Viral Qβ RNA as a high expression vector for mRNA translation in a cell-free system

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Abstract Dihydrofolate reductase (DHFR) mRNA was inserted into Qβ phage RNA instead of its coat protein cistron. Translation of this recombinant mRNA in the *Escherichia coli* cell-free system resulted in the synthesis of DHFR, which was two orders of magnitude higher than that in the case of translation of the control DHFR mRNA. Additionally, it resulted in a significantly enhanced synthesis of Qβ replicase as compared with its synthesis when the original Qβ RNA was used.

Key words: Qβ phage RNA; Recombinant mRNA; Coat protein gene; Cell-free translation; Dihydrofolate reductase gene

1. Introduction

Virus coat protein genes are highly expressed in living cells. In the case of RNA bacteriophages, such as MS2 or Qβ, the coat protein is one of the four phage-encoded proteins (for a review see [1]). It is abundantly synthesized during infection, and it makes up to about 40% of newly formed product upon induction of the appropriate cDNA clones in the cell [2]. It is also strongly produced in vitro when phage RNA is used as a message [3]. The high expression of the phage coat protein cistron is believed to result from special features of both the flanking and the distal structural elements of the phage RNA genome.

In this communication we report that replacement of the coat protein cistron in the bacteriophage Qβ RNA with the *E. coli* dihydrofolate reductase (DHFR) mRNA sequence dramatically (two orders of magnitude) increases synthesis of DHFR in vitro, almost to the level of coat protein gene expression. This approach is proposed for highly effective expression of heterologous sequences in cell-free translation systems.

2. Materials and methods

2.1. Plasmids

Plasmid pQβ7, carrying a full-length copy of Qβ RNA [4], was generously provided by Dr. P. Shaklee, University of North Texas. Plasmid pT7DHFR$_{0.7}$, carrying the DHFR coding sequence, was generously provided by Dr. B. Hardesty, University of Texas at Austin.

The replacement of the phage coat protein gene by the *E. coli* DHFR gene was carried out at the DNA level. A fragment corresponding to the sequence 549–1072 of the *E. coli* DHFR gene [5] was obtained from pT7DHFR$_{0.7}$ by the PCR technique [6]. The PCR primers induced nucleotide substitutions A→C (position 557), A→G (position 561), and G→T (position 1062), producing unique *Nco*I and *Bsp*MII sites. The substitution T→C in position 1342 of the Qβ genome [7], achieved by site-specific mutagenesis using PCR [8], produced a unique *Nco*I site. The resultant construct was digested with *Nco*I and *Bsp*MII. The smaller fragment obtained after digestion was replaced with the DHFR gene-containing *Nco*I/*Bsp*MII PCR fragment, producing the final plasmid pQβDHFR. Cloning manipulations were done by standard methods [9].

2.2. Transcripts

mRNAs were prepared by runoff T7 transcription [10] of *Sma*I-digested pQβ7 and pQβDHFR, and *Hin*dIII-digested pT7DHFR$_{0.7}$. As a result we obtained the genomic Qβ RNA, recombinant QβDHFR RNA, and the control DHFR mRNA, respectively. T7 polymerase was prepared by S. Aksenovich in this laboratory according to the modified procedure [11].

2.3. Cell-free translation system

Translation mixtures were incubated at 37°C for 30 min in a 30 μl volume of 50 mM Tris-HCl, pH 8.2, with 140 mM KOAc, 10 mM $Mg(OAc)_2$, 1 mM EDTA, 0.5 mM ATP, 0.5 mM GTP, 2 mM dithiothreitol, 5 μl of *E. coli* S-30 ribosomal extract [12], 13 μg of *E. coli* tRNA, 5 pmol of mRNA, 300 ng of pyruvate kinase (500 U/mg), 15 U of human placental RNase inhibitor, 0.01% of sodium azide, where 10 mM phosphoenol pyruvate, 300 ng folinic acid, 12 μM [^{14}C]leucine or 2.6 μM [^{35}S]methionine and all the other 19 amino acids in 50 μM amounts were added. The time-course of protein synthesis was monitored by sampling 5 μl aliquots and counting the radioactivity of the material precipitated in hot trichloracetic acid. The translation products were analyzed by gradient polyacrylamide gel-electrophoresis in the presence of SDS [13], followed either by autoradiography or gel lane slicing with subsequent radioactivity measurements of the slices. Translation mixtures without added mRNA were used for taking endogenous protein synthesis into account. These mixtures were analyzed like others, and the counts obtained were subtracted for plotting.

