RNA Species that Replicate with DNA-Dependent RNA Polymerase from *Escherichia coli*†

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Received December 4, 2000; Revised Manuscript Received January 18, 2001

ABSTRACT: An RNA that replicates with core RNA polymerase from *E. coli* and the substrates ATP, CTP, ITP, and UTP, was selected from a random poly(A,U,I,C) library and named EcorpI. Another replicating RNA, EcorpG, was obtained by template-free incubation of holo RNA polymerase and the substrates ATP, CTP, GTP, and UTP. Both RNA species showed typical autocatalytic RNA amplification profiles with replication rates in the range of other RNA replicons. The replication products were heterogeneous in length; the different lengths appeared to be different replication intermediates. Both RNA were single-stranded with much internal base-pairing but low melting points. Their sequences were composed by permutations of certain sequence motives in both polarities separated by short oligo(A) and oligo(U) clusters. There was evidence for 3′-terminal elongation on an intramolecular template. No double-stranded RNA was found, even though base-pairing is certainly the underlying basis of the replication process. The reaction was highly sensitive: a few RNA strands were sufficient to trigger an amplification avalanche.

The success to replicate RNA nonenzymically in vitro has been a milestone in prebiotic chemistry (*1*−*3*). The experimental approach was oriented essentially on the model of DNA replication: a primer hybridized to a single-stranded template is elongated to form a double strand. An autocatalytic replication reaction, however, requires that template and replica can be reused as templates, but how should melting of the RNA double strand be realized? It is remarkable that the mechanism of the enzymic RNA replication as it is realized in the replication of most prokaryotic, probably also of many eukaryotic, RNA viruses is largely different from the mechanism of DNA replication: (i) a primer is not required, (ii) only specific sequences are replicated, and (iii) the replica formed by the replication round does not form a double strand with the template. Since the replica produced is the complement of the template according to the base-pairing rules of Watson and Crick, a temporary RNA double strand at the replication fork is likely, even though it has not been demonstrated. How are the strands then separated? The participation of an RNA helicase is highly unlikely, because in vitro studies of RNA replication have shown that only a single highly purified enzyme, the RNA replicase, is required and a consumption of nucleoside triphosphates other than for incorporation into the replica strand could not be detected. Thus, the energy for the separation of replica and template must come from the replication process itself (*4*). It seems likely that RNA replication was realized in a way different from DNA replication because the RNA chemistry offers special opportunities. One reason for a bias to single-strandedness of the RNA may be the relatively stronger secondary structures of most natural RNAs as compared to DNAs.

It has been shown also that DNA-dependent RNA polymerases have the potential to autocatalytic amplification of RNA when a suitable template is offered. The first example was shown by Biebricher and Orgel (*5*), where an RNA was shown to be amplified by DNA-dependent RNA polymerase from *Escherichia coli*. Since a natural template was not known, a new technique was invented that is now an established routine in evolutionary biotechnology: it was selected from a random sequence library, synthesized by randomly copolymerizing ribonucleotides. It was estimated to be a very rare incident to find a template in the random population. However, the RNA was not precisely characterized. Konarska and Sharp (*6*, *7*) found a replicable contaminant in a commercial batch of RNA polymerase from bacteriophage T7. The genetic origin of these replicating RNA species remained unknown. Recently, we have shown that a large variety of different RNA species that are replicated by DNA-dependent RNA polymerase from bacteriophage T7 is formed when high concentrations of T7 RNA polymerase are incubated with substrate for extended time periods (*8*). The products differed from sample to sample in molecular weight and sequence; their chain lengths ranged from 60 to 120. The mechanism of autocatalytic amplification of RNA by T7 RNA polymerase proved to be analogous to what is observed with viral RNA-dependent RNA polymerases (replicases): Only single-stranded templates were accepted, and both, template and its complementary replica were shown to be released as single strands.

† This work has been supported by the Deutsche Forschungsgemeinschaft (BI521-1).

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10.1021/bi002756g CCC: $20.00
Published on Web 02/22/2001

Promoter sequence elements were not required for replication.

If RNA was the primordial source of genetic information, as now commonly supposed (*2*, *9*), it seems plausible that enzymes catalyzing RNA replication used a similar mechanism to present-day RNA replication mechanisms. The fact that DNA-dependent RNA polymerases amplify specific RNA in a mechanism analogous to RNA replicases suggests that the ability of single strands to replicate may be a more general property of RNA and that the structures of template and replica RNA contribute to a large extent to strand separation (*4*). The high template specificity of all RNA replicases indicates that much of the replication potential is provided by RNA itself. In this paper, we characterize RNA species that are able to be amplified by DNA-dependent RNA polymerase from *E. coli*.

MATERIALS AND METHODS

RNA polymerase from *E. coli* was purified according to the method of Burgess and Jendrisak (*10*). *Core* and *holo* enzymes were separated on a phosphocellulose column (Whatman P11) in phosphate buffer containing 50% glycerol with a gradient of 0−0.5 M NaCl.

