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Synergism in replication and translation of messenger RNA in a cell-free system

(Qβ replicase/recombinant RNA/asymmetric replication)

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ABSTRACT **Combination of the Qβ replicase reaction with the *Escherichia coli* cell-free translation system markedly enhances replication of a recombinant RQ-DHFR RNA consisting of the dihydrofolate reductase (DHFR) mRNA sequence inserted into RQ135$_{-1}$ RNA, an efficient naturally occurring Qβ replicase template. The enhancement is associated with a replication asymmetry previously described for the replication of Qβ phage RNA *in vivo*; the sense (+)-strands are produced in large excess over the antisense (−)-strands. This, in turn, results in increased synthesis of the functionally active DHFR. These effects are not observed when DHFR mRNAs or RQ135$_{-1}$ RNAs are used as templates, if the translation system is not complete, or if it is inhibited by puromycin. The coupled replication–translation of nonviral mRNA recombinants can serve as a useful model for studying the fundamental aspects of virus amplification and can be implemented for large-scale protein synthesis *in vitro*.**

There have been many attempts to exploit the unique capability of the RNA-directed RNA polymerase of phage Qβ (Qβ replicase) to efficiently amplify RNA *in vitro* for large-scale synthesis of mRNAs useful in cell-free translation systems. A homologous (cognate) single-stranded RNA template is copied by the purified Qβ replicase into its single-stranded complement. Both the original and product strands serve as equally efficient templates in the subsequent rounds of replication resulting in exponential RNA amplification under isothermal conditions (1–3). Among cognate RNAs, there are Qβ phage RNA and a number of small RQ RNAs found in the Qβ phage and in the phage-infected cells (4, 5). In contrast, heterologous RNAs do not endure more than one round of replication because of collapse of the template and product strand into a duplex (6–11), which cannot be copied by Qβ replicase (12, 13). Heterologous RNA sequences whose length is within several tens of nucleotides can be made amplifiable by inserting them into actively replicating cognate RQ RNAs (14, 15), but longer inserts often inactivate Qβ replicase templates *in vitro* (16). Surprisingly, some of the inactive RQ-mRNA recombinants appeared to be amplifiable by Qβ replicase in a living *Escherichia coli* cell (17).

We examined the effects of intracellular components on the capability of an RQ-mRNA recombinant to replicate *in vitro* and found that significant stimulation of RNA synthesis is only observed in the presence of a complete functioning translation system. The stimulation is associated with elevated production of the sense (+)-strands over the antisense (−)-strands, which in turn provides more messengers for translation. Thus, coupling of the replication and translation reactions results in their synergistic action.

MATERIALS AND METHODS

Template RNAs. RQ-DHFR RNA recombinants were prepared at the DNA level by inserting the bacterial dihydrofolate reductase (DHFR) mRNA sequence into the sequence of RQ135$_{-1}$(−) RNA (18). The sense (+)- and antisense (−)-strands of RQ-DHFR RNA were obtained by runoff transcription (19) of plasmids pT7RQ135$_{-1}$(−)DHFR(+) or pT7RQ135$_{-1}$(−)DHFR(−), respectively, digested with endonuclease *Sma* I. The plasmids were prepared as follows. A DHFR gene-containing *Sma* I/*Hae* II fragment was excised from plasmid pSP65DHFR generously provided by N. V. Murzina (this institute) and treated with T4 DNA polymerase to produce blunt ends prior to ligation into site *Xho* I of a mutant plasmid pT7RQ135$_{-1}$(−), which was blunt-ended by incubation with Klenow fragment. The original pT7RQ135$_{-1}$(−) was prepared by A. V. Munishkin (this institute) by insertion of a PCR-amplified T7 promoter/RQ135$_{-1}$(−) cDNA construct into plasmid pUC18 between polylinker sites *Hin*dIII and *Sma* I. To generate a unique *Xho* I site within pT7RQ135$_{-1}$(−), the T → C and A → T substitutions at positions 53 and 54 of the RQ135$_{-1}$(−) sequence, respectively, were introduced by oligonucleotide mutagenesis.

