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Reinitiation of translation from the triplet next to the amber termination codon in the absence of ribosome-releasing factor

(ribosome read-through/*in vitro* translation/bacteriophage R17/peptide mapping)

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ABSTRACT **Ribosome releasing factor (RR factor) releases ribosomes from mRNA at the termination codon in *Escherichia coli*. In the absence of this factor, polypeptides with molecular weights very close to the molecular weight of bacteriophage R17 coat protein were synthesized *in vitro* under the direction of a mutant R17 phage RNA having an amber mutation at codon 7 of the coat cistron. The major coat-protein-like product shared all the R17 coat protein sequence except that the seven NH_2-terminal amino acids were missing. The minor product had the complete coat protein sequence starting from formylmethionine except for a probable amino acid substitution at codon 7 (UAG). Addition of RR factor inhibited the synthesis of the major protein. These results indicate that, in the absence of RR factor, the ribosome that has released the NH_2-terminal hexapeptide at the amber codon stays on the mRNA and subsequently reinitiates translation "in phase" immediately after the amber codon without formylmethionine.**

A mutant R17 phage amB_2 has an amber mutation at the seventh triplet of the coat cistron (1), and suppressor tRNA is needed to produce complete coat protein. In the absence of suppressor tRNA, this amB_2 R17 RNA directs synthesis of only NH_2-terminal hexapeptide proximal to the amber codon besides the polypeptides coded for by the other genes. The hexapeptide is released from the ribosome–amB_2 R17 RNA complex by termination factors (2–5) and possibly by an additional factor called "rescue factor" (6). Subsequently, ribosome-releasing factor (RR factor) releases ribosomes from mRNA at the amber codon (7–9). In the absence of this factor, this mutant RNA is translated even without suppressor tRNA (8, 9), leading to synthesis of polypeptide(s) with a molecular weight of 13,800, very close to that of the authentic R17 coat protein.

In this paper, we demonstrate that, in the absence of RR factor, translation is reinitiated "in phase" immediately after the amber codon without formylmethionine, resulting in synthesis of this coat-protein-like product (CPP). The implication of this finding is discussed in connection with possible ribosome movement from one cistron to the next in translation of bacterial polycistronic mRNA.

MATERIALS AND METHODS

***In Vitro* Translation System.** All *in vitro* translations were carried out in a reconstituted system of *Escherichia coli* cell extract from which RR factor had been removed as described (7, 9). Where indicated, 6.0 μg of electrophoretically homogeneous RR factor purified from the ribosomal wash (9) was added per 70 μl of reaction mixture. For the peptide mapping, 62–94 μCi (1 Ci = 3.7×10^{10} becquerels) of [^{35}S]methionine were used per 70 μl of reaction mixture. For the NH_2-terminal analysis by the fluorodinitrobenzene method, a mixture of 16 ^{14}C-labeled amino acids (total, 1.9 μCi/70 μl of reaction mixture) and nonlabeled glutamine, asparagine, cysteine, and tryptophan at 0.01 mM each was added. For determination of the sequence of the NH_2-terminal region of the CPP, a mixture of 18 ^{14}C-amino acids (total, 1.9 μCi/70 μl of reaction mixture) and nonlabeled cysteine and tryptophan at 0.01 mM was used. Incubation was for 35 min at 30°C.

Peptide Mapping. *In vitro* products programmed by amB_2 or wild-type R17 RNA were separated by $NaDodSO_4$/polyacrylamide gel electrophoresis as described (9). The CPPs were isolated, oxidized with performic acid (10), and digested by trypsin (Calbiochem, TPCK-treated) and then by chymotrypsin (Worthington, crystallized three times). Thin-layer electrophoresis at pH 3.5, chromatography, and subsequent autoradiography were performed as described (9).

NH_2-Terminal Analysis by the Fluorodinitrobenzene Method. The CPP containing ^{14}C-labeled amino acids was treated with fluorodinitrobenzene (11) and hydrolyzed in 5.7 M HCl. Dinitrophenyl (DNP) amino acids were then isolated and separated by two-dimensional chromatography on a cellulose thin-layer plate (11, 12). After separation, the region of each DNP-amino acid was scraped off and the radioactivity was determined.

