qβ buffer containing 50 mM $(NH_4)_2SO_4$ and 500 μM each CTP, GTP, UTP, 500 μM [α-^{32}P]ATP (sp. act. 700 Bq/nmol) and 100 nM holo Qβ replicase. Transfers were incubated for 5 min at 30°C and diluted 1000-fold into fresh mixture without template. The reaction was stopped by adding EDTA and formamide (20%) and loaded onto a 12.6% polyacrylamide gel. Electrophoresis was for 24 h at 3.6 V/cm, autoradiography for 24 h. The double band with the lowest mobility is a hybrid double strand between MNV-11 and SV-11; the hybrid can also be formed directly by heating MNV-11 single strand with the corresponding SV-11 single strand. Plus and minus strands of MNV-11 are separated under the electrophoresis conditions. Lower electropherogram: competition in the linear growth phase. The conditions are the same as above except that the template was diluted 50-fold into fresh mixture after 30 min incubation at 30°C. The first transfer was started with a 50-fold dilution of transfer 1 of the upper panel.

show homology neither to SV-11 nor to other known self-replicating RNA species. SV-5 and SV-7 both contain interior tandem and inverse duplications which might have also originated from recombination events. Unfortunately, the ancestors of SV-5 and SV-7 have been lost; therefore comparison of the sequence of salt-resistant RNA species with that of their ancestor was not possible. Sequence duplications are often observed in other self-replicating RNA species: MDV-1 seems to be composed almost entirely of a few oligonucleotide motifs (Biebricher *et al.*, 1981); in RNA species synthesized *de novo* duplications of sequences are also often observed (Biebricher,C.K. and Luce,R., submitted).

SV-11 RNA has a metastable structure

Recombination between a plus and a minus strand inevitably results in a palindromic sequence which may fold into a

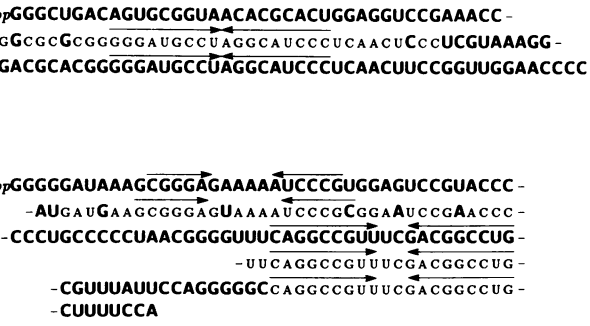

Fig. 3. Sequences of SV-7 (top) and SV-5 RNA (bottom). Tandem duplications are marked with small capitals, inverse duplications with arrows.

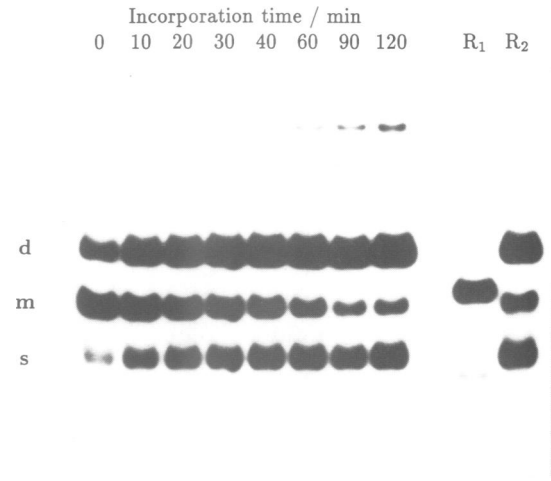

Fig. 4. Pulse–chase experiment SV-11 replication. The replication mixture contained in Qβ buffer 0.3 mM each CTP, GTP and UTP, 0.1 mM [α-^{32}P]ATP (sp. act. 4×10^3 Bq/nmol), 10 nM SV-11 and 100 mM holo Qβ replicase. After pulse labelling for 10 min, the ATP concentration was adjusted to 1 mM (chase) and samples were withdrawn at the times shown, shock frozen and stored until loading them onto a 14% polyacrylamide gel. Note the decay of the metastable band (m) and the increase of the double strand (d) and the stable form of SV-11 (s). At pulse times of 1 min (not shown), only the metastable form is detected.

hairpin structure. In fact, the hairpin structure of SV-11 has been predicted previously from structural studies (Biebricher *et al.*, 1982). It has been shown that the hairpin structure is unable to replicate; pulse–chase experiments (Figure 4) show that the active template is a metastable structure formed during replication. The metastable structures of the SV-11 strands rearranged spontaneously to their stable hairpin structures; under physiological conditions rather slowly, but rapidly at elevated temperature. After prolonged standing or short boiling, the template activity of SV-11 was irreversibly lost. The efficiency of replication of an RNA by Qβ replicase is thus not dependent on the presence of certain elements of the primary sequence alone, but requires specific secondary and tertiary folding of the template chain. Metastable RNA conformations important for the biological activity have also been found with other RNAs (Loss *et al.*, 1991; van Tol *et al.*, 1991).