Control DHFR mRNA containing a 36-nt-long 5′-terminal and a 165-nt-long 3′-terminal untranslated region originating from the *E. coli* chromosome (20) was obtained by runoff transcription with SP6 polymerase of a *Hin*dIII-digested plasmid pSP65DHFR$_{0.7}$ generously provided by B. Hardesty (University of Texas, Austin). Recombinant MDV-CAT RNA (21) was obtained by transcription with T7 RNA polymerase of plasmid pT7-MDV(+)CAT(−) generously provided by Y. Wu and F. R. Kramer (Public Health Research Institute, New York).

Replication. A 30-μl Qβ replicase reaction mixture was prepared on buffer A [100 mM Tris·HCl, pH 7.7/9 mM $MgCl_2$/1 mM EDTA/10% (vol/vol) glycerol] or buffer B (65 mM Tris·HCl, pH 7.5/50 mM KCl/9 mM $MgCl_2$/1 mM EDTA/2 mM dithiothreitol) and contained 15 units of human placental RNase inhibitor; 0.15 unit of pyruvate kinase; 10 mM phospho*enol*pyruvate; 0.5 mM each ATP, GTP, CTP, and UTP; purified Qβ replicase (18); and an unlabeled RNA template as specified. Where appropriate, [^{32}P]UTP was present at a specific activity of 2.5 Ci/mmol (1 Ci = 37 GBq) to label RNA products. Reaction mixtures were incubated at 37°C for the time indicated. RNA products were analyzed in 5-μl aliquots by autoradiography after electrophoresis through a 5% polyacrylamide gel (22) or by measuring the radioactivity of trichloroacetic acid-precipitated material.

Labeled (+)- and (−)-strands were discriminated by annealing them with excess unlabeled (+)-strands (23). ^{32}P-labeled replication products were separated by electrophoresis through a low melting point agarose (22), and the bands corresponding to single-stranded and double-stranded RQ-DHFR RNA were excised, pooled, and eluted. The samples were denatured in the presence of 20 μg of unlabeled RQ-DHFR(+) RNA at 95°C for 10 min in 0.1 mM Tris·EDTA/0.5% SDS and annealed by incubation at 65°C for 20 min in

Abbreviation: DHFR, dihydrofolate reductase.

750 mM NaCl/75 mM sodium citrate, pH 7.5. The labeled (+)-strands (occurring among single strands) were then separated from the labeled (−)-strands (involved in duplexes) by electrophoresis through a 5% polyacrylamide gel (22) and analyzed by autoradiography.

Translation and Coupled Replication–Translation. A complete cell-free translation system was prepared on buffer B and contained in 30 μl: 1.4 A_{260} units of 0.5 M NH_4Cl-washed 70S ribosomes (24), 12 μg of protein of S100 fraction (25), 10 μg of *E. coli* tRNA (Boehringer Mannheim), 0.15 unit of pyruvate kinase, 15 units of human placental RNase inhibitor, 10 mM phospho*enol*pyruvate, 0.5 mM ATP, 0.5 mM GTP, 25 μM each of the 20 amino acids, 0.3 μg of folinic acid, and unlabeled mRNA as specified. Where appropriate 2.5 μM [^{3}H]leucine (79 Ci/mmol) or 10 μM [^{35}S]methionine (20 Ci/mmol) replaced the respective unlabeled amino acids to label the reaction products. The reaction mixtures were incubated at 37°C. Labeled reaction products were assessed by counting the radioactivity of 5-μl samples precipitated with 5% trichloroacetic acid. ^{35}S-labeled polypeptides were analyzed by autoradiography of electrophoretic gels (26).

A coupled replication–translation system contained, in addition to the above, 0.5 mM each CTP and UTP (with or without addition of [^{32}P]UTP) and Qβ replicase as specified.

