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Translation of Bacteriophage Qβ RNA by Cytoplasmic Extracts of Mammalian Cells

(L cells/Krebs ascites cells/coat protein/tryptic peptides/*N*-formylmethionine)

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ABSTRACT **Cytoplasmic extracts from Krebs II mouse ascites cells and from L cells translate messenger RNA from coliphage Qβ with fidelity to produce products that migrate on polyacrylamide gels with those products directed by Qβ RNA in an *Escherichia coli* cell-free system. The mammalian cell extracts correctly initiate and terminate Qβ coat protein synthesis, as shown by: (*i*) [^{3}H]lysine- and [^{3}H]arginine-labeled tryptic peptides derived from the coat-sized product resemble these from authentic Qβ coat protein, (*ii*) Qβ coat (which contains methionine only at the N-terminal end) can be radioactively labeled with methionine only if the methionine is formylated, and (*iii*) L cell extracts directed by Qβ *am*$^{-}$11 (an amber mutant in the coat protein) RNA make no completed coat-sized material, but do make a peptide the size of the authentic amber coat fragment.**

The study of protein synthesis in bacterial systems has benefitted from the use of RNA bacteriophages as a source of large amounts of simple, homogeneous messenger RNA. The reactions involved in translation, the regulation of translation, and—in particular—the mechanisms of suppression of nonsense mutations have been elucidated with these messenger RNAs.

Studies of mammalian cell translation could similarly benefit from the use of simple mRNAs obtained from RNA viruses. However, no viral messenger has been as extensively characterized as mRNAs from RNA phages in terms of their structure and the products for which they code. Furthermore, precisely characterized mutants of mammalian RNA viruses are not available. Therefore, certain questions concerning eukaryotic initiation and termination of protein synthesis can best be explored by the use of phage mRNA in mammalian cell-free extracts.

We report here the translation of Qβ mRNA in cytoplasmic extracts of Krebs II mouse ascites cells and of mouse L-cells. These extracts appear to initiate and terminate correctly protein synthesis with Qβ mRNA to produce authentic phage protein products. Translation of Qβ RNAs containing nonsense mutations in such extracts should be useful in the identification of nonsense suppressors in mammalian cells.

MATERIALS AND METHODS

Growth of Cells. Krebs II mouse ascites cells used to prepare extracts were grown for 7 days in peritoneal cavities of female white mice, strain CD1 (obtained from Charles River Breeding labs of Wilmington, Mass.). L cells were cultured and harvested as described (1).

Preparation of Cell Extracts from ascites cells and L cells have been described by McDowell *et al.* (1).

Preparation of Qβ RNA. Qβ was grown and harvested by procedures used for the purification of bacteriophage f2 (2). Qβ RNA was extracted from purified phage by procedures described for phage f2 (3).

Cell-Free Protein Synthesis. Reaction mixtures (100 or 400 μl) contained—per ml—0.6 ml of cell-free extract, 1 μM ATP, 0.2 μM GTP, 11 μM creatine phosphate, 1 mg of creatine phosphokinase, 30 μM hydroxyethylpiperazine sulfonate (HEPES), pH 7.5, 2.5 μM $Mg(OAc)_2$, 10 μM dithiotheitol, 0.12 μM (each) of 19 unlabeled amino acids, 40 Ci/mmol [^{3}H]leucine, 50 μCi/ml of [^{3}H]lysine—5 Ci/mmol—or [^{3}H]-arginine—5 Ci/mmol—(New England Nuclear Corp.), or 300 μCi/ml of [^{35}S]methionine—40 Ci/mmol—(New England Nuclear Corp.), or [^{35}S]fMet-tRNA [prepared from yeast tRNA, as outlined by Housman *et al.* (4)], and 0.1–0.2 mg/ml of viral RNA. The contents of the reaction vessel were incubated for 10 min at 37°; proteins were precipitated with trichloroacetic acid and prepared for scintillation counting or electrophoresis (5, 6). Reactions catalyzed by *Escherichia coli* extracts have been described (5).