Figure 5 shows the tentative metastable structures of the plus and minus strands of SV-11, which were assumed to be the same as those of homologous parts of the MNV-11 sequence. The structures of the MNV-11 strands shown are supported by nuclease digestion experiments (Biebricher, 1987; Biebricher,C.K. and Luce,R., unpublished). The stable structures of SV-11 were calculated by the algorithm of Zuker and Stiegler (1981). Melting curves of MNV-11 RNA (Biebricher *et al.*, 1982) revealed two domains that melt at different temperatures. The two stems of MNV-11 with the highest stabilities have been duplicated by recombination and are thus present in SV-11 at both the 5′ and the 3′ termini. During RNA replication, parts of the replica and template chains fold before the replica is completed. Rearrangement of the formed metastable structures to the stable hairpin structures requires at least partial melting of the stems found at the termini.

Modification of the 3′ terminus of RNA by Qβ replicase

The proposed formation of SV-11 by a replication error is not directly observable, because it is such a rare event. However, there is biochemical evidence that Qβ replicase can modify RNA by attaching sequences at the 3′ terminus that are complementary to parts of the RNA sequence. Under normal conditions, this reaction was not observed, because the replicase rapidly starts a new round of replication at the 3′ terminus. However, when GTP was replaced by ITP, initiation of a new replica chain cannot occur (Feix and Sano, 1975; Mills and Kramer, 1979). A slow reaction took place shifting the electrophoretic mobility of the MNV-11 RNA to higher values (Figure 6). Complete denaturation of the products by glyoxylation (McMaster and Carmichael, 1977) showed that the modification increased the chain length of the MNV-11 template. The reaction was entirely dependent on the action of replicase: the thermal denaturation profile of the reaction was in complete agreement with the thermal denaturation profile of the replicase activity (Figure 6). Furthermore, the reaction required Mg^{2+} and triphosphates which were incorporated into the chain.

Sequencing the 5′ and 3′ termini of the modified RNA (Donis-Keller *et al.*, 1977) showed that the 5′-terminus was unchanged while the sequence of the 3′ terminus was modified in a heterogeneous, but not random way. Hybridizing the modified strand with the complementary strand and digesting the hybrid with mung bean nuclease restored an unmodified and intact MNV-11 sequence (data not shown) and removed all nucleotides incorporated by the modification reaction; therefore, the modification must be an elongation of the original RNA sequence.

To investigate the nature of the added material, a representative selection of elongated sequences was cloned and sequenced. For this purpose, terminally elongated MNV-11 plus and minus strands were polyadenylated and transcribed into cDNA with the help of a primer containing a $(dT)_8$ stretch having a different nucleotide at the 3′ terminus to achieve phasing of the priming at the correct start position. The phasing failed in most cases, however; instead, a longer oligo(A) region with a G replacing the A after the first eight positions was found. Apparently, the primer bound rather to the 3′ end of the template and the G:A mispair at the 3′ terminus of the primer did not prevent the reverse transcriptase from synthesizing cDNA.

The sequences of the terminal additions were found to be heterogeneous, as predicted from the earlier observations, but were complementary to parts of the original sequence, particularly to the 3′ terminus itself, except for a few A residues inserted without instruction at the 3′ terminus of

