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Template-free generation of RNA species that replicate with bacteriophage T7 RNA polymerase

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A large variety of different RNA species that are replicated by DNA-dependent RNA polymerase from bacteriophage T7 have been generated by incubating high concentrations of this enzyme with substrate for extended time periods. The products differed from sample to sample in molecular weight and sequence, their chain lengths ranging from 60 to 120. The mechanism of autocatalytic amplification of RNA by T7 RNA polymerase proved to be analogous to that observed with viral RNA-dependent RNA polymerases (replicases): only single-stranded templates are accepted and complementary replica strands are synthesized. With enzyme in excess, exponential growth was observed; linear growth resulted when the enzyme was saturated by RNA template. The plus strands, present at 90% of the replicating RNA species, were found to have GG residues at both termini. Consensus sequences were not found among the sequences of the replicating RNA species. The secondary structures of all species sequenced turned out to be hairpins. The RNA species were specifically replicated by T7 RNA polymerase; they were not accepted as templates by the RNA polymerases from *Escherichia coli* or bacteriophage SP6 or by Qβ replicase; T3 RNA polymerase was partially active. Template-free production of RNA was completely suppressed by addition of DNA to the incubation mixture. When both DNA and RNA templates were present, transcription and replication competed, but T7 RNA polymerase preferred DNA as a template. No replicating RNA species were detected *in vivo* in cells expressing T7 RNA polymerase.

Keywords: hairpin structure/molecular evolution/Qβ replicase/RNA replication/T7 RNA polymerase

Introduction

In vivo cellular RNA is exclusively synthesized by transcription from DNA. If RNA was the primordial source of genetic information, as now commonly supposed, enzymes catalyzing RNA replication must have existed. Many RNA viruses use non-canonical RNA synthesis by RNA replication. Biebricher and Orgel (1973) hypothesized that RNA polymerases may serve as replicases if instructed by a proper RNA template; they found that *Escherichia coli* RNA polymerase could select, from a random nucleotide co-polymer, an RNA species that it could amplify to high concentration. Konarska and Sharp (1989) observed that a commercial batch of T7 RNA polymerase amplified an RNA species that was present as a contaminant; this RNA could also be amplified by other T7 RNA polymerase preparations. Screening a large number of batches from various sources showed that such contaminations are rare, but a second replicating RNA was eventually found (Konarska and Sharp, 1990). Sequence analysis showed that both RNA species are able to form a hairpin structure. The genetic origin of these replicating RNA species, however, remains unknown.

The template requirements for RNA transcription and RNA replication are quite different, even though both reactions generally represent template-instructed condensation of nucleoside triphosphates into single-stranded RNA. RNA transcription recognizes a specific promoter sequence that must be in a double-helical conformation for specific initiation (Schick and Martin, 1995). RNA replication requires a single-stranded RNA template (Weissmann, 1974; Biebricher *et al.*, 1982; Biebricher, 1987) and a specific structure rather than a specific sequence is required for template activity of an RNA strand (Biebricher and Luce, 1993; Zamora *et al.*, 1995). Neither RNA transcription nor RNA replication need a primer and both recycle the template after the reaction, i.e. the template may be regarded as a catalyst. However, RNA replication is an autocatalytic process, the RNA replica synthesized by the process also serving as a template, while RNA transcription is not. It seems intriguing that one and the same enzyme should be able to catalyze both RNA transcription and RNA replication, even though the mechanism of RNA replication by T7 RNA polymerase was not investigated in detail. RNA replication by T7 RNA polymerase is usually not detected, because cellular RNA and the transcription products are not templates for RNA replication. RNA replication is thus highly specific for the template and it seems appropriate to assume that the structures of the RNA species that are replicated are directly involved in the replication process.

Investigation of template requirements is usually performed by comparing the properties and sequences of a large number of different templates. Two different strategies have been developed to generate suitable templates: (i) the enzyme is incubated with a random sequence mixture and if a strand with template activity is present, it will be amplified and grow out (Biebricher and Orgel, 1973); (ii) the enzyme is incubated without template for extended time periods and if a random condensation of nucleoside triphosphates happens to produce a template strand by chance, it will be amplified (Sumper and Luce, 1975; Biebricher *et al.*, 1981a,b, 1986; Biebricher, 1987). In both cases the occurrence of a template strand is a highly improbable singular event and thus neither directly observable nor reproducible. The RNA species produced by the latter reaction with Qβ replicase were investigated

in detail (Biebricher, 1987; Biebricher and Luce, 1993). Their chain lengths and sequences were found, as expected, to differ from experiment to experiment. They were extremely short and inefficient templates that bound only weakly to replicase. The reaction times required for detecting RNA in a template-free reaction were much longer than necessary for amplifying a single product strand to macroscopic appearance. Furthermore, the template-free reaction with Qβ replicase required unusually high enzyme and triphosphate concentrations to occur, while amplification of the products did not. Interference of RNA amplification with template-free synthesis thus does not take place and the kinetic properties of template-instructed and template-free reactions by Qβ replicase allow a clear distinction between the two processes. Therefore, even extremely inefficient templates were amplified by Qβ replicase, the only problem being the evolutionary optimization of the template strands during amplification (Zamora *et al.*, 1995).

Here we report that DNA-dependent RNA polymerase from bacteriophage T7 shows an analogous template-free production of a large variety of different RNA species that are able to replicate with T7 RNA polymerase. The RNA replication mechanism uses single-stranded templates and synthesizes a single-stranded replica. The RNA templates are different in sequence, but share a common structure. The reaction has many features in common with the analogous reaction observed previously with Qβ replicase (Biebricher *et al.*, 1981a,b, 1986; Biebricher, 1987; Biebricher and Luce, 1993). The analogy indicates a wider occurrence of RNA replication than previously assumed. A remarkable natural example of a parasitic RNA directing its own replication by a host RNA polymerase are the viroids, the smallest agents of infectious diseases known (Diener *et al.*, 1993).