RESULTS

Recombinant mRNA Constructs. The recombinant RNAs used in this study (RQ-DHFR RNAs) contained the *E. coli* DHFR mRNA within the sequence of RQ135$_{-1}$ RNA (18), which is one of the most efficient Qβ replicase templates. The DHFR gene sequence (20) was inserted at the DNA level into the position corresponding to a loop of RQ135$_{-1}$(−) RNA; as expected, positioning of the insert into this site would minimally disturb the RQ RNA secondary and tertiary structures. To this end, two nucleotide substitutions were introduced into the loop in order to generate the restriction site *Xho* I (in a cDNA sequence) as shown in Fig. 1*A*. The mutant RNA was as effective in amplification by Qβ replicase as the original RQ135$_{-1}$ RNA (data not shown).

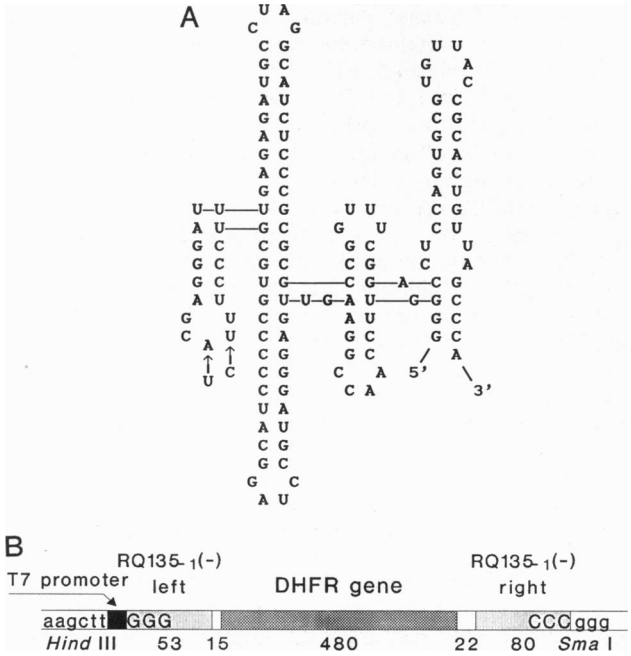

FIG. 1. Construction of recombinant RQ-DHFR RNA. (*A*) Secondary structure of RQ135$_{-1}$(−) RNA with indication (arrows) of nucleotide substitutions that give rise to site *Xho* I in cDNA. (*B*) Scheme of the *Hin*dIII/*Sma* I segment of plasmid pT7-RQ135$_{-1}$(−)DHFR(+). Beginning and end of the transcribed region are marked in capital letters. Numbers at the bottom indicate sizes (nt) of the respective segments.

The sense (+)-strand of RQ-DHFR RNA was obtained by runoff transcription of the *Sma* I-digested plasmid pT7-RQ135$_{-1}$(−)DHFR(+), which is schematically shown in Fig. 1*B*. The transcript began with GGG and terminated with CCC, which is a characteristic of any RQ RNA. The DHFR insert contained, in addition to the 480-nt-long coding sequence, 15 upstream and 22 downstream nucleotides originating from the *E. coli* chromosome (20), which together with the RQ135$_{-1}$-derived segments formed the 68-nt-long 5′-terminal and the 102-nt-long 3′-terminal untranslated regions. The antisense (−)-strand of RQ-DHFR RNA was obtained by transcription of plasmid pT7RQ135$_{-1}$(−)DHFR(−), which differed from pT7RQ135$_{-1}$(−)DHFR(+) by orientation of the DHFR insert.