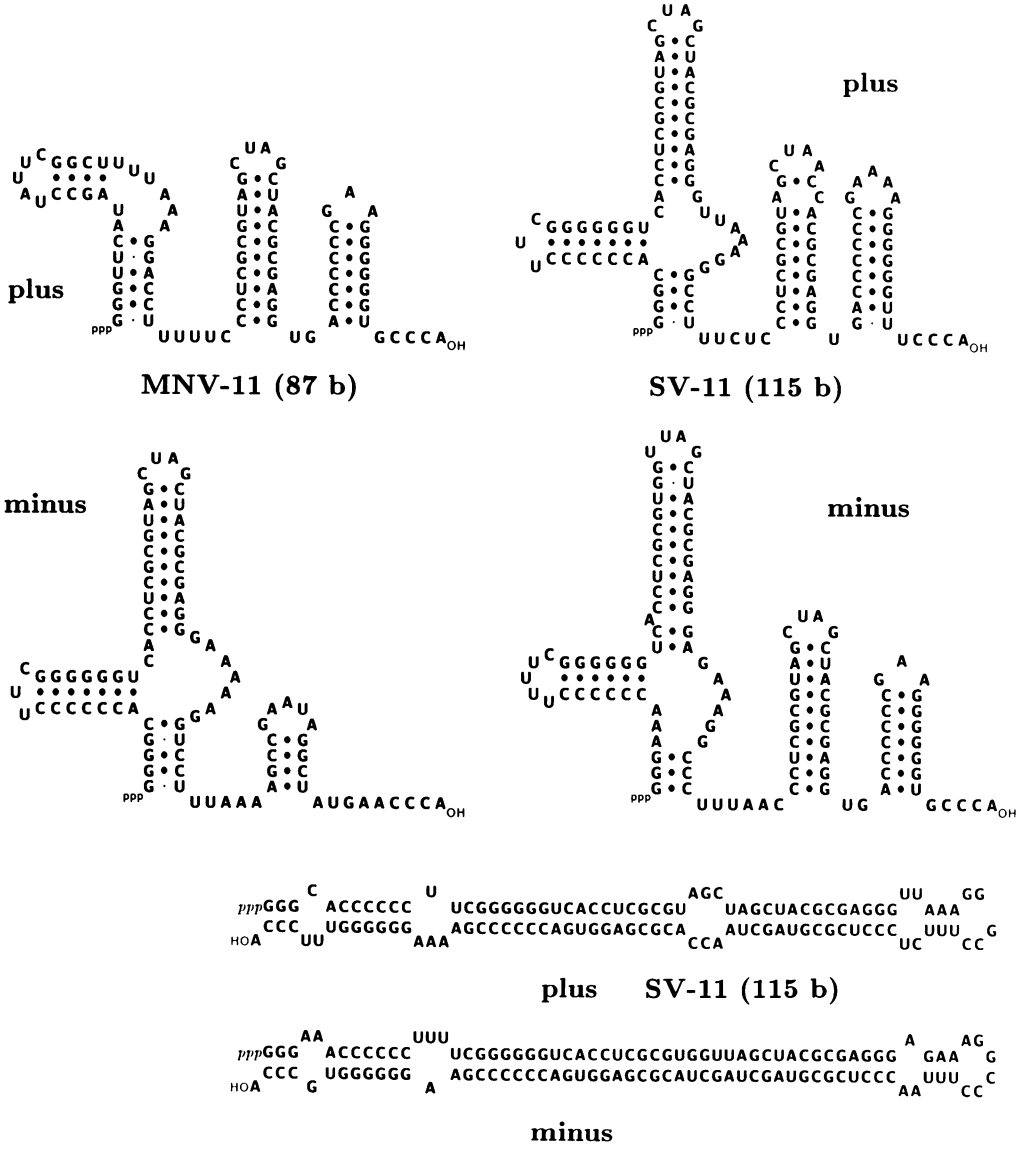

Fig. 5. Secondary structures of MNV-11 and SV-11 plus and minus strands. The metastable structures (top) and the stable forms (bottom) of the SV-11 strands are shown. b, nucleotide chain length.

the sequence (Figure 7, top). In some cases, the terminal elongation was iterated by using the newly added 3′ terminal sequence as template (Figure 7, bottom). Termination of the reaction occurred preferentially at positions where a secondary structure hampered the availability of part of the sequence as template.

The described reaction was not limited to templates of Qβ replicase. RNA that binds only weakly to Qβ replicase like tRNA did not react, but other RNA species that bind more strongly to Qβ replicase, e.g. rRNA, were found to incorporate a few nucleotides at the 3′ terminus of the template. This reaction was at least five orders of magnitude slower than template-instructed incorporation and stopped after addition of a few nucleotides, altering the electrophoretic mobility of longer RNA chains only at a barely detectable level. The sequences of the added 3′ termini to rRNA were not determined, but denaturation studies of the RNA by glyoxylation clearly indicated that initiation of new chains did not occur.