Results

Template-free incorporation

Under the standard conditions of *in vitro* transcription DNA-dependent RNA polymerase from bacteriophage T7 is absolutely dependent on template, i.e. it does not synthesize RNA in the absence of extraneously added template. However, when the transcription mixture was incubated with high concentrations of T7 RNA polymerase (μM) for 20 h at sufficiently high (mM) nucleoside triphosphate concentrations, RNA synthesis was observed to set in after long lag times. Gel electrophoretic analysis of the synthesized RNA (Figure 1, top) revealed defined bands for each sample; the band pattern varied from sample to sample even when identical incorporation conditions were established by subdividing an incorporation mixture into several samples and incubating them under the same conditions. The RNA products were excellent templates for T7 RNA polymerase: upon dilution into fresh incorporation mixture (Mills *et al.*, 1967) the RNA was rapidly amplified. The electrophoretic properties of the amplified RNA products were identical to those of the template RNA. After several serial transfers only one species survived in each population (R in Figure 1, top), but different RNA species were selected in different samples. For each RNA species formed two bands were detected. The upper band disappeared after heating the

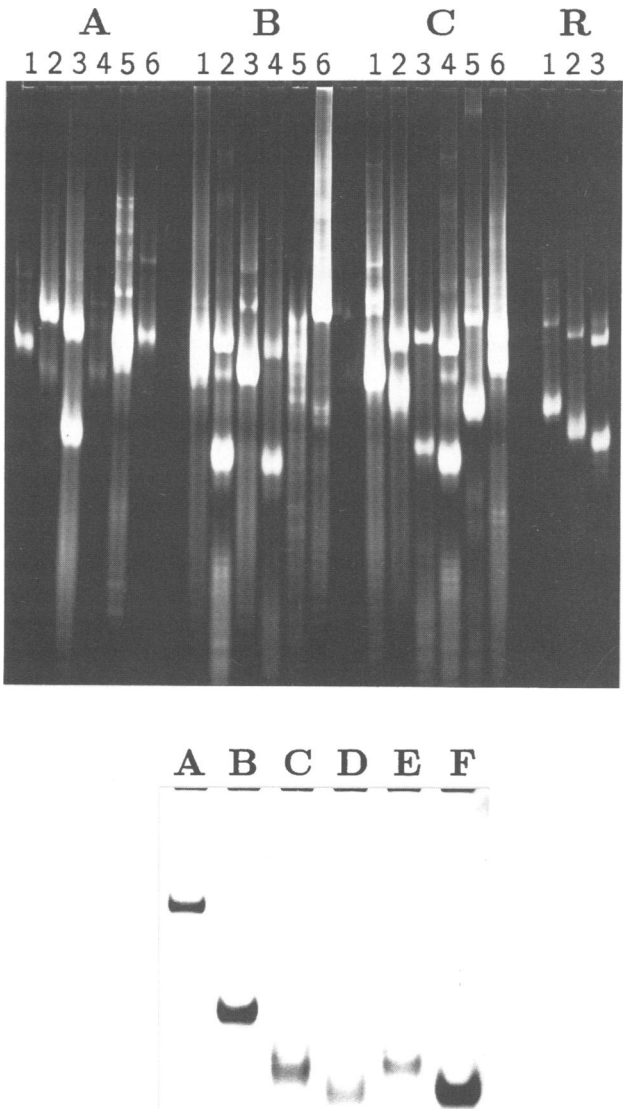

Fig. 1. Electropherogram of RNA synthesized without template with T7 RNA polymerase. Top: standard mixtures were prepared with T7 RNA polymerase [a preparation concentrated by $(NH_4)_2SO_4$ precipitation] at final concentrations of (A) 0.4, (B) 0.8 and (C) 1.6 μM, subdivided into six samples each, incubated at 37°C for 20 h and loaded onto 12% polyacryamide gels. R are the isolated species T7rp1, T7rp2 and T7rp3. Bottom: chain length determination. Species T7rp1 and T7rp2 were glyoxylated, loaded on a 10% electrophoresis gel and stained by the method of McMaster and Carmichael (1977). (A) MDV-1, 221 nucleotides; (B) SV-11, 115 nucleotides; (C) T7rp1, 84 nucleotides; (D) T7rp2, 76 nucleotides; (E) MNV-11, 87 nucleotides; (F) $tRNA^{Phe}$, 76 nucleotides. The chain lengths of T7rp1 and T7rp2 were obtained by interpolation according to the method of Lehrach *et al.* (1977).

samples and reappeared after annealing, thus the minor (upper) bands were double-stranded RNA and the major (lower) bands single-stranded RNA. When the RNA strands were converted to random coils by glyoxylation (McMaster and Carmichael, 1977), only one band remained (Figure 1, bottom). The chain lengths determined from the electrophoretic mobilities of the random coils ranged from 60 to 120 nucleotides.