Replication of Recombinant mRNA Is Enhanced in the Presence of the Cell-Free Translation System. In the purified Qβ replicase reaction, RQ-DHFR RNA replicated inefficiently, as did another recombinant RNA, MDV-CAT (Fig. 2*A*). The latter RNA carries chloramphenicol acetyltransferase mRNA within the MDV-1 RNA (midivariant RNA) sequence (21). Although full-length RNA was synthesized early in the reaction, no significant product accumulation occurred during further incubation (Fig. 2*A*). Varying the Mg^{2+} concentration from 1 to 15 mM or temperature from 20°C to 40°C did not improve the results. Similar kinetics

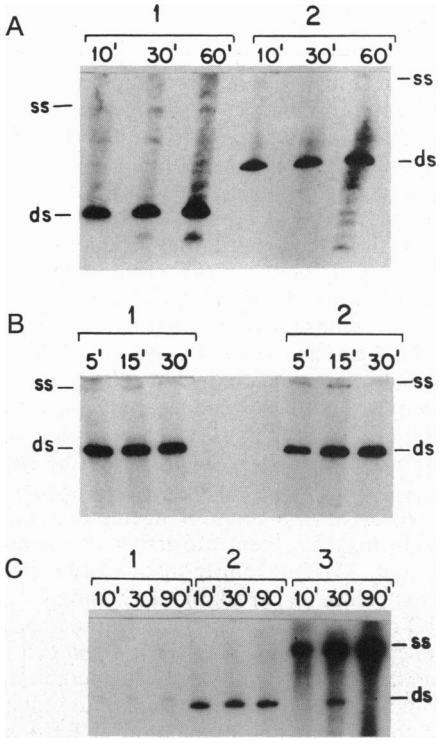

FIG. 2. Electrophoretic analysis of products of recombinant mRNA replication. Locations of the unlabeled single-stranded (ss) and double-stranded (ds) RQ-DHFR RNA and MDV-CAT RNA size markers were revealed by gel staining with toluidine blue. (*A*) RNA synthesis in buffer A by 2 μg of purified Qβ replicase using 1 μg of RQ-DHFR(+) RNA (lanes 1) or MDV-CAT(−) RNA (lanes 2) as a template. (*B*) RNA synthesis in buffer A by 0.5 μg of purified Qβ replicase using 0.1 μg of the (+)-strand (lanes 1) or the (−)-strand (lanes 2) of RQ-DHFR RNA as a template. (*C*) RNA synthesis in buffer B by 1 μg of Qβ replicase using 1 μg of the control DHFR mRNA (lanes 1) or 1 μg of RQ-DHFR(+) RNA (lanes 2 and 3) in the absence (lanes 1 and 2) or in the presence (lanes 3) of the complete cell-free translation system.

were observed whether the reaction was initiated with the (+)- or (−)-strands of RQ-DHFR RNA (Fig. 2*B*). At the same time, insertion of DHFR mRNA into the RQ135$_{-1}$ sequence had a strong stimulating effect on early RNA synthesis (see lanes in the center and on the left of Fig. 2*C*). This suggests that the presence of the elements of RQ135$_{-1}$ RNA improved recognition of the recombinant RNA by Qβ replicase.

Fig. 2*C* shows that supplementing the Qβ replicase reaction with a complete cell-free translation system from *E. coli* resulted in a drastic increase in RNA synthesis on the RQ-DHFR template (see lanes in the center and on the right of Fig. 2*C*). This is consistent with the observation that RQ-mRNA recombinants can efficiently replicate *in vivo* (17), as the cell-free translation system includes many cellular components. In contrast, the translation system did not promote RNA synthesis on the control DHFR mRNA template (data not shown).

Replication of Recombinant mRNA Is Enhanced due to Involvement of the Sense Strands in Translation. The fast early RNA synthesis by purified Qβ replicase on both the RQ-DHFR(+) and the RQ-DHFR(−) RNAs suggests that the single-stranded recombinant RNAs (as formed during plasmid transcription) are good Qβ replicase templates. The subsequent slow kinetics indicates that copying from these templates makes them less accessible for further replication, presumably because of annealing with the product strands.