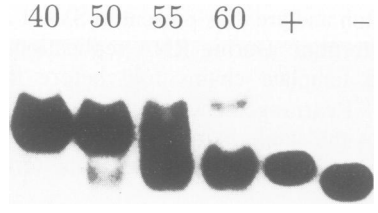

Fig. 6. Terminal elongation of MNV-11 by Qβ replicase heated to different temperatures. The reaction conditions are described in Materials and methods. The enzyme stock solution was heated for 10 min to the indicated temperatures (40, 50, 55 or 60°C) and used at 100 nM and 30°C for the terminal elongation and for replication assays. The replication activities of the heated enzyme probes in relation to untreated enzyme were 100% at 40°C, 50% at 50°C, 15% at 55°C and 0% at 60°C. The RNA was separated by gel electrophoresis and stained with ethidium bromide. + and – are untreated plus and minus strands of MNV-11.

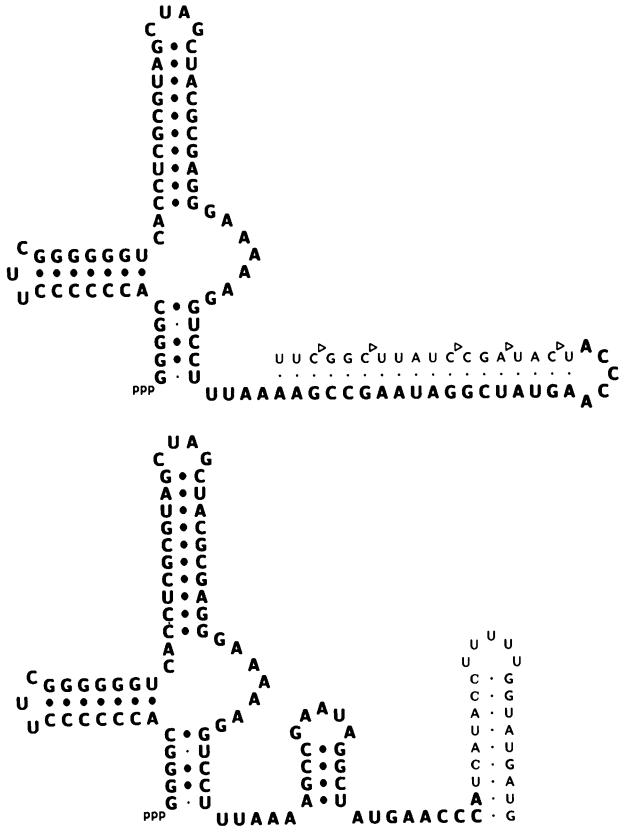

Fig. 7. Sequences of the terminally elongated MNV-11 minus strand. MNV-11 minus strand were elongated by Qβ replicase and cloned into PUC18 as described in Materials and methods and different clones were sequenced. **Top**: the open triangles designate 3′ ends of different clones. **Bottom:** a loop formed as shown on the left has undergone another elongation round.

Discussion

RNA recombination has been observed with a variety of animal and plant viruses (King *et al.*, 1982; Lai *et al.*, 1985; Allison *et al.*, 1990; van der Kuyl *et al.*, 1991; Weiss and Schlesinger, 1991; Lai, 1992); it is generally assumed to proceed by a copy choice mechanism. RNA splicing (Zaug and Cech, 1986) is another process which can work in principle—as has been shown *in vitro* (Woodson and Cech, 1989)—both as an intramolecular and an intermolecular recombination. Recombination among prokaryotic RNA viruses has been observed recently (Palasingam and Shaklee, 1992) and RNA recombination is the probable source of several short-chained RNA species replicated by Qβ replicase (Munishkin *et al.*, 1988) originated presumably *in vivo*. Direct observation of the recombination mechanism is nearly impossible, because the events are rare and unreproducible. We describe here the reproducible formation of an SV-11 from MNV-11 by a recombination process *in vitro*, taking place under conditions of high ionic strength and long incubation time. Preincubation of MNV-11 without enzyme at the typical conditions of non-enzymatic transesterification (Zaug and Cech, 1986) did not increase the frequency of SV-11 formation. Therefore, the recombination we found is probably an irregular event of the replication process itself, where the replica–replicase complex may disengage from the template and rebind to another template or—preferentially—to the same strand at another position (Figure 8). Elongation may be resumed if the 3′-terminal nucleotides of the aborted replica chain are complementary to the template. High ionic strength of the medium was found to be necessary for all recombination processes, probably by lowering the processivity of the replicase and increasing the yield of abortive replica pieces. This model readily explains the formation of SV-11 from MNV-11: when replicating MNV-11 plus strand, the nascent minus strand may fall off preferentially after the AU-rich portion following the second stem region (Figure 1) and may rebind to another (complete) minus strand to produce the observed recombinant. Alternatively, the reaction could also use an abortive replica directly as template; however, strand separation and production of a metastable product would then be difficult.