Selection of the Replicons. The AUIC copolymers were prepared as described previously (*5*). AUIC-mix contained 50 mM Tris-HCl buffer (pH 7.5), 200 mM KCl, 2 mM $MgCl_2$, 3 mM $MnCl_2$; 0.2 mM dithiothreitol; 0.75 mM ATP, CTP, ITP, UTP each; 1 μM RNA polymerase and the indicated amounts of template. For the selection experiments, serial transfer experiments were performed that contained additionally 100 μg of AUIC copolymer and 200 μg of tRNA and incubated for 3 h at 37 °C. One-tenth of the incubated mixture was transferred to a fresh incubation sample. Only the first five transfers contained copolymer and tRNA; the later transfers contained sufficient template to sustain the reaction.

The AUGC-mix contained 50 mM Tris-HCl buffer (pH 7.5), 200 mM KCl, 10 mM $MgCl_2$, 0.2 mM dithiothreitol, 0.75 mM ATP, CTP, GTP, UTP each, 1 μM RNA polymerase and the indicated amounts of template. One hundred microliter samples were incubated without template overnight, and the products were analyzed by electrophoresis.

Cloning and Sequencing. EcorpI RNA was isolated and a short oligo(A) tail was attached as described previously (*12*). After retrotranscription with reverse transcriptase from AMV and TTTTTTTTTTV (V = A/G/C) as primer, the cDNA was isolated and converted to double-stranded DNA by the method of Gubler and Hoffmann (*11*). The DNA was made blunt with T4 DNA polymerase, provided with *Bam*HI-linkers, ligated into *Bam*HI-cut pUC18, and used for transforming appropriate hosts. The sequences were determined with the help of the Sequenase kit (USB) according to the instruction of the supplier or by the dye-terminator cycle-sequencing method in a 373A DNA sequencer (Applied Biosystems).

EcorpG RNA was isolated, oligoadenylated, and retrotranscribed using GGGATCCCTTTTTTTTTTV as primer. To the cDNA, an oligo(dA) tail was attached by terminal transferase and dATP. Second strand synthesis was performed with *E. coli* DNA polymerase I and GGGGATCCCTTTTTTTTTTV. The double-stranded cDNA was digested with *Bam*HI and ligated into pUC18.

For expression of EcorpG in plasmids, a modified procedure according to Biebricher and Luce (*12*) was used. Retrotranscription was performed with GGGGATCCCTTTTTTTTTTV as primer. Second strand synthesis was performed with Sequenase and with a primer composed of the phosphorylated, annealed, and ligated oligonucleotides TTTAGTGAGGGTTAATTAAGCT and TAATTAACCCTCACTAAAGAA. After digestion with *Bam*HI and *Hind*III, the DNA was ligated into the plasmid pGL18 and used for transformation.

Run-off Transcription by T3 RNA Polymerase. Plasmids were isolated and digested with *Gsu*I (Fermentas). Ten micrograms of linearized plasmid DNA were incubated in 250 μL of AUGC-mix and 4 μg of T3 RNA polymerase and incubated for 60 min at 37 °C. The mixture was extracted with phenol and precipitated by addition of 500 μL of ethanol. The RNA was separated from the plasmid by gel HPLC[1] with a TSK G3000 SWXL column (Pharmacia, Freiburg) and desalted by a NAP10 column (Pharmacia).

RESULTS

Generation of the Replicons. Two general methods have been employed previously to generate replicons for RNA-dependent and DNA-dependent RNA polymerases: (i) selection from a library of random RNA sequences; (ii) prolonged incubation of RNA polymerase at high concentration with substrate without addition of template. In the simplest case, the RNA polymerase contains already a replicable impurity. However, even in the absence of replicable templates, Qβ replicase (*12*, *13*) and DNA-dependent RNA polymerase from bacteriophage T7 (*8*) are capable to create a replicon: more or less random polynucleotides are produced by slow condensation of the nucleotides and replicable RNA molecules are amplified and optimized.

It was reported that replicating RNA could be selected from a random poly(A,U,I,C) library, provided that Mg^{2+} was replaced by Mn^{2+} and GTP by ITP. We repeated the procedure as described (*5*) and selected a replicon. Electrophoresis of the selected replicon indicated a rather heterogeneous RNA distribution containing two maxima. When the RNA fractions were separately eluted from the gel, amplified, and analyzed again by gel electrophoresis, the multiband pattern was regenerated during amplification, indicating that the different gel bands belong to the same RNA species named EcorpI (Figure 1). The electrophoretic mobility of EcorpI was similar to the RNA species replicating with Qβ replicase or T7 RNA polymerase which have chain lengths of about 100 nucleotides.