This assumption is supported by experiments in which the newly synthesized (+)- and (−)-strands were separately assayed in the reaction products by annealing the labeled RNA products with a large excess of the unlabeled RQ-DHFR(+) RNA (23). The labeled (−)-strands became entrapped in duplexes, while most of the labeled (+)-strands remained single stranded and, therefore, were easily separated from the (−)-strands by gel electrophoresis. As shown in Fig. 3*A*, the products of the purified Qβ replicase reaction initiated with the RQ-DHFR(+) transcripts consist mainly of the (−)-strands, whereas the (+)-strands are barely detectable. It follows that the (−)-strands synthesized by purified Qβ replicase cannot serve as Qβ replicase templates, in contrast to the (−)-strands formed in transcription (which produces single strands). On the contrary, in the presence of the translation system the (+)-strands are produced in a large amount, indicating that the (−)-strands synthesized in the first round are readily available for copying in the subsequent rounds.

In view of these observations, the ability of recombinant RNAs to efficiently replicate *in vivo* (17) and in the presence of the cell-free translation system (Fig. 2*C*) could be attributed to their interaction with some cellular RNA-binding proteins, which prevents the template and product strands from annealing. However, in the presence of an incomplete translation system with either the S100 fraction (containing the soluble *E. coli* proteins) or the ribosomes being omitted, neither enhanced replication of the RQ-DHFR RNA nor significant synthesis of the (+)-strands was observed. Moreover, the stimulating effect of the complete translation system on RQ-DHFR RNA replication disappeared in the presence of puromycin, a specific inhibitor of protein synthesis (Fig. 3*A*). The results suggest that replication of RQ-DHFR RNA is enhanced because of involvement of the sense (+)-strands in translation. This conclusion is further supported by the observation that replication of the precursor RQ135$_{-1}$ RNA that does not contain a protein-coding sequence was neither stimulated in the presence of the translation system nor significantly inhibited by puromycin (Fig. 4).

Replication of Recombinant mRNA in the Coupled Replication–Translation Reaction Is Asymmetric. In contrast to the "symmetric" replication of RQ135$_{-1}$ RNA in the Qβ replicase reaction where the complementary strands are synthesized in roughly equal amounts (18), the (+)-strands of the recombinant RQ-DHFR RNA are produced in excess over the (−)-strands in the coupled replication–translation reac-