Amino Acid Sequence Determination by the Edman Degradation. For the Edman degradation, 150 μg of RNase A as a carrier and 20 μl of phenylisothiocyanate were added to the 200-μl sample containing ^{14}C-labeled CPP. Subsequent steps were carried out as described (13, 14). Phenylthiohydantoin (>PhNCS) amino acids were finally separated by two-dimensional chromatography as described (15). The position of each standard >PhNCS amino acid marker was detected under a UV lamp, and the radioactive spot was identified by superimposing the autoradiogram on the chromatogram.

RESULTS

Peptide Mapping of CPP. When amB_2 R17 RNA was translated to an *in vitro* system lacking RR factor, a significant amount of polypeptides with molecular weights of about 13,800 was synthesized (9). Two-dimensional peptide mapping analysis (Fig. 1) revealed that the CPP polypeptides had all the methionyl peptides found in the digest of the normal coat protein except for the peptides 1 and 2; these were present in much smaller amounts in the CPP polypeptides. Because polypeptides 1 and 2 were negatively charged and totally missing from the coat protein isolated from the R17 virion (Fig. 2), they must have come from the NH_2 terminus of the coat protein synthesized *in vitro* which retains formylmethionine. In agreement with the fact that the virion coat protein contains two methio-

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Abbreviations: RR factor, ribosome-releasing factor; CCP, coat-protein-like product; DNP, dinitrophenyl; >PhNCS, phenylthiohydantoin.

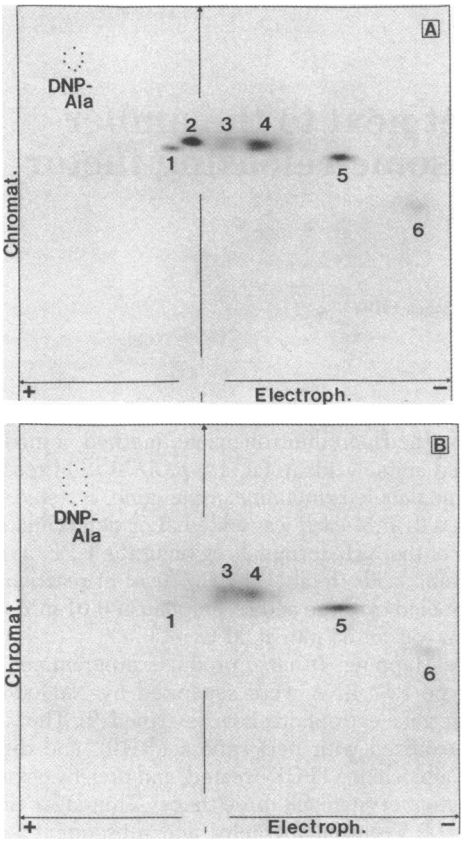

FIG. 1. Peptide mapping of coat protein and CPP synthesized *in vitro*. (*A*) R17 coat protein programmed by wild-type RNA was synthesized in the RR factor-free translation system containing [^{35}S]methionine and isolated by NaDodSO$_4$/polyacrylamide gel electrophoresis. It was then digested with trypsin and chymotrypsin. An aliquot containing 5×10^4 cpm was analyzed by two-dimensional peptide mapping on a cellulose thin-layer plate. The autoradiogram shown was obtained after 36-hr exposure. Six spots were detected. Spots 1 and 2 are formylmethionyl peptides from the NH_2 terminus of the coat protein. The other spots are internal methionyl peptides. (*B*) CPP programmed by amB$_2$ R17 RNA was synthesized in the RR factor-free translation system and analyzed by the same procedure as in *A*.

nine residues (16), two major spots were found in the digest of the virion coat protein (spots 4 and 5 in Fig. 2). The additional spots 3 and 6 in Fig. 1*A* were due to either incomplete digestion or abnormal cleavage by trypsin and chymotrypsin. These additional spots were also observed in the digest of the virion coat protein when autoradiography was prolonged (data not shown). Similarly, spots 1 and 2 are of the same origin because prolonged digestion with the same enzymes converted spot 2 to spot 1 (data not shown). Other investigators also have reported extra spots in the coat protein digest (17, 18).