The template-free reaction was highly dependent on the enzyme batch used, in particular on the concentration of the enzyme stock solution. Enzyme concentrated by dialysis

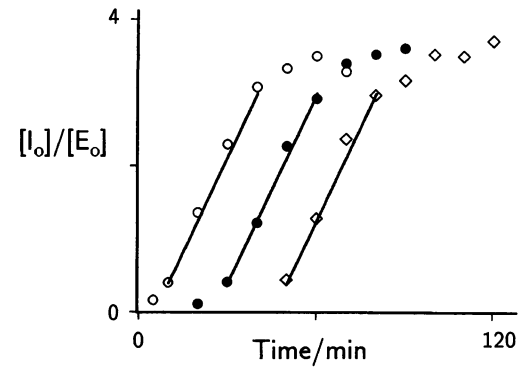

Fig. 2. Nucleotide incorporation profiles of replicating RNA. T7rp1 was incubated with 1 μM T7 RNA polymerase and 0.5 mM NTP at initial concentrations of 0.1 μM (○), 1 nM (●) and 10 pM (◇). The synthesized RNA strands (I_o) per enzyme molecule (E_o) were calculated from the [α-^{32}P]ATP incorporation. The overall replication rate in the linear growth phase, ρ, was calculated from the slope to be 1.47×10^{-3}/s; the replication rate in the exponential phase, κ, from the time displacement caused by the dilution to be 3.84×10^{-3}/s (Biebricher, 1986). From these data one can calculate the the average time required for binding the enzyme and synthesizing the replica strand τ_E = 80 s; release of enzyme from the inactive complex requires τ_D = 600 s. At 0.2 μM T7 RNA polymerase $\kappa = 1.28\times10^{-3}$/s and $\rho = 1.09\times10^{-3}$/s, i.e. the average time required for enzyme binding and replica synthesis rose to ~400 s, while the enzyme release time remained the same.

against 50% glycerol and 0.1 M NaCl and stored at a concentration of 1 mg/ml required enzyme concentrations of at least 1 μM to generate RNA products without template in 20 h. Enzyme concentrated by ion exchange chromatography and stored at 0.5 mg/ml in 0.2 M $(NH_4)_2SO_4$ did not show any template-free reaction. Enzyme concentrated by ammonium sulfate precipitation (under special precautions to avoid introduction of impurities) and stored at 2 mg/ml was especially potent in template-free productions: 400 nM concentrations sufficed to produce RNA after 20 h (Figure 1). Despite such differences in ability to catalyze template-free RNA synthesis, the enzyme preparations had equal specific activities, as determined by the transcription assay. It is possible that only enzyme aggregates were able to catalyze the early reactions required to generate replicatable RNA. Both template-free and RNA-dependent RNA synthesis by T7 RNA polymerase were strongly affected by elevated ionic strength: addition of 20 mM $(NH_4)_2SO_4$ or 50 mM NaCl to the reaction mixture inhibited template-free and RNA template-dependent RNA synthesis entirely. The salt introduced into the reaction mixture by addition of the enzyme stock solution had thus to be taken into account to obtain reproducible results.

Replication kinetics

Template-instructed RNA growth profiles are shown in Figure 2. After a short lag time a linear increase in the RNA was observed. Serial dilutions by a factor of 100 resulted in identical profiles with equidistant displacements on the time axis, typical for template-instructed, exponential growth of the RNA introduced as template (Biebricher *et al.*, 1981a,b; Biebricher, 1986). As in the case of RNA amplified by Qβ replicase, overall replication rates in the exponential growth phase were much higher than in the linear growth phase, indicating that the rate limiting step in the total cycle is dissociation of template from enzyme after completion of a replication round. At a ratio of RNA to enzyme as low as 3:1, deviation from linear growth became noticeable, indicating strong product inhibition. Eventually, growth levelled off at a molar ratio of RNA to enzyme of ~4. The specific rate coefficients were found to be lower than those of optimized RNA species replicated by Qβ replicase, in the same range as those of the RNA species synthesized without template by Qβ replicase (Biebricher and Luce, 1993).

The rate coefficients of the overall replication rates of T7 RNA polymerase in the exponential phase were essentially independent of enzyme concentration at enzyme concentrations exceeding 0.8 μM. Below this concentration the rate of exponential replication dropped rapidly. Analysis of the replication kinetics (Biebricher, 1986) indicates that the contribution of the enzyme binding rate to the overall replication rate is negligible at high enzyme concentrations, but significant at concentrations below 0.5 μM. With Qβ replicase similar results were found at a saturation threshold near 0.2 μM. The kinetics of RNA replication catalyzed by T7 RNA polymerase and Qβ replicase are thus quite similar.

Double-strand formation and plus/minus asymmetry

In contrast to RNA replication by Qβ replicase, where concentration growth in the late linear phase is predominantly that of double-stranded product, the growing RNA concentration synthesized by T7 RNA polymerase continued to be mainly single-stranded RNA. Annealing of the RNA species did not further increase the proportion of double-stranded RNA (not shown), indicating that one strand was produced in large excess. This conclusion was corroborated by analysis of the 5′-termini: while for the lower band, corresponding to the single strand, G was exclusively found as the terminal nucleotide, for the upper band, corresponding to double strands, equimolar amounts of G and C were found. The strand found in excess and having the 5′-G-terminus was arbitrarily designated the plus strand. When [γ-^{32}P]GTP was offered as substrate, stoichiometric amounts of ^{32}P were found in the plus strands of all RNA species tested, showing that the 5′-termini begin with pppG-. Many rather short oligonucleotides with 5′-terminal pppG were also produced as side products of the reaction (data not shown); apparently abortive synthesis is frequent.

The sequences of the 5′-ends were determined by removing the terminal phosphates and labeling the 5′-termini with [γ-^{32}P]ATP and kinase. Analysis by the wandering spot method (Silberklang *et al.*, 1977) gave for the investigated species, T7rp3, predominantly GGGA-AAA, GGAAAA and some minor spurs. Analysis of the double-stranded band gave equal amounts of (G)GGA-AAA and CCAA, indicating that the minus strand begins with CC. The 5′-termini of minus strands were, however, rather heterogenous. The 3′-termini were not determined; when assuming fully paired complements, both termini of the plus strands would have GG; those of the minus strands CC. The termini were the same for all replicating species we investigated and for those found previously (Konarska and Sharp, 1989); we thus conclude that the ends are invariant.

When the template activity of dsRNA was kinetically investigated, RNA grew out with a lag time corresponding to a residual content of 1% ssRNA, suggesting that double strands are neither templates nor intermediates in replication.