Disc Gel Electrophoresis. 7.5% Polyacrylamide gels containing 0.1% Na dodecyl sulfate and 8 M urea were prepared as described by McDowell and Joklik (7). Gels were run 20 hr at 4 mA/gel. The gels were sliced into 1-mm fractions; each fraction was incubated in toluene–PPO–POPOP scintillation fluid containing 3.5% NCS tissue solubilizer (Amersham–Searle) overnight before it was counted.

High-Voltage Paper Electrophoresis. For analysis of the tryptic peptides, protein samples were prepared and run at pH 3.5 (6).

RESULTS

Stimulation of protein synthesis by Qβ mRNA and the effect of antibiotics

The addition of Qβ mRNA to a cell-free extract from Krebs II mouse ascites cells stimulates protein synthesis from 3- to 15-fold. Polypeptide synthesis is maximal at RNA concentrations of about 200 μg/ml, whereas higher concentrations of RNA result in an inhibition (Fig. 1). Per microgram of messenger RNA, Qβ RNA is about 25% as efficient in directing protein synthesis as is globin mRNA. Since, as is shown below, the major product of Qβ RNA is the Qβ coat protein, which repre-

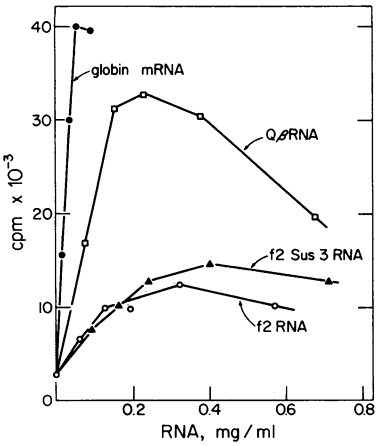

FIG. 1. Protein synthesis in extracts from mouse ascites cells. Reactions (50 μl) contained [³H]leucine and the indicated concentrations of messenger RNAs. f2 Sus 3 RNA contains an amber mutation in the beginning of the coat gene (23). Globin mRNA was a generous gift of Dr. Gary Temple. Incubation was at 30° for 40 min.

sents only 20% of the coding capacity of the RNA, the globin message and the Qβ coat cistron function equally well in this system. Protein synthesis is linear for 20 min and ceases at about 40 min (Fig. 2).

RNA from bacteriophage f2 also stimulates protein synthesis in the extracts, but is a much poorer message than is Qβ RNA (Figs. 1 and 2). Analysis of the product coded for by phage f2 RNA is currently in progress.

Protein synthesis by these extracts is mediated by cytoplasmic, rather than mitochondrial, ribosomes. Drugs known to inhibit cytoplasmic protein synthesis, such as anisomycin (8), cycloheximide (9), and emetine (10), abolish the activity of these extracts (Table 1). By contrast, drugs that inhibit only bacterial or mitochondrial protein synthesis, such as chloramphenicol (11), have no effect upon the activity of these extracts with phage Qβ RNA or f2 RNA.

Size of the products

Radioactively-labeled polypeptide material (with [³H]lysine) made by ascites extracts with Qβ RNA as template was analyzed on 7.5% polyacrylamide gels in order to determine the size of the reaction product. Authentic Qβ protein, labeled with [¹⁴C]lysine and made in an *E. coli* cell-free extract directed by Qβ RNA, was included as a marker (Fig. 3, *top*). The products made in the ascites extract appear to be the same size, and occur in about the same proportions, as the products derived from the *E. coli* cell-free extract. The major product of the Qβ-primed ascites extract is a peptide of the same size as authentic Qβ coat protein.