2.4. RNA decay assay

The mRNAs labeled with [^{14}C]UTP (10,000 cpm/μg) were incubated in the *E. coli* translation mixture (see above). Aliquots were taken and analyzed after phenol/chloroform extraction by electrophoresis in 5% polyacrylamide gels in the presence of 7 M urea with subsequent autoradiography [9]. The half-life of an mRNA was determined from the autoradiograph as the time when the original intensity of the main mRNA band was reduced two-fold.

3. Results and discussion

3.1. Recombinant construct

The recombinant mRNA used in this study, namely Qβ DHFR RNA, contained the *E. coli* DHFR coding sequence instead of the coat protein cistron within the phage Qβ RNA. The replacement was done at the DNA level using *Nco*I and *Bsp*MII restriction sites (Fig. 1). In introducing the *Nco*I site into the DHFR sequence, two point mutations were made, one of which caused the second DHFR gene codon, ATC, to be changed to GTC. This corresponds to the substitution of the second DHFR amino acid Ile→Val. With the introduction of the *Nco*I site into pQβ7, the substitution T→C was made at position −2 from the coat protein gene start codon. The final construct, pQβDHFR, obtained after the replacement, is

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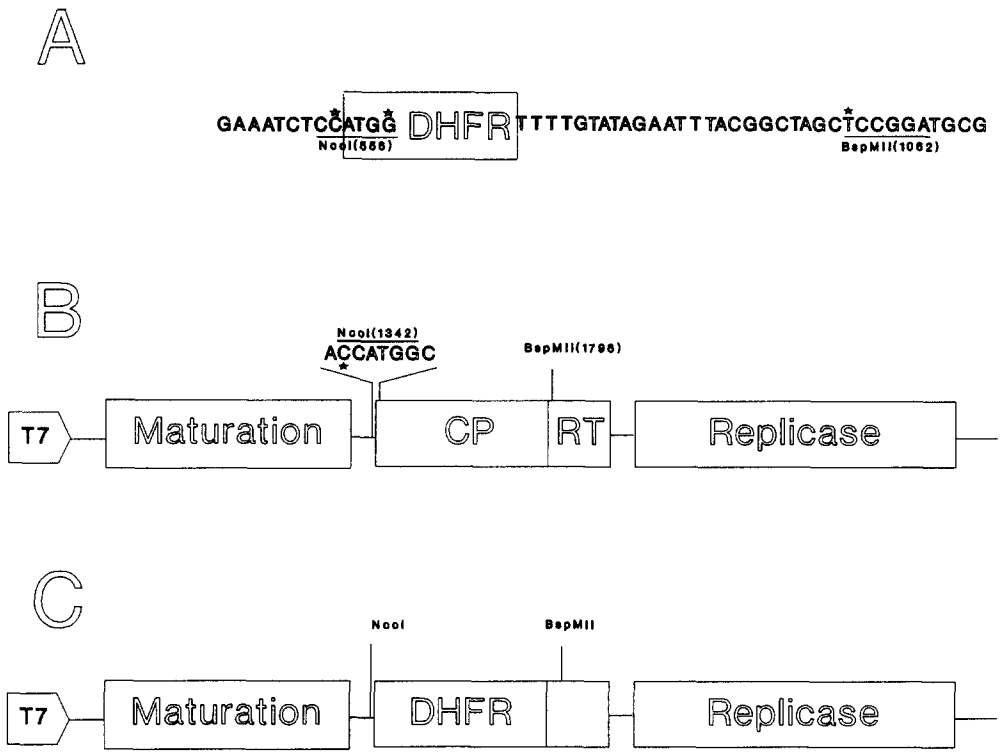