Cloning and sequencing of replicating species

The presence of invariant ends suggested application of a general cloning procedure similar to that developed for cloning the RNA species replicating with Qβ replicase (Biebricher and Luce, 1993). It involves polyadenylation of the RNA, phased priming of reverse transcription of the RNA and performing second strand cDNA synthesis with T7 DNA polymerase lacking 3′ exonuclease activity using a primer containing the double-stranded T3 promoter with a single-stranded 3′-overhang corresponding to the invariant two 5′-terminal nucleotides (see Materials and methods). However, the hairpin structure of the replicatable RNA species (see next paragraph) caused difficulties in both first strand and second strand cDNA synthesis. Reverse transcription with the reverse transcriptases from AMV and HIV-1 yielded heterogeneous cDNA that was unsuitable for cloning; only with modified MuMoLV reverse transcriptase (Superscript) were specific cDNA bands obtained, most of which, however, were shorter than full-length cDNA products. In particular, reverse transcription of species with greater chain lengths yielded mainly abortive synthesis products. Presumably incomplete cDNA strands are pushed out from partial DNA–RNA hybrids by reformation of the more stable RNA hairpins when the reverse transcriptase dissociates prematurely.

Priming of the single-stranded cDNA for second strand synthesis was not successful. The method of Gubler and Hoffmann (1983) was also not suitable, because the abortive first strand products proved to serve, due to the partially palindromic sequence, as excellent primers for the 5′-ends of the second strand and thus produced wrong termini. Therefore, the abortive first strand products had to be removed and the RNA–DNA hybrid obtained in the first-strand cDNA synthesis was cleaved instead with RNase T1 and then primed with a partially double-stranded T3 promoter sequence, as described in Materials and methods. This cloning method gave satisfactory results.

The sequences obtained are shown in Figure 3. As was found for RNA replicating with Qβ replicase, the sequences did not show a consensus sequence, except for the invariant ends. Remarkably, one of the species, T7rp2, contained a partial T7 promoter sequence; that part of the promoter sequence forming the transcript 5′-terminus, however, was incorrect. While the presence of a T7 promoter in one species seems unlikely to be a coincidence, it is not clear what its role may be. The presence of the promoter sequence fragment is not necessary for RNA replication, because other species that do not contain it replicate just as efficiently. It is also not sufficient for RNA synthesis, because incubating equimolar or excess amounts of single-stranded or double-stranded RNA containing a full T7 promoter sequence, obtained from an RNA species replicating with Qβ replicase (Zamora *et al.*, 1994), with T7 RNA polymerase under normal transcription conditions did not result in detectable RNA synthesis.

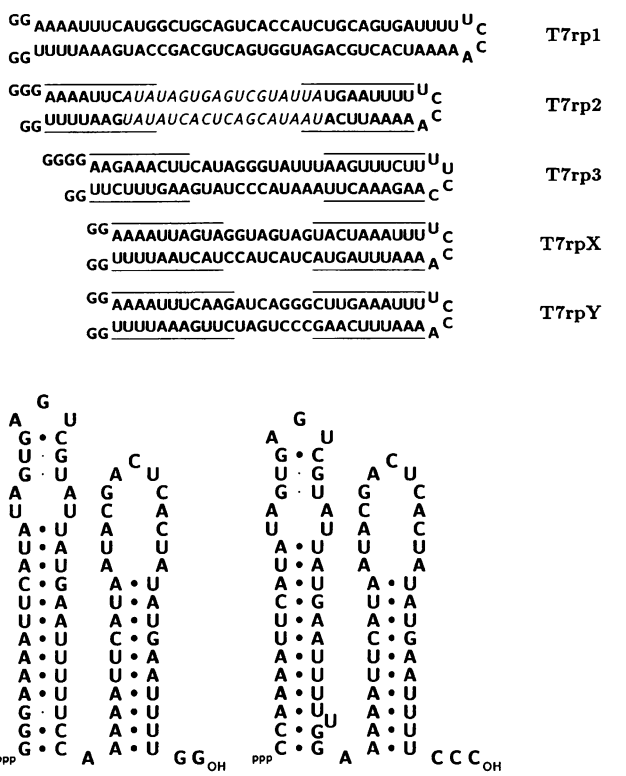

Fig. 3. Top: sequences of RNA species replicated by T7 RNA polymerase, represented as hairpin loops. The nucleotides homologous to the T7 promoter sequence in T7rp2 are shown in italic. The two lowest sequences (T7rpX and T7rpY) are those reported by Konarska and Sharp (1990). Bottom: hypothetical alternative structures of T7rp2 plus and minus strands.

Considering the differences in geometry of DNA (B form) and RNA (A form) this result is not surprising.

To confirm that the RNA sequences found were indeed replicated by T7 RNA polymerase, T7rp3 RNA was synthesized *in vitro* by the method of Milligan and Uhlenbeck (1989) using T3 RNA polymerase; the synthetic products were templates for T7 RNA polymerase and indistinguishable in their properties from the products synthesized by replication. Likewise, transcription from cDNA clones containing T7rp1 cDNA produced fully replicatable T7rp1 RNA.