Template-free incubation of holo RNA polymerase from *E. coli* (at 1 μM concentration) with the normal incorporation mixture, containing Mg^{2+} as the divalent ion and the natural four nucleoside triphosphate (method 2), also produced replicons (Figure 2). In contrast to the experiments with Qβ replicase and T7 RNA polymerase, different template-free incubation samples resulted in nearly identical results and were heterogeneous in length. Most likely, the RNA polymerase preparation contains nucleic acids that are used as partial templates that are then amplified and optimized. The

[1] Abbreviations: HPLC: high performance liquid chromatography; Tris: tris-(hydroxymethyl)-aminomethane; cpm: counts per minute.

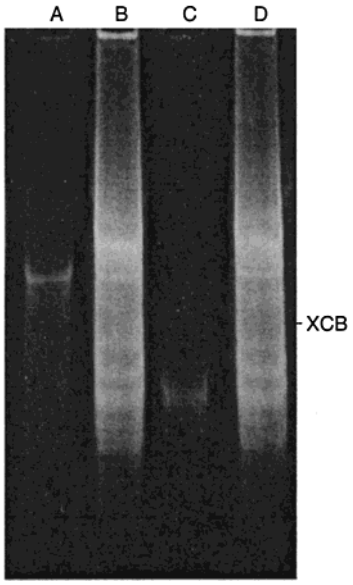


FIGURE 1: Electrophoretic analysis of EcorpI. EcorpI was grown in an AUIC-incubation mix with 0.3 μM core RNA polymerase from *E. coli*. Twenty micrograms of EcorpI were separated by electrophoresis on 12% polyacrylamide gels. The upper and the lower bands were cut out and electroeluted. Aliquots of both fractions were incubated for 1 h at 37 °C in an AUIC-mix with 0.3 μM core RNA polymerase and analyzed by polyacrylamide gel electrophoresis. A: upper band of EcorpI; B: replication products using A as template, C: lower band of EcorpI; D: replication products using C as template. XCB is the position of the dye marker Xylene cyanol blue FF.

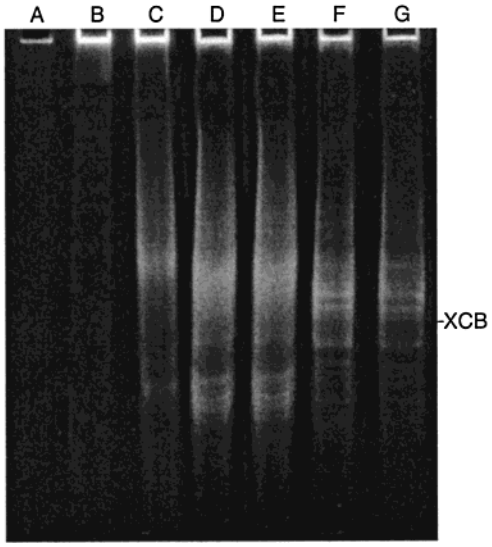
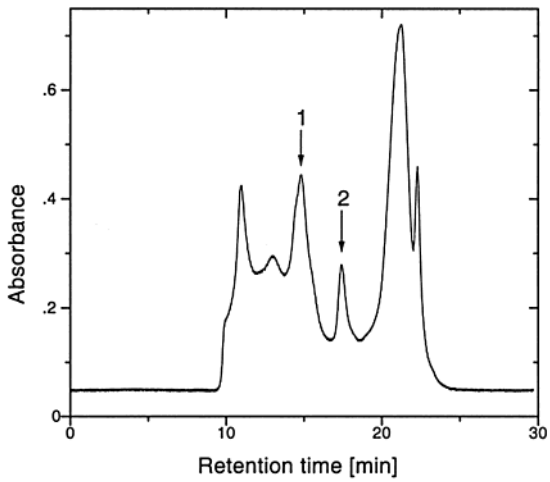


FIGURE 2: Electropherogram of the products of template-free incubation in an AUGC-mix with 1 μM holo RNA polymerase from *E. coli*. A: after 3 h incubation at 37 °C; B: after 48 h incubation; C: 1. serial transfer (90 min, 1 μM holo RNA polymerase); D: 2. serial transfer (90 min, 1 μM holo RNA polymerase); E: 3. serial transfer (90 min, 1 μM holo RNA polymerase); F: 4. serial transfer (90 min, 0.5 μM holo RNA polymerase); G: 5. serial transfer (3 h, 1 μM holo RNA polymerase).

presence of replicable RNA in the polymerase preparation was ruled out (see below). As in template-free incubations with Qβ replicase and T7 RNA polymerase, a substantial amount of the early products were of high molecular weight that barely entered the polyacrylamide gel. In contrast to the other RNA polymerases, however, the high molecular weight material was still present after several serial transfers, proving