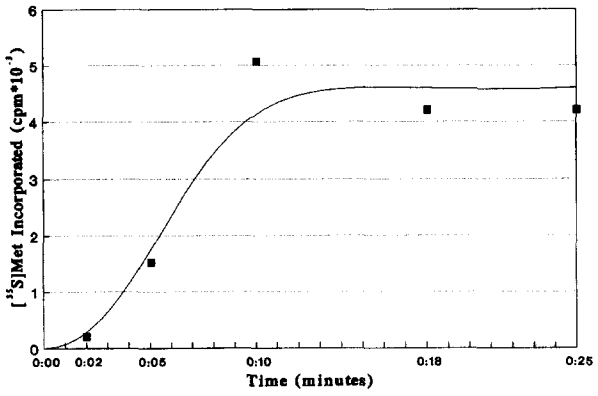
Fig. 1. General scheme of the replacement of the Qβ coat protein gene with the *E. coli* DHFR gene. The restriction sites used are shown. The numbers in parentheses described the location of the sites in the appropriate DNA sequences. Asterisks indicate point mutations. Open boxes show the coding regions. (A) The PCR fragment of the DHFR gene. (B) Scheme of the plasmid containing the Qβ cDNA with one point mutation. Genes coding for maturation protein, coat protein (CP), read-through protein (RT), and replicase subunit are indicated. (C) Scheme of the final plasmid pQβDHFR.

schematically shown in Fig. 1C. In addition to the DHFR coding sequence, it contained 24 nucleotides from the 3′ UTR of the DHFR gene, while 50 nucleotides after the stop codon of the coat protein gene were removed together with the coat protein gene.

3.2. Cell-free translation

The results of cell-free translation experiments demonstrate that recombinant Qβ DHFR RNA-programmed synthesis of DHFR is 50- to 150-times higher than in the case of translation of the control DHFR mRNA (Figs. 2 and 3). The amount of synthesized DHFR is only 4- to 5-times less than that of the Qβ RNA-programmed coat protein (Fig. 4). The resultant DHFR is enzymatically active, displaying a specific activity of 0.65×10^{-4} U/pmol (DHFR assay was conducted as in [14]).

The effect described can not be explained only by the different RNase sensitivities of the control and the recombinant DHFR mRNAs. Indeed, studies of DHFR mRNA stability showed that the half-life of the recombinant mRNA is 5- to 6-times longer than that of the control mRNA (bars A and C in Fig. 5). At the same time, another recombinant mRNA, RQ-DHFR RNA [15], possessing approximately the same



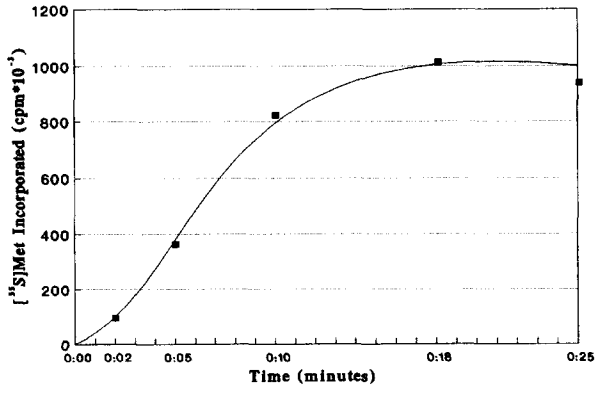


Fig. 2. Time–course of protein synthesis in the *E. coli* cell-free translation system with 2.6 μM [35S]methionine directed by the control DHFR mRNA (top graph) and by the recombinant QβDHFR RNA (bottom graph).