Structural analysis of replicating species

The electrophoretic mobility ratios of corresponding single- and double-stranded forms of the RNA species were essentially constant (Figure 1) and independent of ionic strength; the mobility of the single-stranded forms corresponded to that of double strands of about half the chain length. Such electrophoretic behavior is typical of hairpin structures (Biebricher *et al.*, 1982). Indeed, all sequences we found to be replicated by T7 RNA polymerase were also found to be nearly palindromic. The sequence halves forming the hairpin, however, can themselves form shorter hairpins. In principle, as noted already by Konarska and Sharp (1990), alternative structures are possible (Figure 3, bottom), although at substantially higher energies. Because the smaller hairpins may form during synthesis, one has to consider the possibility of metastable structures (Biebricher *et al.*, 1982; Biebricher and Luce, 1992). However, the electrophoretic mobilities

of the single-stranded bands were not affected by heating and rapid or slow cooling cycles, thus there is no evidence for metastable structures. Both limited RNase A digestion (Figure 4, top) and temperature gradient gel electrophoresis (Figure 4, bottom) supported the presence of full-length hairpin structures. The melting profiles resolved into several parallel bands with only a few spurs of different shapes. Therefore, in complete agreement with the cloning and sequencing data, the sequence heterogeneity of the RNA population of a single incubation was large at the positions near the ends, while the base paired stem was rather conserved. The hairpin stems had A_n-U_n clusters on both ends of the stem, allowing 'breathing' or partial melting of the stem, which presumably helps to open the RNA structure during replication.

Template specificity of T7 RNA polymerase

The competition between RNA synthesis by replication and by transcription was investigated by offering equimolar amounts (20 nM) of a plasmid directing synthesis of the RNA species MNV-11 by transcription (Rohde *et al.*, 1995) and the replicating species T7rp1 to an excess of T7 RNA polymerase (1 μM) and measuring the amounts of the RNA species after gel electrophoretic separation. At first, transcription from DNA was strongly preferred over RNA replication. With time RNA synthesis by replication increased and finally overwhelmed transcription. T7 RNA polymerase seems to prefer DNA templates by binding them with a higher efficiency. During the reaction, however, the concentration of DNA templates did not change, while RNA templates were amplified and thus at later times the excess RNA template outcompeted the DNA template.

The template-free generation of replicatable RNA is inhibited by DNA. A standard incorporation mixture was incubated with 0.5 μM T7 RNA polymerase and 50 μg/ml pUC18 DNA (which does not contain a T7 promoter sequence) for 20 h at 37°C, resulting in synthesis of a very heterogeneous RNA. This RNA was not accepted as a template when offered in various dilutions to a standard incorporation mix.

The template activity of the RNA species for other DNA-dependent RNA polymerases was investigated. RNA polymerase from bacteriophage T3 was able to amplify T7rp1 RNA at much reduced efficiency, but not T7rp2 and T7rp3 RNA. RNA polymerase from *E.coli* was able to generate RNA in template-free reactions (A.Wettich and C.K.Biebricher, unpublished results), but not to amplify the species replicated by T7 RNA polymerase. RNA polymerase from bacteriophage SP6 was not active in amplification of the various T7rp species. The species replicating with T7 RNA polymerase were not accepted as templates for Qβ replicase and the tested RNA species that replicate with Qβ replicase were not amplified by T7 RNA polymerase.

The origin of the replicating RNA

The RNA species arising from the template-free reaction with T7 RNA polymerase were not present in the original incorporation mixture, as shown by the following arguments. (i) At high T7 RNA polymerase concentrations (1 μM) amplification of a single strand of RNA to macroscopic appearance required <100 min, while no macroscopic synthesis could be detected even after 4 h incubation in the absence of exogenously added template. At low enzyme concentrations (100 nM) no template-