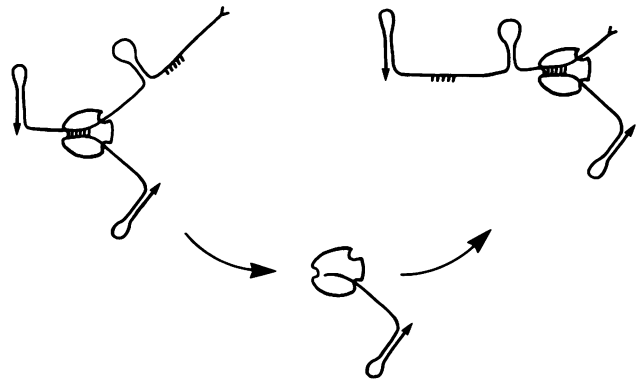

Fig. 8. Model of recombination. Replica and enzyme temporarily leave the template and rebind to the same or another RNA. Since rebinding must involve formation of a short double helix between the 3′ terminus of the replica and the new template, recombination requires a short sequence homology (see also Lai, 1992).

The reaction adding nucleotides to the 3′ terminus of an RNA raises some questions in connection with the 'nonspecific replication' reported to be catalysed by Qβ replicase under a variety of nonphysiological conditions such as addition of Mn^{2+} ions (Palmenberg and Kaesberg, 1974; Obinata *et al.*, 1975; Vourmakis *et al.*, 1976), abnormally high concentrations of GTP (Blumenthal, 1980) or primers (Feix, 1976; Feix and Hake, 1975). In some cases, very low yields of incorporation are reported or can be calculated from the published data and it is not entirely clear whether a replica has really been formed. We cannot contribute to an understanding of the reported nonspecific replication, because we failed to get nonspecific replication with our highly purified replicase preparations; only terminal addition of a few nucleotides was observed. The reaction stopped after adding a few nucleotides to the 3′ terminus; complete hairpins were never found. The cloning procedure for elongated MNV-11 (*Bst*EII digestion) selected against these molecules, but the electrophoretic mobilities also indicated that the elongation reaction aborted before long hairpins were produced. Possibly the difficulty of strand separation during replication is responsible for the premature stall.

The 'copy choice' model of RNA replication (for a review see Lai, 1992) is in agreement with the results presented here and with the results of homologous RNA recombination by phage Qβ mutants *in vivo* (Palasingam and Shaklee, 1992). The recombination rates found for Qβ RNA *in vivo* are higher than those we found *in vitro*; however, this is easily

explained: (i) there is only a short sequence homology stretch between MNV-11 plus and minus strands where recombination may take place and (ii) apparently only one recombinant can compete with the parent species.

RNA recombination is probably a ubiquitous phenomenon. In contrast to DNA recombination, however, RNA recombination is not catalysed by a specific genetic apparatus, as is shown in this *in vitro* study which involved only highly purified replicase. A number of scientists have proposed that RNA recombination may go back to a stage of early evolution where RNA rather than DNA was the genetic material. This idea is further supported by the finding that recombination may take place even in the absence of protein enzymes (Woodson and Cech, 1989).

Materials and methods

Materials

Preparation of Qβ replicase devoid of self-replicating RNA impurities has been described previously (Sumper and Luce, 1975; Biebricher *et al.*, 1986). All replicase preparations were regularly checked for the absence of intraneous templates. Poly(A) polymerase was prepared from *Escherichia coli* K12 according to Sippel (1973). T7 RNA polymerase was isolated from an overproducing strain (Davanloo *et al.*, 1984), kindly obtained from F.W.Studier, by the method of Grodberg and Dunn (1988). Modified T7 DNA polymerase ('Sequenase') devoid of 3′-exonuclease activity (Tabor and Richardson, 1987) was obtained from USB. All other enzymes were obtained commercially. Preparation of the single strands of MNV-11 and SV-11 has been described previously (Biebricher *et al.*, 1982). Oligonucleotides were synthesized by the phosphite method using a Pharmacia Gene Assembler.