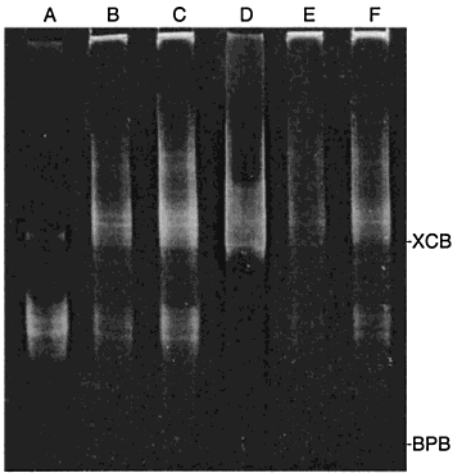


FIGURE 3: Replication products of fractionated EcorpG. Top: HPLC gel chromatography of EcorpG on a TSK G3000 SWXL column using 0.1 M potassium phosphate buffer (pH 7.0)/0.1 M NaCl/1 mM EDTA as eluent. The first peak is high molecular weight RNA followed by fractions 1 and 2 of the replicating RNA. The trailing peak contains the nucleoside triphosphates. Bottom: Electropherogram of the products. A: 1 μg of RNA peak 2; B: Products of replication with 1 μM holo RNA polymerase after 60 min, using A as template; C: Replication products after 120 min; D: 1 μg of RNA peak 1; E,F: replication products using D as templates after 60 and 120 min, respectively. XCB, BPB are the positions of the dye markers Xylene cyanol blue FF and Bromophenol blue.

that the material is produced in the replication process. The other bands were migrating in the range of other RNA replicons, corresponding to chain lengths of 50−100 nt. When the RNA products where separated by gel permeation HPLC (Figure 3, top) and amplified by RNA polymerase from *E. coli*, the original heterogeneous pattern, including the high molecular weight material, was restored (Figure 3, bottom), indicating that the different lengths are different replication intermediates. Heating and rapid cooling (melting) or long incubation at 45 °C (annealing) did not change the replication pattern (not shown); hence the presence of double-stranded RNA is unlikely. Temperature gradient gel electrophoresis (*15*) of the replicon (Figure 4) showed that the different electrophoresis bands have similar melting behavior and have strong secondary structures that break down at similar temperatures, indicating that they are sequentially related one to another. The absence of discontinuities in the temperature lines confirms that double strands are absent.

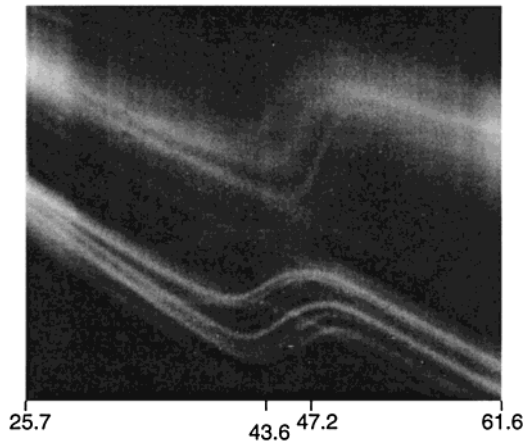


FIGURE 4: Temperature gradient polyacrylamide gel electrophoresis of EcorpG. Electrophoresis from top to bottom, temperature gradient from left to right. Numbers are temperatures in °C.

Temperature gradient gel electrophoresis of EcorpI gave analogous results, but the melting points were somewhat lower.

Replication Kinetics of the RNA Replicons. The growth profiles of the replicating replicons EcorpI and EcorpG showed (Figure 5) the typical pattern of other RNA replicons, e.g., the RNA species that are replicated by Qβ replicase (*16*) or T7 RNA polymerase (*8*): the RNA is amplified exponentially in excess of enzyme; when the RNA polymerase is saturated with template, linear growth is observed. Eventually, the concentration profile levels into a maximal RNA concentration. Dilution of the template results in a displacement of the profile on the time axis (Figure 5). The displacement allows a precise measurement of the overall replication rate in the exponential growth phase; the overall replication rate in the linear growth phase is determined by the slope of the linear part of the profile (*17*). The overall replication rates of EcorpG were in the range of other RNA replicons, while the replication of EcorpI proceeded at a higher rate (Table 1), possibly because of the weaker energy content of the I:C base pairs.

However, in contrast to those RNA species, both EcorpI and EcorpG have similar rates in the exponential and linear growth phases, suggesting large differences in the replication mechanism. Apparently, the liberation of the enzyme from the template at the end of a replication round is not the rate-limiting step but rather initiation of new strands and/or chain elongation. In agreement with this conclusion, the replication rates were found to depend strongly on the enzyme concentration. The standard enzyme concentrations used for replication experiments, 300 nM core RNA polymerase for EcorpI and 1 μM holo RNA polymerase for EcorpG, were thus much higher than for transcription experiments. A sharp transition between exponential growth to linear growth at an RNA-to-enzyme ratio of approximately 1 indicated strong binding of enzyme to template. This was confirmed by nitrocellulose filtration of enzyme−template complexes. Product inhibition was not as strong as observed with Qβ replicase or T7 RNA polymerase: More than 50% of the nucleoside triphosphate were incorporated. For EcorpI, the precipitation of inorganic pyrophosphate as the manganese salt may contribute to a reduced product inhibition.