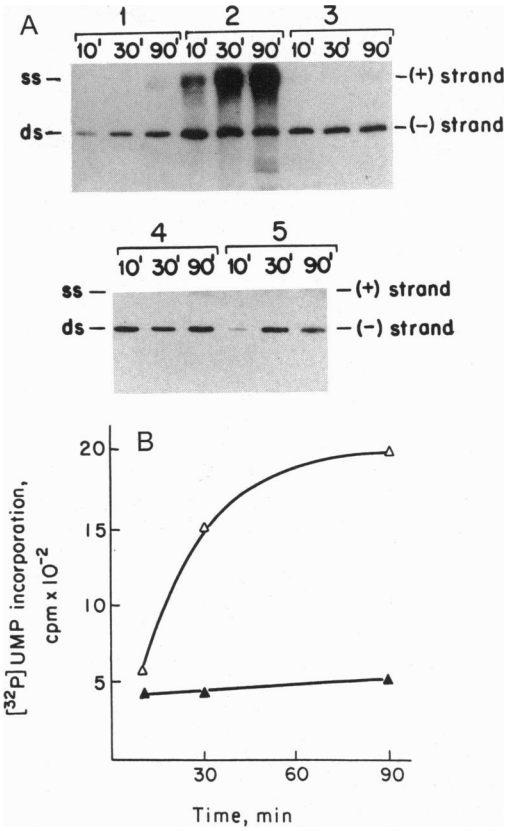

FIG. 3. Analysis of the (+)- and (−)-strands of RQ-DHFR RNA in replication products. (*A*) Separation of the labeled (+)- and (−)-strands by gel electrophoresis after annealing with excess unlabeled (+)-strands. Reactions with 1 μg of Qβ replicase were carried out in buffer B and were initiated with 1 μg of RQ-DHFR(+) RNA. (*Upper*) RNA synthesis by the purified Qβ replicase alone (lanes 1), in the presence of the complete cell-free translation system without further additions (lanes 2), or with the addition of 0.5 mM puromycin (lanes 3). (*Lower*) RNA synthesis in the presence of an incomplete translation system lacking the S100 fraction (lanes 4) or ribosomes (lanes 5). (*B*) Time course of accumulation of (+)-strands (△) and (−)-strands (▲) in the coupled replication–translation reaction. Bands corresponding to the (+)- and (−)-strands (*A*, lanes 2) were excised, and their radioactivity was determined by the Cerenkov method.

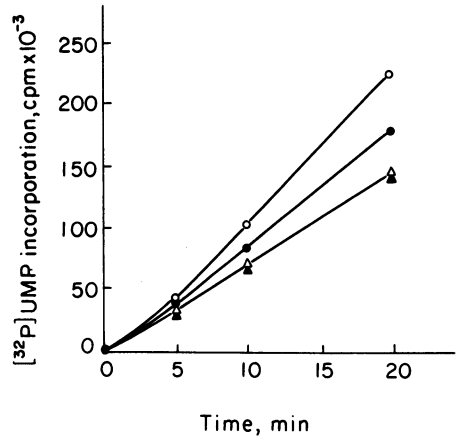

FIG. 4. Replication of RQ135$_{-1}$ RNA in the absence (○, ●) or presence (△, ▲) of the complete cell-free translation system and in the absence (○, △) or presence (●, ▲) of 0.5 mM puromycin. Reactions with 1 μg of Qβ replicase were carried out in buffer B and were initiated with 20 ng of RQ135$_{-1}$(−) RNA.

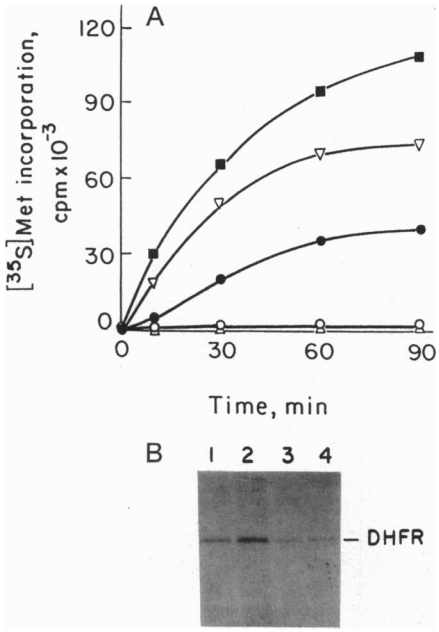

FIG. 5. Synthesis of DHFR in the cell-free translation system. (*A*) Protein synthesis in the reactions programmed with 1 μg of RQ-DHFR(+) RNA without (●, ○) or with the addition of 1 μg (▽, △) or 2 μg (■) of Qβ replicase, and in the absence (●, ▽, ■) or presence (○, △) of 0.5 mM puromycin. (*B*) Electrophoretic analysis of the products synthesized up to 90 min in the reactions programmed with 1 μg of RQ-DHFR(+) RNA (lanes 1 and 2) or 1 μg of the control DHFR mRNA (lanes 3 and 4) without (lanes 1 and 3) and with (lanes 2 and 4) addition of 2 μg of Qβ replicase.

tion despite the later onset of their synthesis in the reaction initiated with the (+)-strands. Fig. 3*B* shows that the amount of (−)-strands synthesized early in the reaction remains essentially unchanged, whereas the amount of (+)-strands continuously increases in the course of the reaction. Up to 90 min, the (+)-strands accumulate in 5-fold excess over the (−)-strands.

Interestingly, a similar (10:1) ratio of the (+)- and (−)-strands of Qβ RNA is observed during Qβ phage development *in vivo* (27). This similarity is striking since in the *in vitro* reaction there is no packaging of (+)-strands of RQ-DHFR RNA into virions, which was believed to be the major reason for unequal synthesis of the Qβ RNA (+)- and (−)-strands in the cell (28).