RNA replication

Qβ buffer contained 50 mM Tris−HCl (pH 7.5), 10 mM $MgCl_2$, 0.1 mM dithioerythritol, 10% glycerol and the indicated concentrations of nucleoside triphosphates and salt. Standard conditions were 0.5 mM ATP, CTP, GTP, UTP and 100 nM Qβ replicase.

Limited polyadenylation of RNA

30−75 pmol RNA (1−2.5 μg) were dissolved in 50 μl polyadenylation buffer [50 mM Tris−HCl (pH 7.9); 0.25 M NaCl; 50 μM [^{3}H]ATP] and incubated with 5 U poly(A) polymerase (1 μl, saturating the RNA) for 2 min at 37°C. The reaction was started with 0.5 μl 1 M $MgCl_2$ and the incubation continued for 5 min at 37°C. The reaction was stopped with 2.5 μl 0.5 M EDTA and the RNA isolated by phenol extraction and ethanol precipitation. On average, 10 A residues were attached to the 3′ termini of single- and double-stranded RNA under these conditions.

Cloning of MNV-11 and SV-11

50 pmol polyadenylated single-stranded RNA or 20 pmol polyadenylated double-stranded RNA and 100 pmol first strand primer (GAATTCTAGGGATCCATTTTTTTGGG) were dissolved in 40 μl H_2O, heated for 1.5 min to 100°C and quickly cooled in a dry ice mixture. 5 μl 10 × retrobuffer (final concentration 50 mM Tris−HCl, pH 8.3; 10 mM $MgCl_2$; 10 mM DTT), 2 μl 25 mM each dATP, dCTP, dGTP and dTTP were added and the volume was adjusted to 50 μl and incubated with 25−50 U AMV reverse transcriptase for 60 min at 42°C. The mixture was extracted with phenol and the nucleic acids precipitated with ethanol. The yield of cDNA was 15−30% of the RNA input. The RNA was hydrolysed by heating the residue in 200 μl 20% piperidine for 3 h at 50°C. After lyophilization the residue was dissolved in 20 μl water and purified by repeated ethanol precipitation followed by Sephadex G50 superfine chromatography or by gel electrophoresis. The cDNA was heated to 100°C for 1 min and rapidly cooled. 100 pmol phosphorylated second strand primer (TATAGTGAGTCGTATTAAGCTTAATACGACTCACTATAGGG) were added, the mixture brought to 50 mM Tris−HCl (pH 7.5), 10 mM $MgCl_2$, 1 mM DTT and 20 μM each dATP, dCTP, dGTP and dTTP and the volume adjusted to 50 μl. The mixture was incubated with 0.5 μl Sequenase for 10 min at 4°C and for 10 min at 37°C. The sequenase was destroyed by heating for 15 min at 65°C and the DNA incubated for 1 h at 37°C with 5 U each of *Hin*dIII and *Bam*HI. The DNA was collected by phenol extraction and ethanol precipitation and purified by electrophoresis or by repeated ethanol precipitation. cDNA was ligated into 200 ng *Bam*HI- and *Hin*dIII-cut vector pUC18 DNA (Yanisch-Perron *et al.*, 1985) by standard methods (Maniatis *et al.*, 1982) and used for transformation of *E.coli* HB101 made competent by the method of Hanahan (1983).

Terminal elongation of RNA by Qβ replicase

The reaction mixture contained 0.2 mM ATP, CTP, UTP and 1 mM ITP in Qβ buffer, 100 nM Qβ replicase and 500 nM RNA. Incubation was for 16 h at 30°C. The modified MNV-11 RNA was isolated by phenol extraction and ethanol precipitation and polyadenylated as described above. First strand cDNA synthesis was performed with GTCTAGAAGATTTTTTTT-$(A,G,C)_1$; unprimed second strand cDNA synthesis was according to Gubler and Hoffmann (1983). *Bam*HI linkers were attached and the DNA was digested with *Bam*HI and *Bst*EII and ligated into *Bam*HI and *Bst*EII-cleaved pUC18 containing the MNV-11 cDNA and T7 promoter sequences constructed as described above.

Sequencing

Double-stranded plasmid DNA was sequenced by the dideoxy chain termination method (Sanger *et al.*, 1977) using Sequenase (USB) according to the instructions of the supplier.

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