To analyze the 5′-terminal nucleotides of the replicating species, the RNA was treated with alkaline phosphatase and

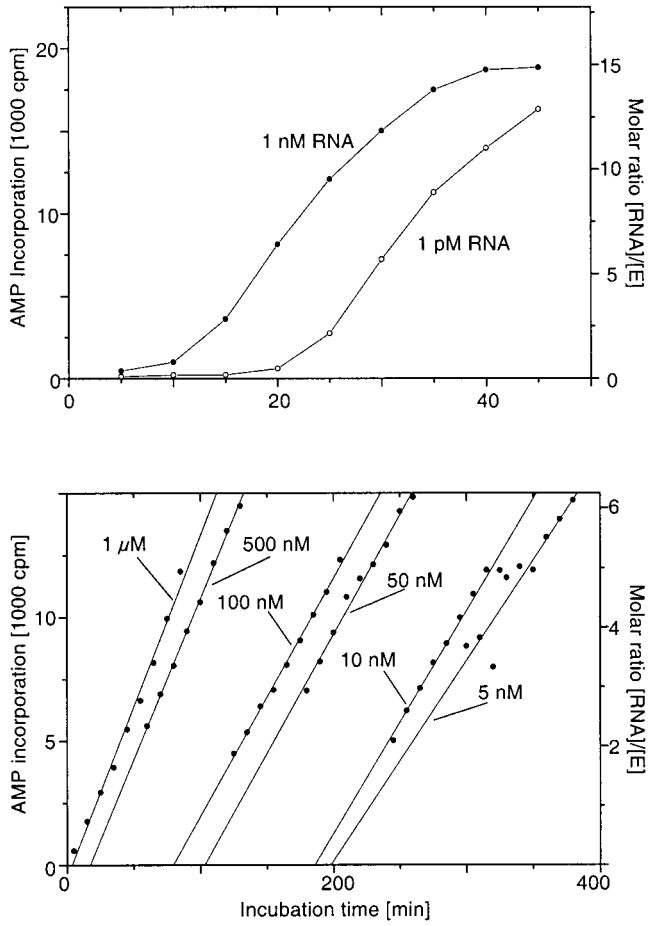


FIGURE 5: Top: Growth profiles of EcorpI with 0.3 μM core RNA polymerase in AUIC-mix containing [^{14}C]-ATP at different template concentrations. The RNA concentration was estimated from the radioactivity by assuming on average 25 A residues per template strand. Bottom: Growth profiles of EcorpG with 1 μM holo RNA polymerase in AUGC-mix containing [^{14}C]-ATP at different template concentrations. The RNA concentration was estimated from the radioactivity by assuming on average 25 A residues per template strand.

Table 1: Replication parameter of EcorpI and EcorpG[a]

	EcorpI	EcorpG	T7rp1
ρ [s^{-1}]	1.0×10^{-2}	0.7×10^{-3}	1.5×10^{-3}
κ [s^{-1}]	1.1×10^{-2}	0.8×10^{-3}	3.8×10^{-3}
k_D [s^{-1}]	3.0×10^{-2}	1.8×10^{-3}	1.7×10^{-3}
k_E [s^{-1}]	1.5×10^{-2}	1.2×10^{-3}	1.3×10^{-2}

[a] For comparison, RNA species T7rp1 replicated by T7 RNA polymerase is shown (*8*). The exponential growth rate κ was determined from the time displacement Δt of the growth profile caused by the dilution factor f_{dil} according to $\kappa = \log f_{dil}/\Delta t$. The overall replication rate in the linear growth phase, ρ, was determined from the slope of the linear part of the growth profile, assuming an average content of 25 A residues per template strand. The calculation of the composite rates for chain elongation k_E and template liberation k_D was done as described for replicons of Qβ replicase (*17*). These values can only give crude estimates due to the differences in the replication mechanisms.

labeled with polynucleotide kinase and [γ-^{32}P]-ATP and digested with alkali. For EcorpI nearly exclusively labeled A was found, and for EcorpG about 5% 5′-terminal U, 45% A, and 50% G were found. If phosphatase treatment was omitted, only a small fraction of RNA strands were labeled. On the other hand, [γ-^{32}P]-ATP was not incorporated into replicating EcorpI or EcorpG. Apparently, the 5′-adenyl

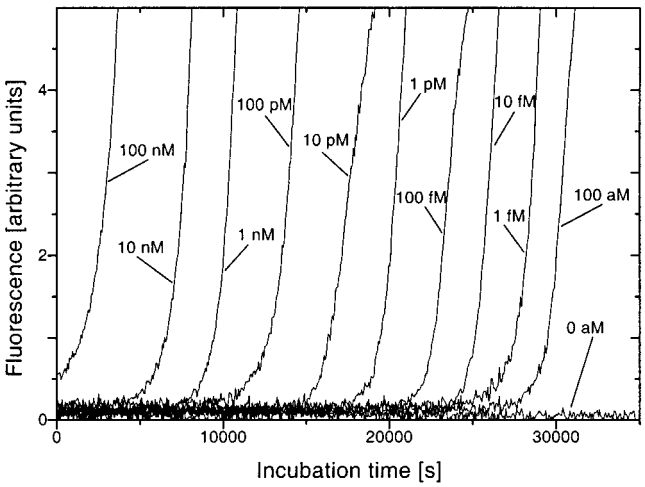


FIGURE 6: Growth profiles in the multichannel fluorimeter. 25 μL samples of AUGC-mix containing 1 μM RNA polymerase and 3 μM thiazole orange were inoculated with EcorpG at serial dilutions and incubated for >12 h at 37 °C. Fluorescence excitation was at a wavelength of 488 nm; emission (in arbitrary units) was recorded at wavelengths above 515 nm. The electrophoretic pattern of all samples was checked and found to be normal.