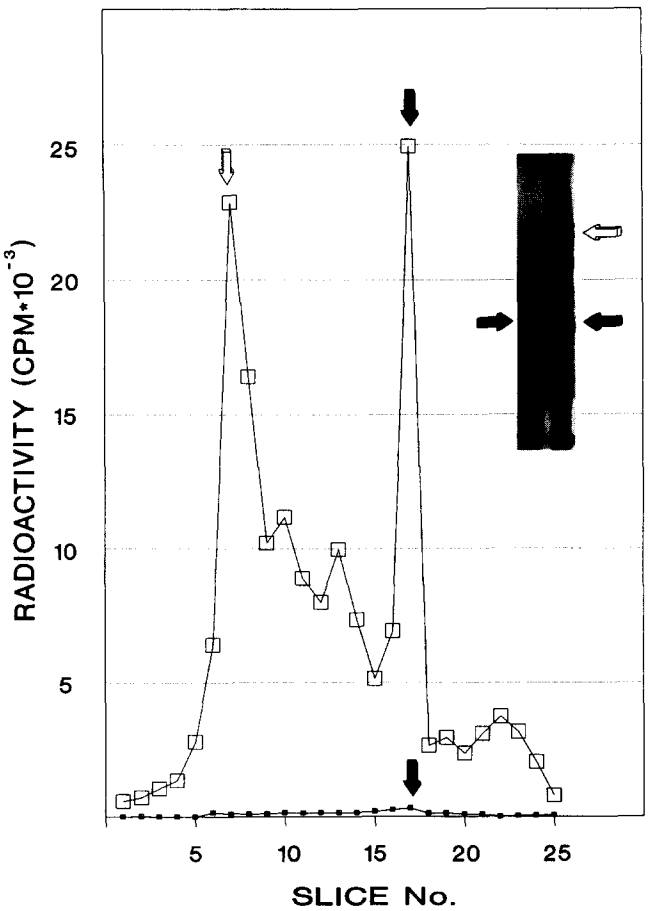


Fig. 3. Synthesis of proteins directed by the control DHFR mRNA (points) and the recombinant QβDHFR RNA (open boxes), analyzed by SDS gradient-PAGE. Inset shows the corresponding autoradiograph. Cell-free translation was conducted with 2.6 μM [^{35}S]methionine. Arrows indicate protein peaks and respective bands on the autoradiograph. Black arrow corresponds to DHFR, open arrow to the replicase subunit.

stability as Qβ DHFR RNA (bars B and C in Fig. 5), was translated only 3- to 4-times better than the control DHFR mRNA in the S-30 system [16]. These results demonstrate that Qβ RNA can serve as a structural frame for a foreign mRNA sequence which not only protects the coding sequence from ribonucleases but also strongly enhances the translation itself in vitro. The two-order enhancement is also not due to the

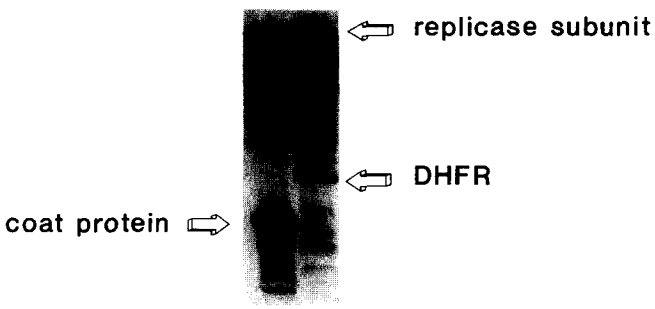


Fig. 4. Expression of Qβ RNA (left) and the recombinant QβDHFR RNA (right) in the *E. coli* cell-free translation system with 12 μM [^{14}C]leucine.

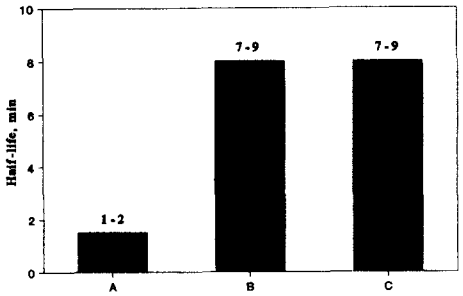


Fig. 5. Diagram showing half-lives of (A) the control DHFR mRNA, (B) the recombinant RQ-DHFR RNA (data taken from Fig. 1B of [16]), and (C) the recombinant QβDHFR RNA in the *E. coli* cell-free translation system.

effect of the sequences flanking the coat protein cistron: the construct comprising the DHFR coding region, a 98 nt upstream sequence and a 193 nt downstream sequence of the recombinant Qβ DHFR RNA, was translated only 3- to 5-times better than the control DHFR mRNA, similar to RQ-DHFR RNA (data not shown).

The replacement of the coat protein cistron for the DHFR mRNA sequence also results in a 5- to 6-fold increase in Qβ replicase subunit synthesis compared to that in the Qβ RNA-directed synthesis (Fig. 4). This can be explained either by the absence of coat protein-induced repression of the replicase cistron translation [17] or by changes in the overall Qβ RNA conformation [18]. The S and M sites in Qβ RNA, which are necessary for its replication [19], were not eliminated by the replacement. This suggests that the recombinant Qβ DHFR RNA may be replicable by Qβ replicase. Thus, on the grounds of an increased level of endogenously synthesized Qβ replicase, autocatalytic replication–translation systems using recombinant Qβ mRNAs can be proposed. This work is in progress.

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