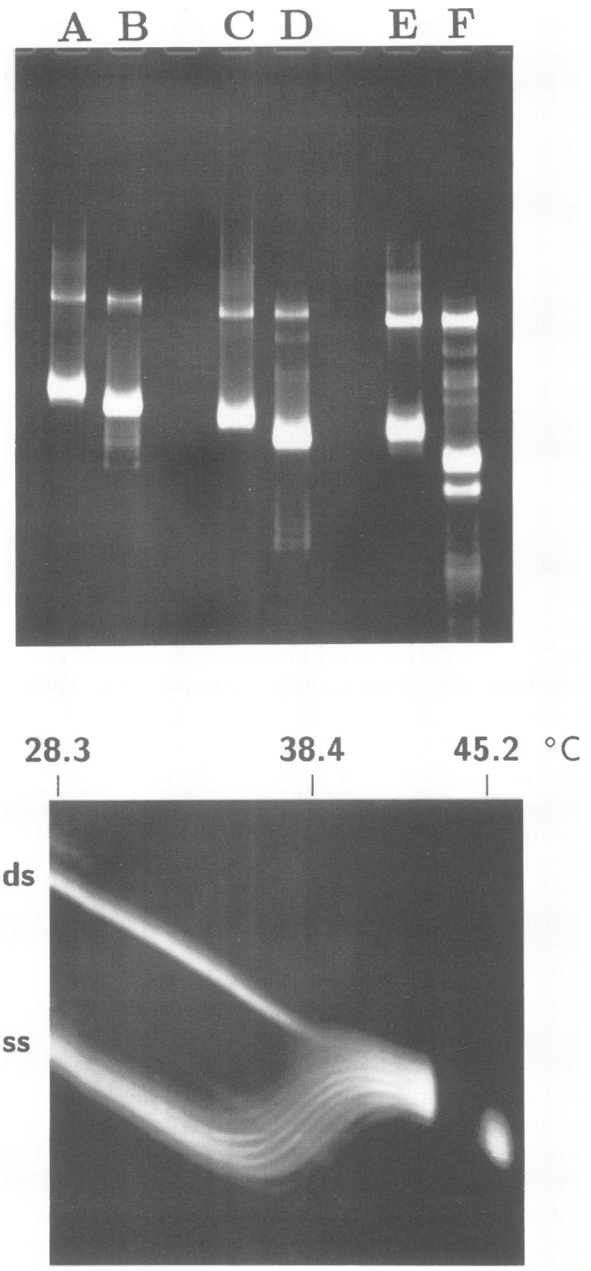

Fig. 4. Structural analysis of RNA species T7rp1. Top: electropherogram of replicating RNA species after limited digestion with RNase A. T7rp1 (A), T7rp2 (C) and T7rp3 (E) were incubated with 50 nM RNase A for 10 min at 37°C (B, D and F) and loaded onto a standard gel. XCB and BPB are the positions of the dyes xylene cyanol blue and bromophenol blue respectively. The lengths of the stems resistant to RNase are consistent with the structures shown in Figure 3, top. Bottom: temperature gradient gel electrophoresis pattern of T7rp3. The apparatus has been described previously (Rohde *et al.*, 1995). The 12% acrylamide gels contained 7 M urea in 0.1 M Tris–borate. The double-stranded T7rp3 seems to decompose into single strands 1–3°C below the melting temperature of the single-stranded secondary structures. This phenomenon is due to the fact that melting of the hairpin is fully reversible, while melting of the double strands is practically irreversible under the electrophoresis conditions. Analogous profiles were found for the other species. The average hairpin melting points (in 7 M urea) were: T7rp1, 57.5°C; T7rp2, 34.5°C; T7rp3, 38.4°C.

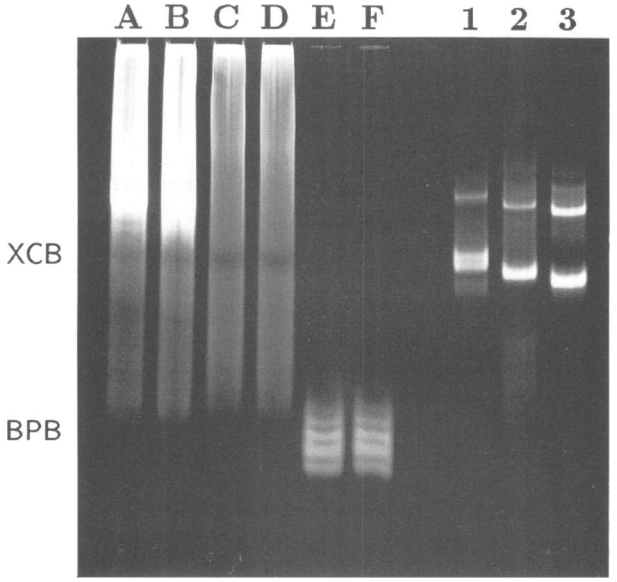

Fig. 5. Condensation of nucleoside triphosphates in the absence of template. T7 RNA polymerase (1 μM) was incubated (two independent reactions each) for 20 h with the following substrates: (A and B) 1 mM each ATP, CTP, ITP and UTP; (C and D) 1 mM each ATP, GTP and UTP; (E and F) 1 mM each ATP, CTP and GTP. The samples were analyzed by polyacrylamide gel chromatography. (1–3) T7rp1, T7rp2 and T7rp3.

free synthesis was detected, while template-instructed replication did take place. (ii) After pre-incubation for 30 min and subdivision into several test tubes each incorporation led to selection of RNA species with unrelated sequences. (iii) The times required for onset of synthesis covered a wide range. (iv) When replication was inhibited, e.g. by omitting one nucleoside triphosphate or replacing GTP by ITP, nucleoside triphosphates were slowly condensed in a more or less random synthesis (Figure 5; Biebricher *et al.*, 1986). It is remarkable that long chain condensation products were synthesized when omitting CTP or replacing GTP with ITP, while omitting UTP resulted in synthesis only of short oligonucleotides. The products with ITP were slowly amplified by T7 RNA polymerase (under standard conditions with GTP), but produced mainly heterogeneous RNA with chain lengths of several hundred nucleotides. RNA produced in the absence of CTP or UTP was not accepted as template. Fingerprints of these products produced a complicated oligonucleotide pattern ruling out repetitive, slipping structures (data not shown).

Konarska and Sharp (1989) found replicating RNA as an impurity of their RNA polymerase preparation; this finding suggests that during the long induction period of the overproducing strain used for preparing the enzyme a similar reaction may take place within the cell. In Qβ-infected cells replicatable material is present late in the infection process (Banerjee *et al.*, 1967) and after induction of *E.coli* strains expressing Qβ replicase (C.K.Biebricher and R.Luce, unpublished results). However, when we isolated intracellular RNA from induced cells of *E.coli* strain BL21 (pAR1219) overproducing T7 RNA polymerase (Davanloo *et al.*, 1984), we neither detected RNA bands not found in uninduced cells nor were we able to select from it replicatable RNA by amplification with T7 RNA polymerase. Presumably the reaction is inhibited *in vivo* by the high concentration of nucleic acid binding to T7 RNA polymerase.

Discussion

In vivo RNA is synthesized normally by transcription from DNA. The double-stranded DNA template is unwound by local melting, a single-stranded RNA is synthesized and the DNA double strand reforms. Accurate start points for transcription are provided by promoter sequences on the double-stranded DNA template. It has been shown that only the promoter sequence must be double-stranded, while the transcribed template strand may be single-stranded and re-used several hundred times (Milligan and Uhlenbeck, 1989). This suggests that recognition of the promoter sequence requires the fixed geometry of the double helix, even though promoter recognition involves predominantly only one strand of the double helix (Schick and Martin, 1995). It seems likely that the newly synthesized RNA strand is peeled from the template without the production of a double strand, but it has not been excluded that a RNA–DNA hybrid is formed during the first transcription round that can be used as template for the following transcription rounds. Strand separation is a crucial step in the replication mechanism of RNA viruses, e.g. leviviruses or picornaviruses, because double strands cannot be used as template. In this paper we have shown that the similarities between RNA replication by RNA-dependent and DNA-dependent RNA polymerases are striking. It is puzzling that DNA-dependent RNA polymerase can also accept RNA as template, albeit with entirely different criteria for template activity. In RNA replication a certain structure of the RNA is necessary for replication and the structure likely participates in the replication process (Biebricher *et al.*, 1981a). RNA species replicated by Qβ replicase require a 'leader' hairpin at the 5′-terminus (Biebricher and Luce, 1993; Zamora *et al.*, 1995); we hypothesized that it is involved in strand separation. The same feature is also found for RNA species replicated by T7 RNA polymerase (Figure 3, bottom), but the structure is later converted into an almost perfect hairpin. The requirement for the hairpin structure is not understood; one might think that a signal in a double-stranded region is recognized, but a conserved sequence in the double-stranded region could not be detected. Hairpin structures are not replicated by Qβ replicase (Biebricher and Luce, 1992) and we found that the requirements for template activity of an RNA species are different for replication by T7 RNA polymerase than by Qβ replicase. Breaker and Joyce (1994) reported the emergence of a replicating species called RNA 'Z' requiring both RNA polymerase and reverse transcriptase; its replication mechanism appears to be greatly different.