residues are not formed by condensation of two triphosphates as in the normal initiation of new RNA chains by DNA-dependent and RNA-dependent RNA polymerases. However, [γ-^{32}P]-GTP was incorporated efficiently into replicating EcorpG. The electrophoretic mobility of the [γ-^{32}P]-GTP incorporation products was identical to the mobility of the replicating RNA; in addition, a rather large amount of short oligonucleotides were also observed (data not shown). If 5′-OH termini of EcorpG were blocked prior to phosphatase treatment by treatment with polynucleotide kinase and [γ-S]-GTP, only A and G were labeled; therefore, the terminal U was probably formed by the normal hydrolysis reaction between the 5′-OH and the 3′-phosphate.

A short pulse-label with [α-^{32}P]-ATP in the incubation mixture labeled preferentially the band with the lower mobility, presumably the longer intermediate. After a chase with excess unlabeled ATP, the equilibrium distribution of the RNA products was obtained (data not shown). Isolation of the enzyme-bound RNA on nitrocellulose filters followed by electrophoresis showed that the RNA with the shorter chain length was preferentially bound to enzyme. A longer RNA chain as an intermediate for the formation of a shorter one must necessarily involve a chain break within the replication cycle.

As in other RNA replication systems, the assay is extremely sensitive to the presence of minute amounts of RNA template. Serial dilutions of template were performed in a multichannel fluorimeter developed in this department (*18*). Recorded was the fluorescence of the intercalating dye Thiazol orange. Incorporation measurements showed that the growth profile were not significantly affected by addition of the dye. Figure 6 shows that the displacements on the time axis remain approximately constant until a dilution of 10^{-16} M, corresponding to approximately 1500 template strands. A sample without adding template did not give a signal within the recording time of >12 h; therefore, the enzyme preparation was not contaminated with replicable RNA. Contamination with nonreplicable RNA, however, cannot be ruled out. The equidistant displacements on the time axis prove that a single RNA strand is used in a replication round and cooperative effects among different template strands are absent.

FIGURE 7: Sequences of cDNA clones of EcorpI. The motives and their polarities are indicated. Smaller symbols designate mutations.

Cloning and Sequencing of Replicating Species. Since the terminal sequences of the EcorpI RNA were not known, cloning and sequencing of the cDNA of the replicating RNA were done by the method of Gubler and Hoffmann (*11*). The 5′-termini were lost by this method. As expected, the cDNA clones had different chain lengths. However, the sequences were strongly related one to another: two common motives, 12 and 18 nucleotides long, occurred both in plus and minus polarities (Figure 7). The motives are not related to the known promoter sequences; this is not surprising, because the promoter sequences are recognized by the σ subunit of RNA polymerase that is missing in the *core* enzyme used for these experiments. Sometimes the motives occurred in mutated form, probably by an replication error. It is remarkable that mutated motives were found repeatedly and in both polarities within the same RNA strand, indicating that parts of the strands may have worked as intramolecular templates (Figure 7, panels c and e).

For cloning of the EcorpG RNA species, the cDNA strand was elongated at its 3′-terminus with terminal transferase and dATP to avoid loss of the 5′-terminal sequence (see Materials and Methods). Figure 8 shows the sequences of some clones. The sequence was essentially composed by oligo(A) and oligo(U) clusters and the complementary motives GAAU or AUUC. The sequence lengths ranged from 25 up to 140 nucleotides. We deduced as the probable sequence-initiating motive GAAU and used it for a cloning procedure designed to give accurate full-length cDNA behind a T3 promoter to allow reproduction of the RNA sequence by in vitro transcription by T3 RNA polymerase. Three RNA sequences were identified, called EcorpG1, EcorpG2, and

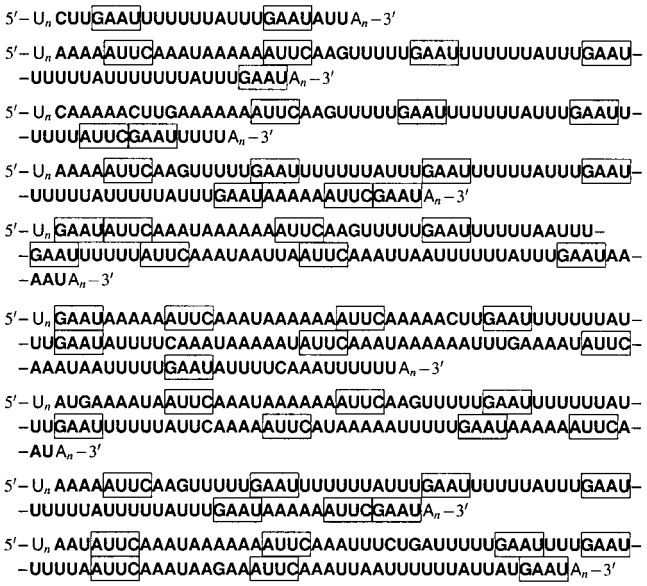


FIGURE 8: Sequences of cDNA clones of EcorpG.