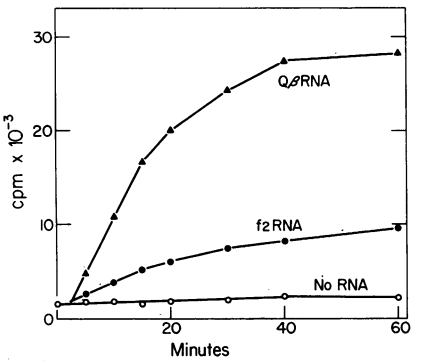

FIG. 2. Time course of protein synthesis. Reactions (250 μl) contained no RNA or 50 μg of either f2 or Qβ RNA. Incubation was at 30°. 25-μl Aliquots were removed at indicated times, and protein radioactivity was determined.

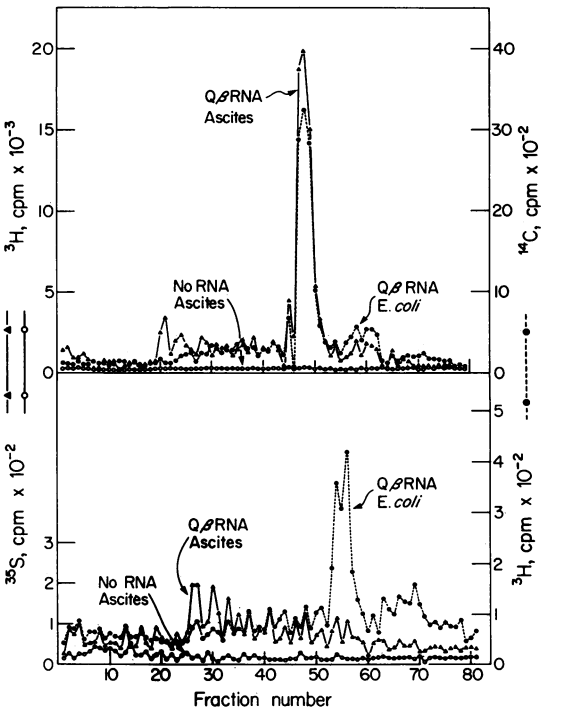

FIG. 3. Acrylamide gel electrophoresis of Qβ-directed products. Ascites extract-directed reactions (50 μl *top*, 100 μl *bottom*) contained 0.16 mg/ml of Qβ RNA and [³H]lysine (*top*) or [³⁵S]-methionine (*bottom*). *E. coli* extract-directed reactions (50 μl *top*, 100 μl *bottom*) contained 400 μg/ml of Qβ RNA and [¹⁴C]lysine (*top*) or [³H]methionine (*bottom*). Incubation was at 30° for 40 min. Samples were prepared for electrophoresis as described (7).

TABLE 1. *Drug sensitivity of ascites cell-free protein synthesis*

	cpm of [³H]leucine/reaction		
	f2 RNA	Qβ RNA	No RNA
− Drug	13,200	51,500	3100
+ Cycloheximide (81 μM)	2,200	6,400	—
+ Emetine (110 μM)	1,200	3,100	—
+ Sparsomycin (2.3 μM)	1,700	4,300	—
+ Anisomycin (140 μM)	1,100	1,100	—
+ Chloramphenicol (140 μM)	13,700	49,200	—
+ Streptomycin (80 μM)	13,500	50,800	—

Reactions (44 μl) with an extract from ascites cells contained [³H]leucine, either f2 RNA (4 μg) or Qβ RNA (5 μg), and antibiotics as indicated. Incubation was at 30° for 40 min.

