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# Expression and stability of recombinant RQ-mRNAs in cell-free translation systems

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**Abstract**

Expression of dihydrofolate reductase (DHFR) and chloramphenicol acetyltransferase (CAT) mRNAs in cell-free *Escherichia coli* translation systems is greatly enhanced as a result of their insertion into RQ135 RNA, a naturally occurring satellite of phage Qβ. The enhancement is due to protection of the recombinant mRNAs against endogenous ribonucleases and to an increased initial rate of translation in the case of the RQ-CAT mRNA.

*Key words:* Qβ phage; Satellite RQ RNA; Recombinant mRNA; Cell-free translation; mRNA stability

## 1. Introduction

RQ RNAs are small satellites of phage Qβ. They are formed by recombinations between RNAs occurring in the Qβ phage-infected *Escherichia coli* cells [1–3], are capable of amplification by Qβ replicase (RNA-directed RNA polymerase of phage Qβ) [4], are incorporated into Qβ phage particles, and can propagate in a number of phage generations [5]. Initially, they were identified as '6S RNAs' in the infected *E. coli* cells [6] and as RNA species 'spontaneously' synthesized in vitro by purified Qβ replicase, and were designated as Qβ RNA 'variants' [7]. Recently, we have shown that the 'spontaneous' synthesis is caused by airborne RQ RNA molecules [4,8] and that RQ RNAs do not necessarily descend from the Qβ RNA sequence. In particular, RQ135(+) RNA, one of the most efficient Qβ replicase templates, is mainly composed of sequences homologous to the 23 S ribosomal RNA [2].

In a previous study we have shown that RQ135 RNA can impart the ability of amplification by Qβ replicase in vitro to DHFR mRNA inserted at one of its internal loops [9]. Replication of the RQ-DHFR mRNA recombinant proceeds efficiently if coupled to translation. Otherwise, replication is limited to one round resulting in a dead duplex formed by the annealed (+) and (−) strands. The translating ribosomes prevent duplex formation by sequestering the (+) strands and induce replication asymmetry that also characterizes the replication of Qβ RNA in vivo: the (+) strands are accumulated in large excess over the (−) strands, and this results in an increased synthesis of DHFR [9]. Thus, the behavior of the recombinant RQ-DHFR mRNA in the cell-free replication–translation system mimics the replication of Qβ phage in vivo, although no part of RQ-DHFR mRNA has originated from the phage genome.

This paper reports other properties acquired by mRNAs inserted into the RQ135 sequence which strengthen the resemblance of the resulting recombinants to the phage genome, namely enhanced expression in *E. coli* cell-free translation systems and elevated resistance to the endogenous ribonucleases.

## 2. Materials and methods

RQ mRNA recombinants were constructed at the DNA level by inserting the corresponding genes into the *Xho*I site of mutant RQ135$_{-1}$ cDNA and prepared by runoff plasmid transcription with T7 RNA polymerase as described [9]. Plasmids pT7RQ135$_{-1}$(−)DHFR(+) (coding the recombinant RQ-DHFR mRNA) and pSP65DHFR$_{0.7}$ (used for synthesis of the control DHFR mRNA with SP6 RNA polymerase) were the same as in [9]. Plasmid pSPT19CAT (generously provided by Dr. Yarchuk of this Institute) was prepared by ligation, at site *Sal*I plasmid pSPT19 (Pharmacia), of the CAT gene-carrying *Sal*I cartridge excised from plasmid pCM-1 (Pharmacia). This plasmid was used for synthesis of the control CAT mRNA with T7 RNA polymerase (following digestion with *Bam*HI) and also as a source of the CAT gene for

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*Abbreviations:* DHFR, dihydrofolate reductase; CAT, chloramphenicol acetyl transferase.


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construction of plasmid pT7RQ135$_{-1}$(−)CAT(+) coding for the recombinant RQ-CAT mRNA.

30 μl cell-free translation reactions [9] contained 25 pmol of the corresponding mRNA and either 1.75 $A_{260}$ units of crude S30 extract [10] (the 'S30 system') or 1.4 $A_{260}$ units of 70 S ribosomes (obtained by mixing equimolar amounts of 30 S and 50 S ribosomal subparticles [11]) plus 12 μg of protein fraction S100 [12] (the 'S100 system'). Protein synthesis was monitored by sampling 5 μl aliquots and either counting the radioactivity of [$^{35}$S]Met (170 Ci/mmol) in the trichloroacetic acid-precipitated material, or autoradiography of the electrophoretic patterns of the $^{35}$S-labeled products [9]. mRNA decay was monitored in the parallel translation reactions by analyzing the Toluidine blue-stained electrophoretic patterns of phenol/chloroform-extracted 6 μl aliquots [9].