Template-free synthesis by T7 RNA polymerase required high concentrations of the polymerase and enzyme preparations stored at different concentrations behaved differently, while normal conditions sufficed for DNA- or RNA-instructed RNA synthesis. How a large variety of different RNA replicators are assembled in the absence of template is not understood; as found (and stated) in previous works on template-free synthesis by Qβ replicase (Biebricher *et al.*, 1981b, 1986, 1993; Biebricher,

1987), while the presence of replicatable RNA as an impurity can be ruled out, partial instruction by oligo- or polynucleotides cannot. Others assumed the origin of replicatable RNA to be the presence of an RNA with extremely low replication rate as an impurity in the Qβ replicase preparation (Moody *et al.*, 1994). While this is not impossible, it seems unlikely that efficient templates are removed during the purification process (cells expressing Qβ replicase contain replicating RNA) while inefficient ones, which usually bind much more weakly, should remain. Whatever the earliest events, an evolving process is required, as argued earlier (Biebricher, 1987): if a randomly synthesized RNA happens to trigger replication, no matter how slowly, it is amplified and optimized. It seems unlikely that template-free RNA synthesis plays an important role *in vivo*; the evolutionary emergence of 'selfish' replicating entities that do not contribute to the fitness of the organism but are selected due to their autonomous parasitic fitness is a frequent phenomenon in biology. The evidence presented in this paper suggests that parasitic replicators may be formed by such evolutionary processes in many systems *in vitro* and *in vivo*; they have a still largely untapped potential for use as an antiviral strategy.

Materials and methods

Materials

Poly(A) polymerase was prepared as described previously (Rohde *et al.*, 1995).

T7 RNA polymerase was isolated from an overproducing strain (Davanloo *et al.*, 1984), a kind gift from Dr F.W.Studier, by the method of Grodberg and Dunn (1988). Extreme care was taken to avoid introduction of impurities. The final stock solutions were concentrated by ultrafiltration, dialysis against 50% glycerol or precipitation with an equal volume of saturated $(NH_4)_2SO_4$ solution and dissolution of the precipitate to give a final concentration of 2 mg/ml.

T3 RNA polymerase and SP6 RNA polymerase were isolated from overproducing strains, kindly provided by Dr W.T.McAllister (Morris *et al.*, 1986; Jorgensen *et al.*, 1991), and isolated by the same procedure used for T7 RNA polymerase. SP6 readily precipitated from concentrated solutions. RNA polymerase from *E.coli* was purified according to the procedure of Burgess and Jendrisak (1975).

The concentrations of the RNA polymerases were calculated from the protein concentrations and the molecular weights, assuming 100% active protein.

Production and isolation of the RNA templates

The reaction mixture contained 50 μM Tris–HCl, pH 7.5, 10 mM $MgCl_2$, 1 mM dithiothreitol, 1 mM each ATP, CTP, GTP and UTP and the indicated concentrations of T7 RNA polymerase and template. After incubation at 37°C the RNA was extracted with phenol and precipitated with 2 vol ethanol.

Cloning and sequencing of the RNA species

The oligonucleotide primers were synthesized by the phosphite method in a Pharmacia Gene Assembler and purified according to the instructions of the manufacturer. The oligonucleotide used for first strand cDNA synthesis was pCCGGATCCCTTTTTTTTTTCC; for second strand cDNA synthesis an equimolar mixture of pTAATTAACCCTCACTAAAGG and pTTTAGTGAGGGTTAATTAAGCT was annealed, ligated and isolated by gel electrophoresis to give pTTTAGTGAGGGTTAATTAAGCTTAATTAACCCTCACTAAAGG, a double strand with 3′ GG overhangs.

An aliquot of 50 pmol polyadenylated RNA and 150 pmol first strand primer was incubated with 1 mM each dATP, dCTP, dGTP and dTTP and 600 U 'Superscript' reverse transcriptase (Gibco BRL) in 60 μl mix (supplied with the kit) for 50 min at 42°C. The DNA–RNA hybrid obtained was isolated and cleaved with 20 ng preboiled RNase T1 and purified by polyacrylamide gel electrophoresis and gel electroelution. The product was incubated with 30 pmol second strand primer, 0.1 mM dATP, dCTP, dGTP and dTTP, 0.15 mM β-NAD, 1 U *E.coli* ligase and 32.5 U modified T7 DNA polymerase (Sequenase; US Biochemical) in 45 μl 40 mM Tris–HCl buffer, pH 7.5, 20 mM $MgCl_2$, 3.5 mM dithiothreitol and 0.1 M NaCl for 30 min at 37°C. The double-stranded DNA was purified by polyacrylamide gel electrophoresis and gel electroelution, digested with *Hin*dIII and *Bam*HI restriction enzymes and ligated into a modified pUC18 vector. After transformation the plasmids were isolated by standard procedures.