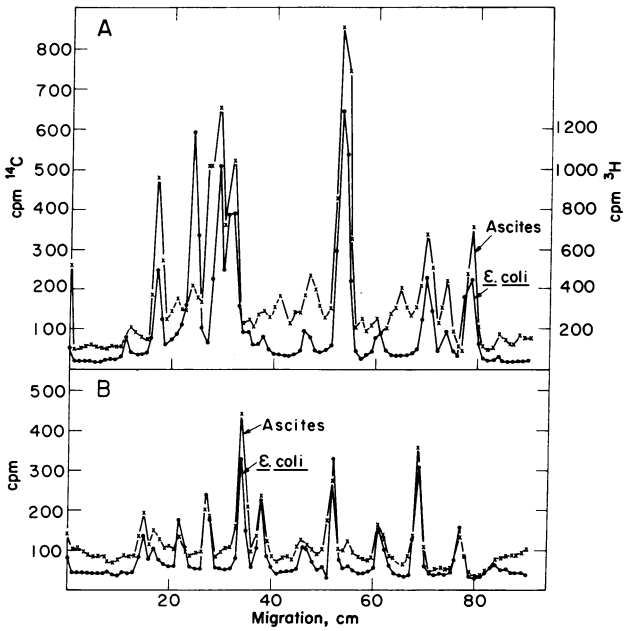

FIG. 4. Trypsin digest of Qβ coat synthesized *in vitro*. Ascites reactions (400 μl) contained 0.15 mg/ml of Qβ RNA and [^{3}H]-lysine (*top panel*), or [^{3}H]arginine (*bottom panel*). *E. coli* reactions (200 μl) contained 0.18 mg/ml of Qβ RNA and [^{14}C]lysine (*top panel*) or [^{14}C]arginine (*bottom panel*). Incubation was at 30° for 40 min. The ascites and *E. coli* products were mixed. The profile of the tryptic peptides derived from the coat protein is shown. The origin is at the *left* and the cathode is the *right*.

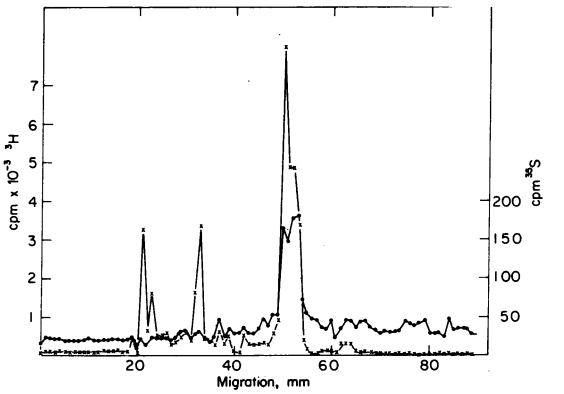

FIG. 5. Polyacrylamide gel electrophoresis of products made in L-cell extracts from Qβ RNA labeled with [^{35}S]fMet-tRNA$_f$. L-cell extract reactions (100 μl) contained 100 μg/ml of Qβ RNA and [^{35}S]fMet-tRNA$_f$. *E. coli* reactions (5 μl) contained 400 μg/ml of Qβ RNA and [^{3}H]lysine. Incubation was at 30° for 40 min. ×, [^{3}H]lysine, *E. coli* extract with Qβ RNA; ●, [^{35}S]-methionine, L-cell extracts with Qβ RNA.

Identification of Qβ coat-sized polypeptides

Three types of experiments show that the coat-sized polypeptide made in mammalian extracts is, in fact, Qβ coat protein. First, Qβ coat protein contains no methionine residues (12), except the one found transiently as the initiator at the N-terminus (13). Qβ coat protein made in *E. coli* extracts does contain labeled methionine (Fig. 3, *bottom*); other experiments have shown that all the radioactivity is at the N-terminus, in the sequence formyl-[^{35}S]methionyl-alanyl-lysine (13).

Since mammalian cells initiate protein synthesis with nonformylated methionyl-tRNA (4, 14, 15), and the initiator methionine residue is usually removed rapidly (4, 14, 15), one would expect Qβ coat polypeptides made in the ascites extract to contain no methionine radioactivity. In fact, Qβ coat-sized polypeptide material is not labeled with [^{35}S]methionine (Fig. 3, *bottom*). Note that the larger Qβ polypeptides made are labeled with [^{35}S]methionine.

Previous work of Housman *et al.* (4) has shown that N-terminal initiator methionine is not cleaved from nascent globin chains if the methionine is formylated. Similarly, if the Qβ-directed ascites reactions contain [^{35}S]formylmethionyl-tRNA$_f$, the coat-sized polypeptide is radioactively labeled (not shown).