## 3. Results

The recombinant RQ-mRNAs used in this work contained *E. coli* DHFR and CAT mRNAs inserted between positions 53 and 54 of the (−) strand of RQ135$_{-1}$ RNA [2]. In addition to the 480 nt- and 657 nt-long sequences coding for DHFR and CAT, the inserts contained, respectively, the 15 nt- and 37 nt-long 5′ terminal, and the 22 nt- and 93 nt-long 3′ terminal untranslated segments. The total lengths of the recombinant molecules were 650 nt (RQ-DHFR mRNA) and 920 nt (RQ-CAT mRNA). The control mRNAs, DHFR mRNA (680 nt) and CAT mRNA (823 nt), contained the 36 nt- and 61 nt-long 5′ terminal, and the 165 nt- and 105 nt-long 3′ terminal untranslated regions, respectively.

The expression of these mRNAs was studied in two cell-free translation systems, S30 and S100, prepared from *E. coli* cells (see section 2). The S30 system contained crude *E. coli* extract and thus mimicked intracellular conditions, whereas the S100 system was deprived of most of the endogenous ribonucleases (mainly associated with ribosomes [13]) by washing the ribosomal subparticles with 0.5 M $NH_4Cl$ [11].

In the S30 system, the expression of each of the RQ-mRNA recombinants was much more efficient than that of the respective control mRNAs (Fig. 1A). Parallel examination of mRNA integrity showed that the enhanced expression of the recombinant mRNAs correlated with their approx. 5-fold longer lifetimes (Fig. 1B). This suggests that the attachment of the RQ135 sequence to the mRNAs improves their expression by protecting them against ribonucleases occurring in the S30 extract. However, the enhancement of expression was not proportional to the stabilization effect. Moreover, despite the equal stabilizing effects of the RQ135 moiety on DHFR and CAT mRNAs, the increase in protein synthesis (the plateau level) was 5-times greater in the case of CAT mRNA (15- to 20-fold; Fig. 1A, right) than with DHFR mRNA (3- to 4-fold; Fig. 1A, left). It follows that the observed increase in the expression level cannot be solely due to the mRNA protection.

In order to discriminate between the protective action of the RQ135 moiety and the direct stimulation of mRNA translation, mRNA expression was studied in the S100 system (Fig. 2). In this system the mRNAs degrade much slower than in the S30 system (cf. Figs. 2B and 1B) which allows the initial translation rates to be

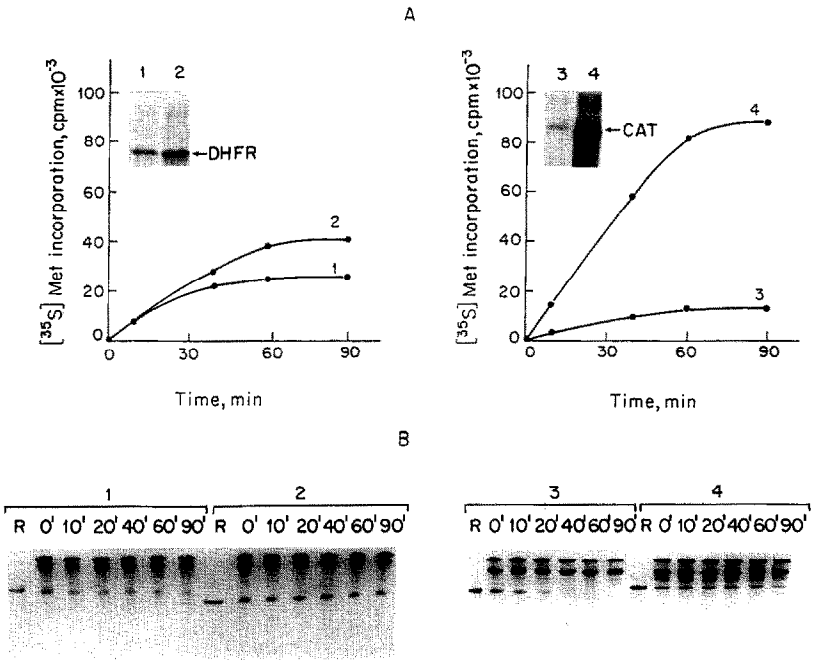


Fig. 1. Expression of DHFR mRNA (1), RQ-DHFR mRNA (2), CAT mRNA (3) and RQ-CAT mRNA (4) in the S30 system. (A) Time–course of protein synthesis and electrophoretic pattern of the products synthesized up to 30 min (inset). (B) Time–course of mRNA decay during translation. The corresponding mRNA transcripts were used as size markers (lanes R). The intense bands at the top of the gel correspond to ribosomal 23 S and 16 S RNAs.