Sequences were determined by the dideoxy method using Sequenase according to the protocol of the supplier or with an Applied Biosystems 373A DNA sequencer. Computer manipulation of the sequences (searching for sequence homologies in the GenBank or the EMBL bank, mapping, etc.) was with the GCG package of the University of Wisconsin at the facilities of the Gesellschaft für Wissenschaftliche Datenverarbeitung, Göttingen.

Transcription by T3 RNA polymerase

The transcription mixture (25 μl) contained 50 mM Tris–HCl buffer, pH 7.5, 10 mM $MgCl_2$, 1 mM dithiothreitol, 1 mM each ATP, CTP, GTP and UTP, 60 U T3 RNA polymerase (US Biochemical) and 1.5 pmol template. Incubation was for 60 min at 37°C. The templates used were dAATTAACCCTCACTAAAGGGAAGAAACTTCATAGGGTATTTA-AGTTTCTTTTCCAAGAAACTTAAATACCCTATGAAGTTTCTTGG, dCCAAGAAACTTCATAGGGTATTTAAGTTTCTTGGAAAAGAAA-CTTAAATACCCTATGAAGTTTCTTCCCTTTAGTGAGGGTTAATT and an annealed mixture of the two oligonucleotides. Only the double-stranded template gave a defined product with the mobility of T7rp3. For determination of the template activity of RNA an aliquot of the transcript was used after appropriate dilution without further purification. T7rp1 RNA was obtained by transcription from plasmid DNA containing T7rp1 cDNA according to the described cloning procedure and digested with restriction endonuclease *Gsu*I and used for amplification without further purification.

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References

Banerjee,A.K., Eoyang,L., Hori,K. and August,J.T. (1967) *J. Mol. Biol.*, **45**, 181–193.
Biebricher,C.K. (1986) *Chemica Scripta*, **26B**, 51–57.
Biebricher,C.K. (1987) *Cold Spring Harbor Symp. Quant. Biol.*, **52**, 299–306.
Biebricher,C.K. and Luce,R. (1992) *EMBO J.*, **11**, 5129–5135.
Biebricher,C.K. and Luce,R. (1993) *Biochemistry*, **32**, 4848–4854.
Biebricher,C.K. and Orgel,L.E. (1973) *Proc. Natl Acad. Sci. USA*, **70**, 934–938.
Biebricher,C.K., Eigen,M. and Luce,R. (1981a) *J. Mol. Biol.*, **148**, 369–390.
Biebricher,C.K., Eigen,M. and Luce,R. (1981b) *J. Mol. Biol.*, **148**, 391–410.
Biebricher,C.K., Diekmann,S. and Luce,R. (1982) *J. Mol. Biol.*, **154**, 629–648.
Biebricher,C.K., Eigen,M. and Luce,R. (1986) *Nature*, **321**, 89–91.
Biebricher,C.K., Eigen,M. and McCaskill,J.S. (1993) *J. Mol. Biol.*, **231**, 175–179.
Breaker,R.R. and Joyce,G.F. (1994) *Proc. Natl Acad. Sci. USA*, **91**, 6093–6097.
Burgess,R.R. and Jendrisak,J.J. (1975) *Biochemistry*, **14**, 4634–4638.
Davanloo,P., Rosenberg,A.H., Dunn,J.J and Studier,F.W. (1984) *Proc. Natl Acad. Sci. USA*, **81**, 2035–2039.
Diener,T.O., Owens,R.A. and Hammond,R.W. (1993) *Intervirology*, **35**, 186–195.
Grodberg,J. and Dunn,J.J. (1988) *J. Bacteriol.*, **170**, 1245–1253.
Gubler,U. and Hoffmann,B.J. (1983) *Gene*, **25**, 263–269.
Jorgensen,E.D., Durbin,R.K., Risman,S.S. and McAllister,W.T. (1991) *J. Biol. Chem.*, **266**, 645–651.
Konarska,M.M. and Sharp,P.A. (1989) *Cell*, **57**, 423–431.
Konarska,M.M. and Sharp,P.A. (1990) *Cell*, **63**, 609–618.

Lehrach,H. Diamond,D., Wozney,J.M. and Boedtker,H. (1977) *Biochemistry*, **16**, 4743–4751.
McMaster,G.K. and Carmichael,G.G. (1977) *Proc. Natl Acad. Sci. USA*, **74**, 4835–4838.
Milligan,J.F. and Uhlenbeck,O.C. (1989) *Methods Enzymol.*, **180**, 51–62.
Mills,D.R., Peterson,R.L. and Spiegelman,S. (1967) *Proc. Natl Acad. Sci. USA*, **58**, 217–224.
Moody,M.D., Burg,J.L., DiFrancesco,R., Lovern,D., Stanick,W., Lin-Goerke,J., Mahdavi,K., Wu,Y. and Farrell,M.P. (1994) *Biochemistry*, **33**, 13836–13847.
Morris,C.E., Klement,J.F. and McAllister,W.T. (1986) *Gene*, **41**, 193–200.
Rohde,N., Daum,H. and Biebricher,C.K. (1995) *J. Mol. Biol.*, **249**, 754–762.
Schick,C. and Martin,C.T. (1995) *Biochemistry*, **34**, 666–672.
Silberklang,M., Gillum,A.M. and RajBhandary,U.L. (1977) *Nucleic Acids Res.*, **4**, 4091–4108.
Sumper,M. and Luce,R. (1975) *Proc. Natl Acad. Sci. USA*, **72**, 162–166.
Weissmann,C. (1974) *FEBS Lett.*, **40** (suppl.), S10–S18.
Zamora,H., Luce,R. and Biebricher,C.K. (1995) *Biochemistry*, **34**, 1261–1266.

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