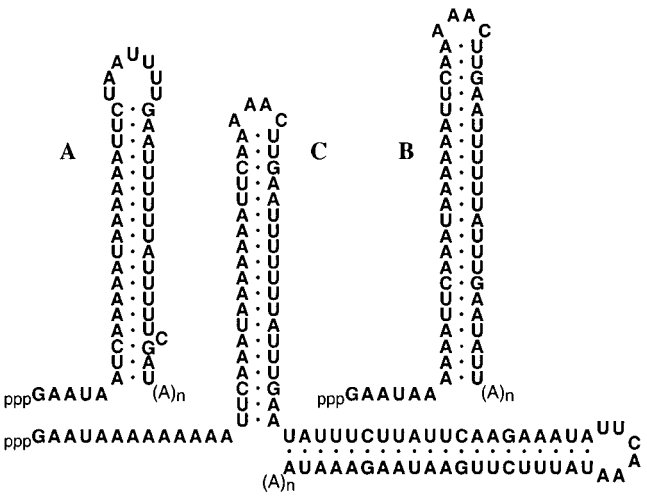


FIGURE 9: Calculated secondary structures of representatives of the EcorpG RNA population. The 3′-terminal A-clusters were attached by the cloning procedure and do not belong to the sequence. A: EcorpG1, B: EcrorpG3, C: EcorpG2.

EcorpG3, with chain lengths of 51, 56, and 103 nucleotides, respectively. Their calculated secondary structures contained large hairpins with unpaired 5′-termini. The secondary structures (Figure 9) are in agreement with the results from the temperature gradient polyacrylamide gel electrophoresis. Because of the composition by repetitive motives several alternative foldings with similar energies are possible. Note that full-length complementary strands are unlikely because the sequences do not contain AUUC motives at their 3′-termini.

To prove that the identified RNA molecules do serve as templates for *E. coli* RNA polymerase, the transcibed RNA was incubated under standard replication conditions. It could be shown that each of the three sequences was amplified by the RNA polymerase in an autocatalytic way, although at a lower rate than the original EcorpG (Figure 10). While serial dilution of EcorpG by a constant factor produced shifts of the profile on the time axis by constant time intervals, the growth profile displacements of EcorpG1 were larger at the beginning getting smaller and smaller, until the displacement

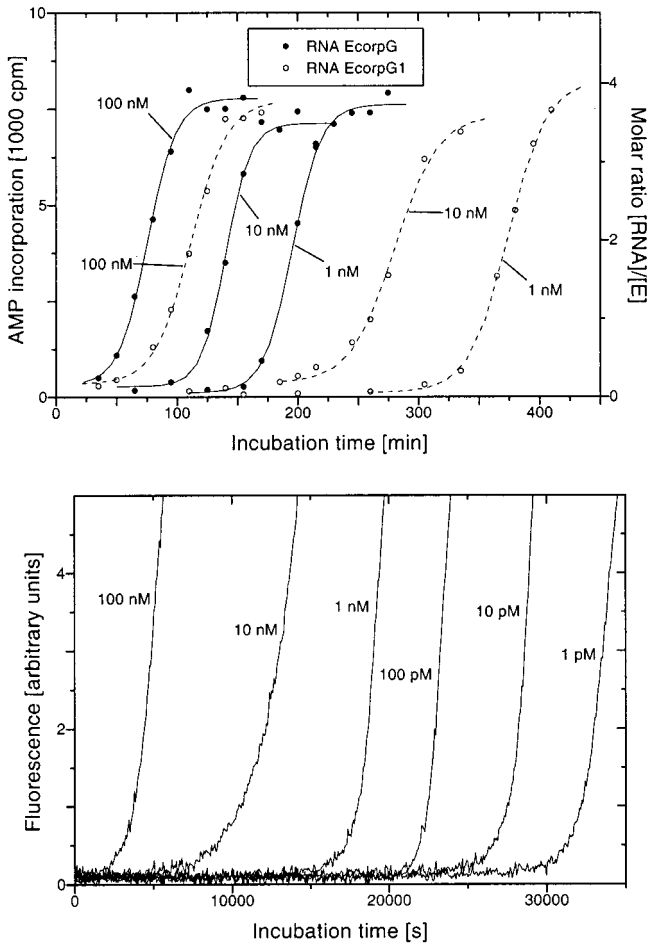


FIGURE 10: Growth profiles of RNA transcripts from cDNA clones. EcorpG1 transcript was prepared as described in the methods part and used as template. Top: Measurement of [^{14}C]-AMP incorporation. Conditions as in Figure 5. Bottom: Measurements in the multichannel fluorimeter. Conditions as in Figure 6.

of EcorpG was reached. The increase in the rate of the exponential amplification suggests an optimization process. Concomitant with the increase in rate was the restoration of the electrophoretic pattern of EcorpG.