Second, analysis of trypsin digests of the coat-sized polypeptide made in ascites extracts showed that this material is authentic Qβ coat protein.

Reaction products directed by Qβ RNA in ascites extracts, labeled with [^{3}H]arginine, were run on 7.5% polyacrylamide gels. [^{14}C]Arginine-labeled material, directed by Qβ RNA in *E. coli* extracts, was included in the same gel. After electrophoresis, the region of the gel containing authentic Qβ coat protein was eluted with a solution of 0.5% Na dodecyl sulfate. The polypeptide material was digested with trypsin, and the tryptic peptides were resolved by high-voltage paper ionophoresis at pH 3.5. After electrophoresis, the paper was cut into 1-cm sections; the labeled material was eluted with 0.1 N NaOH and counted (Fig. 4, panel *B*). Lysine-labeled tryptic peptides were analyzed similarly (panel *A*). The pattern of arginine-labeled tryptic peptides derived from reactions with ascites extracts precisely coincides with that of arginine-labeled tryptic peptides obtained from authentic Qβ coat made in *E. coli* extracts. This result strongly indicates that ascites extracts make authentic Qβ coat protein. Moreover, all lysine-labeled tryptic peptides of authentic Qβ coat are present in ascites reaction products with one exception. The peptide present in *E. coli* products, but absent in ascites products (slice 24), runs in the position of the N-terminal fMet-Ala-Lys tryptic peptide (13). Since mammalian protein initiates with methionine, and not with formylmethionine (4, 14–16), the

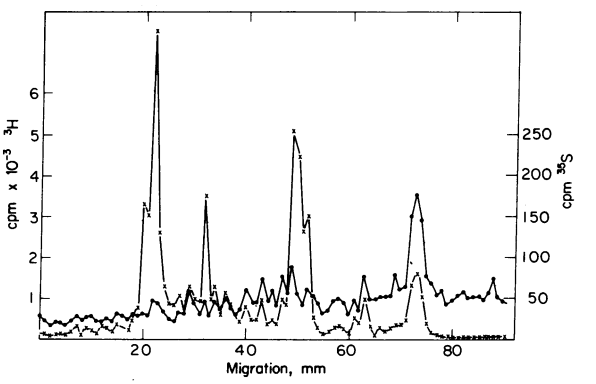

FIG. 6. Polyacrylamide gel electrophoresis of products made from Qβ am-11 in L-cell extracts. L-cell reactions (100 μl) contained 100 μg/ml of Qβ *am*-11 RNA and f-[^{35}S]Met-tRNA$_f$. *E. coli* products were a mixture of a 5-μl reaction containing 400 μg/ml of Qβ RNA and [^{3}H]lysine and a 20-μl reaction containing 400 μg/ml of Qβ *am*-11 RNA and [^{3}H]lysine. Incubation was for 40 min at 30°. ×, *E. coli* extract with Qβ RNA and Qβ *am*-11 RNA; ●, L-cell extract with Qβ *am*-11 RNA.

ascites reaction product would be expected to lack the fMet-Ala-Lys peptide.

Translation of amber mutants

As a third way of identifying coat protein, we used as a message Qβ RNA that contains an amber (UAG) chain-termination mutation in the coat gene. In extracts of Su⁻ *E. coli*, Qβ *am*-11 directs the synthesis of a peptide fragment (17) containing *N*-formylmethionine, and the N-terminal 36 amino acids of the Qβ coat (A. Ball and P. Kaesberg, personal communication). No completed coat protein is made. In extracts of ascites or L-cells labeled either with [^{3}H]lysine or [^{3}H]-arginine, *am*-11 RNA directs the synthesis of much less coat-sized polypeptide than does wild-type Qβ RNA. Contrary to expectation, no material the size of the amber fragment could be found (data not shown). However, if proteins synthesized in mammalian extracts contain an N-terminus blocked with *N*-formylmethionine, then peptide material the size of the amber fragment is stable and can be visualized on polyacrylamide gels.