Replication of Recombinant mRNA Enhances the Coupled Translation. The addition of Qβ replicase to the cell-free translation system programmed with the (+)-strands of RQ-DHFR RNA resulted in elevated production of DHFR (Fig. 5*A* and Fig. 5*B*, lanes 1 and 2) whose activity was proportional to the amount of protein synthesized (Table 1). The increase can only be attributed to the RQ-DHFR RNA amplification as the addition of Qβ replicase had no effect on the translation system programmed with the control DHFR mRNA (Fig. 5*B*, lanes 3 and 4).

Table 1. Enzymatic activity of DHFR synthesized to 90 min in a cell-free system programmed with RQ-DHFR(+) RNA

Qβ replicase added, μg	Protein synthesized, pmol	DHFR activity*	
		Total, units × 10^6	Specific, units × 10^4/pmol
0	0.078	8.4	1.1
1	0.144	17.7	1.2
2	0.195	28.2	1.4

Amount of synthesized protein in pmol (per 30-μl reaction mixture) was calculated proceeding from the specific activity of the incorporated [^{3}H]leucine and the number of leucine residues in the DHFR molecule (20).
*Assayed as described (29).

DISCUSSION

The results of this study show that combining replication and translation of a RQ-mRNA recombinant in one test tube results in genuine coupling of the two reactions. They actively influence each other; moreover, they display a synergistic action as the total effect cannot be achieved unless the two reactions run together.

The observed stimulating action of RQ-DHFR RNA translation on its replication and vice versa is unexpected and is opposite what was known about the interplay between replication and translation of the (+)-strand of Qβ phage RNA. In the latter case, the two processes are mutually inhibitory, and this has been attributed to competition between Qβ replicase and ribosomes for the same RNA template, which they read in mutually opposite directions (30).

A clue to this controversy seems to lie in two observations of this work: (*i*) the constrained entrance of RQ-DHFR RNA into the second round of replication in the absence of a coupled translation system, presumably because of duplex formation, and (*ii*) the asymmetry in accumulation of the RQ-DHFR RNA (+)- and (−)-strands in the coupled replication–translation reaction. The involvement of the (+)-strands in translation seems to have two opposite consequences: inhibition of their copying into the (−)-strands due to competition between Qβ replicase and ribosomes and promotion of replication due to suppression of (+)- and (−)-strand annealing. When the efficiently replicating Qβ RNA is used as a template, only the inhibitory effect is seen (30). When the poorly replicating RQ-DHFR RNA is used, the promoting effect becomes apparent, but inhibition of (−)-strand synthesis leads to replication asymmetry. The resulting prevalent accumulation of the (+)-strands benefits the translation reaction, although some of these strands are engaged in synthesis of the (−)-strands. The stimulating action of replication on the coupled translation is apparent with RQ-DHFR RNA but is not seen when Qβ RNA is used as a template, presumably because of the presence in the latter case of the special regulatory mechanism whereby Qβ replicase displaces ribosomes from Qβ RNA by competitive binding at the coat cistron initiation site (30).

This study demonstrates that coupled replication–translation of heterologous mRNA recombinants *in vitro* can serve as a useful model for studying the fundamental aspects of virus amplification in the cell. These RNAs are free from viral regulatory elements and can be amplified and expressed in the absence of virus-specific factors whose action may obscure the basic mechanism. It is interesting in this regard that no part of RQ-DHFR RNA is of Qβ phage origin, since RQ135 RNA used for the construction of this recombinant is formed entirely by a recombination of heterologous sequences (18).

The expression of heterologous mRNAs in a coupled replication–translation reaction can also be implemented for large-scale synthesis of desired proteins in cell-free continuous-flow reactors (31–33). The continuous production of the sense strands by Qβ replicase would compensate for the mRNA losses due to degradation and thereby extend the reaction lifetime.

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