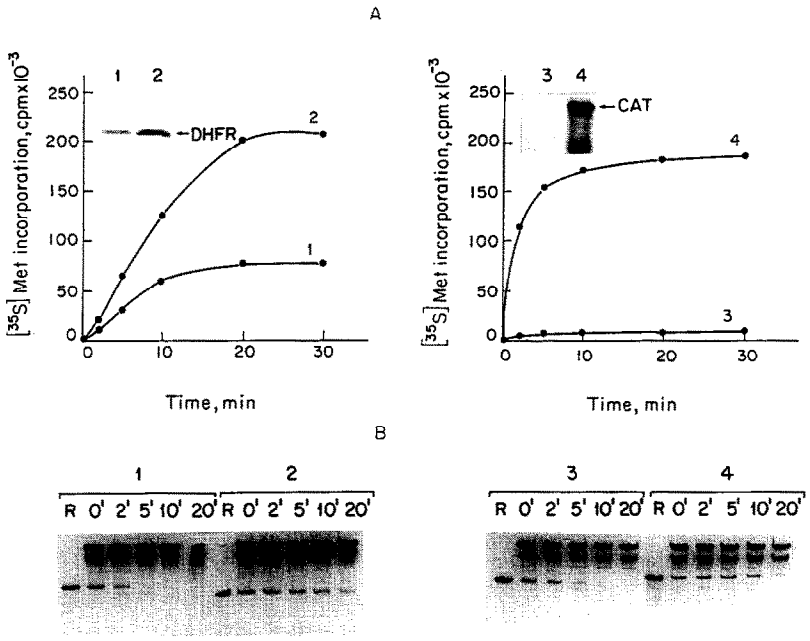


Fig. 2. Expression of DHFR mRNA (1), RQ-DHFR mRNA (2), CAT mRNA (3) and RQ-CAT mRNA (4) in the S100 system. (A) Time–course of protein synthesis and electrophoretic pattern of the products synthesized up to 90 min (inset). (B) Time–course of mRNA decay during translation. For other details see legend to Fig. 1.

compared. The comparison shows that while the initial translation rates for the RQ-DHFR mRNA and the control DHFR mRNA are indistinguishable, the RQ-CAT mRNA is translated at a 5-times higher initial rate than the control CAT mRNA (Fig. 2A).

## 4. Discussion

In a previous study we showed that the insertion of DHFR mRNA at an internal site of RQ135 RNA makes it amplifiable in a coupled, cell-free replication–translation system and leads to a higher protein yield due to the synthesis of additional copies of the sense strands [9]. An increased protein production in a coupled replication–translation system over the non-coupled translation system has also been observed for CAT mRNA embedded within another RQ RNA, MDV-1 (RQ223) [14]. This work extends the earlier observations by showing that in addition to replicability, RQ RNA imparts to an mRNA insert a higher resistance to ribonucleases and can also increase the rate of its translation, possibly at the initiation step. The resulting gross enhancement of mRNA expression in a coupled replication–translation system is thus the sum of these three separate effects.

The protective action of the RQ RNA moiety can readily be explained by the high resistance to ribonucleases of the RQ RNAs themselves. These RNAs possess highly developed secondary structures and, presumably, stable molecule 'stalks' formed by the base-paired 3′ and 5′ termini [1,2,15]. The latter feature can be important for RNA protection against 3′-exoribonucleases which are abundant in bacterial cells and play a major role in mRNA turnover in vivo [16]. There is ample evidence that mRNAs can be efficiently stabilized by 3′ terminal hairpin structures, both in vivo and in vitro [16–18]. The reason for the increase in the translation rate in not as apparent. RQ RNAs have been selected for their high replication rates and lack of any protein-coding function [7]. We can indicate two possibilities explaining the stimulation by the RQ135 moiety of the translation of the CAT mRNA insert. (i) As RQ135 RNA possesses a very stable tertiary structure [2] it should keep the 3′ and 5′ ends of the embedded mRNA together. This could induce a more favorable conformation of the mRNA moiety and promote translation, e.g. by exposing the ribosome-binding site. (ii) Another possibility could be accounted for by the fact that the (−) strand of RQ135 RNA, in which the mRNAs have been inserted, contains three long segments complementary to the 23 S ribosomal RNA [2] that could provide for additional binding sites of the recombinant RQ-mRNAs on the ribosome.

The replicability, elevated ribonuclease resistance, and high translation rates of the RQ-mRNA recombinants make them attractive templates for large-scale protein synthesis in vitro [19,20]. The high performance of such templates in a continuous-flow cell-free replication–translation system has recently been demonstrated [14,21].

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