DISCUSSION

Replication of RNA by the DNA-dependent RNA polymerase from *E. coli* is obviously a nonphysiological reaction. It shares with similar reactions by Qβ replicase and T7 RNA polymerase the properties that the reaction is highly specific for the RNA and its mechanism does not involve a double strand as a replication intermediate. It appears that probably any RNA polymerase can serve as an RNA replicase, provided a suitable template RNA is presented. There is also an in vivo example how a parasitic RNA makes use of this potential: viroid RNA does not code for an RNA-dependent RNA polymerase, but directs the host RNA polymerase II to replicate it (*19, 20*). The RNA replication kinetics follows the same principles: one or a few template strands are autocatalytically amplified by the enzyme (*21*).

However, RNA replication by RNA polymerase from *E. coli* shows some features not found in the RNA replication by Qβ replicase (*22*) or T7 RNA polymerase (*8*):

(i) RNA replicating with RNA polymerase from *E. coli* does not have a clearly defined sequence with a certain length

but is composed rather by a variable permutation of a few motives.

(ii) Replication does not begin at the 3′-terminus of a template producing a fully complementary replica, but produces a mixture of shorter and longer strands. When the mixture is fractionated, each fraction is triggering replication; after a few replication rounds, the original pattern is restored, indicating that the different fractions represent different ages in the replication cycle.

(iii) Errors in a motive led to the propagation of the error in the same strand, suggesting an intramolecular template-directed elongation of the 3′-terminus. This mechanism is supported by the kinetic evidence for a single template molecule and by the large inhibition caused by periodate oxidation of the 3′-terminus. A similar reaction was observed with Qβ replicase when initiation of new strands was suppressed by replacing GTP by ITP (*23*).

(iv) A large part of the RNA products lacks a 5′-triphosphate end of the RNA but contains a 5′-terminal adenosine monophosphate. Pulse-labeling marked preferentially long RNA strands that were later chased into shorter molecules. This required cutting of longer sequences into shorter ones by a specific hydrolysis reaction producing 5′-pA termini. Incorporation of GTP indicates starting a new chain; incorporation of GTP by transesterification (*24*, *25*) seems highly unlikely.

(v) Amplification of poly(AU) or poly(IC), poly(A):poly-(U) (*26*−*28*) has been reported to proceed by a slippage mechanism (*29*). A slippage mechanism for the replicating RNA described in this work seems impossible, because the repeated motives are too long. However, the presence of repeated motives in both polarities opens the possibility of refolding the RNA to alternative structures. Refolding requires melting of the RNA, and indeed the melting points are low. The presence of two neighboring strong base pairs in the EcorpG sequence is extremely rare. The repeating motives are longer in EcorpI where strong base pairs do not exist. It is also remarkable that fully palindromic sequences corresponding to perfect hairpins or fully complementary sequences were not found. However, despite the many mechanistic hints, the replication mechanism by RNA polymerase from *E. coli* is not yet fully understood.

It is remarkable that an enzymatic in vitro amplification system of double-stranded RNA is not yet known. All known DNA-dependent and RNA-dependent RNA polymerase synthesize single-stranded RNA. RNA replicated by T7 RNA polymerase (*8*) or Qβ replicase (*30*−*31*) have a high degree of intramolecular base-pairing as is required also for RNA templates in vivo (*32*−*33*). The high intramolecular base pairing is found also in the EcorpI and EcorG sequences described in this work. Replicating RNA species often have a leader stem at their 5′-termini that may initiate and facilitate the strand separation during replication (*31*). This is not observed in EcorpG and EcorpI, probably because initiation does not begin at the 3′-terminus.

Double-stranded RNA when formed is unable to replicate with replicases (*30*). The hypothetical "RNA world" (*9*) certainly requires single-stranded RNA molecules, because only these can form the defined tertiary structures of high complexity required for highly selective binding and catalysis. Primitive RNA replication mediated by ribozymic polymerases (*34*−*36*) should thus not work by completion of a partially double-stranded template−primer complex to a perfect double strand, because this mechanism would not provide suitable RNA products. We propose that primitive RNA replication (i) may have been also highly selective in accepting RNA templates and (ii) proceeded by strand separation and not by completion of a template−primer complex.

ACKNOWLEDGMENT

We are indebted to Dr. Tobias Ederhof for his help in the experiments in the multichannel fluorimeter apparatus and to Rüdiger Luce for many valuable suggestions.

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BI002756G