Fig. 5 shows that when protein synthesized in extracts of L cells with wild-type Qβ RNA and labeled with yeast [^{35}S]-fMet-tRNA$_f$ is analyzed by acrylamide gel electrophoresis, formyl-[^{35}S]methionine radioactivity is found in the position of Qβ coat protein. This result is identical with that obtained with ascites extracts (noted above).

In the experiment depicted in Fig. 6, marker proteins were a mixture of *E. coli* reactions primed with Qβ RNA or Qβ *am*-11 RNA; protein labeled by [^{35}S]fMet-tRNA$_f$ made in L-cell extracts with Qβ *am*-11 RNA as template yields material that comigrates with the fragment of the coat protein synthesized from Qβ *am*-11 template in *E. coli* extracts (slice 73). No labeled material was found to comigrate with the complete Qβ coat protein synthesized in *E. coli* extracts with wild-type Qβ template (slice 49).

DISCUSSION

Translation of Qβ RNA

The work described here shows that the prokaryotic messenger derived from Qβ RNA phage is translated with fidelity in cytoplasmic extracts of Krebs II mouse ascites cells. The products directed by Qβ RNA in ascites extracts are the same size, and are made in about the same proportions, as authentic Qβ proteins made in *E. coli* extracts. Tryptic peptide analysis of the coat-sized polypeptide made in ascites extracts has verified that ascites extracts make authentic Qβ coat. Furthermore, the fact that Qβ coat protein made in mammalian cell extracts can be labeled with radioactive methionine only if the methionine is protected from cleavage by formylation indicates that mammalian ribosomes correctly initiate coat synthesis with an N-terminal methionine. In addition, experiments not detailed here have shown that chymotryptic digestion of f-[^{35}S]Met-labeled material yields a peptide inseparable from the coat N-terminal peptide made in *E. coli* extracts: f-[^{35}S]Met-Ala-Lys-Leu (13).

The fact that the major product of the mammalian systems is precisely the size of authentic Qβ coat protein argues that the ascites ribosomes are also correctly terminating coat protein synthesis. In support of this conclusion is the finding that L-cell extracts directed by RNA containing an amber mutation (*am*-11) in the coat gene synthesize little, if any, completed coat protein, but synthesize only the appropriate N-terminal fragment of the coat. Qβ RNA was correctly translated in these extracts without the addition of tRNA or other factors to the extract.

Internal initiation

As a result of their studies of poliovirus-directed protein synthesis *in vivo*, Jacobson and Baltimore (18) have proposed that all mammalian mRNAs are monocistronic. Poliovirus mRNA exists as a large polycistronic messenger, rather than containing internally located initiation sites; one long "polycistronic" polypeptide is made and is subsequently cleaved to the poliovirus proteins. Alternatively, messenger RNAs of other RNA viruses, such as Reovirus (19, 20), apparently exist as single monocistronic RNA species. Such a requirement for monocistronic mRNA differs considerably from the situation in bacterial systems, in which polycistronic message may be initiated at internally located sites (5, 6, 21, 22). However, the results presented above argue that mammalian ribosomes can recognize and initiate properly at the internally located initiation site of Qβ coat protein. Therefore, if mammalian systems only contain various forms of monocistronic RNA, the reason for this property cannot be an inability of mammalian ribosomes to use internally located initiation sites. The requirement for monocistronic message, if real, must reside at some other level of gene expression, such as transcription, control of transcription, or message processing after transcription.

Usefulness of the system

The system described here should be useful in characterizing properties of eukaryotic translation. For example, this system would readily allow one to study the response of eukaryotic ribosomes to other nonsense mutations. Such a system allows one to screen for cell lines that suppress amber mutations. Su⁺ and Su⁻ cell lines would be invaluable in the genetic analysis of viral and cellular functions.

Aviv *et al.* (24) have also showed that Qβ RNA will direct the synthesis of Qβ coat protein in ascites extracts.

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