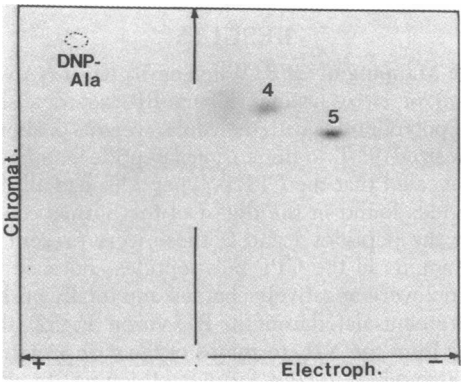

FIG. 2. Peptide mapping of R17 coat protein synthesized *in vivo*. Virion coat protein was isolated by NaDodSO$_4$/polyacrylamide gel electrophoresis from R17 phage grown in the presence of [^{35}S]methionine and subjected to the same peptide mapping as in Fig. 1.

It is important to note the existence of the formylmethionyl peptide (spot 1) as a minor component in the digest of the CPP (Fig. 1*B*). This suggests that the CPP is actually made up of two kinds of polypeptides. The major one is similar to the authentic coat protein except that the NH_2-terminal region is missing. The minor one has the entire sequence of coat protein including the formylmethionine of the NH_2 terminus. However, this minor polypeptide presumably has an amino acid substitution at the amber codon, although the *in vitro* system used in this experiment did not contain any suppressor tRNA.

Quantitative Relationships of Various Polypeptides in CPP. The peptide analysis presented in the preceding section indicated that the CPP consists of at least two populations, one containing total coat protein sequence including formylmethionine at the NH_2 terminus (polypeptide Cf, standing for *c*oat with *f*ormylmethionine) and the other lacking the NH_2-terminal region of coat protein (polypeptide Cd, standing for *c*oat with *d*eletion). A third group that might exist is a polypeptide that is deformylated but still retains the NH_2-terminal methionine (polypeptide Cm, standing for *c*oat with unblocked *m*ethionine). When the quantities of Cf, Cm, Cd, and NH_2-terminal hexapeptide (fMet-Ala-Ser-Asn-Phe-Thr) of the coat protein were computed (Table 1), it was clear that approximately 4 times more Cd was synthesized in the absence of RR factor. Furthermore, in the absence of RR factor, the quantity of the hexapeptide synthesized was comparable to that of Cd, suggesting that most ribosomes that synthesized the hexapeptide ran through the amber codon and initiated the synthesis of Cd without dissociating from mRNA.

Upon addition of RR factor, the amount of the hexapeptide was significantly increased, and much more hexapeptide than Cd was synthesized. This indicates that over 90% of ribosomes fell off the mRNA at codon 7 (UAG) of the coat cistron in response to the action of RR factor. Table 1 also indicates that the minor components in the CPP—Cf and Cm—were not influ-

Table 1. Quantitative relationships of various CPP polypeptides synthesized under the direction of amB$_2$ RNA

Peptide	Product, pmol × 10^3	
	Without RR factor	With RR factor
Cf	5.3	4.0
Cm	1.2	1.1
Cd	30.4	8.4
NH_2-Terminal hexapeptide	51.6	165.0

The content of formylmethionine was determined by analysis of Pronase digests of [^{35}S]methionine-labeled CPP. The amount of NH_2-terminal unblocked methionine was measured by the first cycle of the Edman degradation. By using these data, the quantities of polypeptides Cf, Cm, and Cd were then computed as follows. The quantities of Cf and Cm are equivalent to those of formylmethionine and NH_2-terminal unblocked methionine, respectively. Subtraction of the radioactivities of formylmethionine and NH_2-terminal unblocked methionine from the radioactivity of total CPP (corresponding to Cf + Cm + Cd) would yield the amount of Cd. The quantities of Cf, Cm, and Cd presented in this table correspond to the product synthesized from 40 μg of amB$_2$ R17 RNA in the absence or presence (1.7 μg) of purified RR factor, and they were corrected for the recovery in each step of the isolation procedure. The hexapeptide was assayed as described (9).

enced by the presence of RR factor. In other words, RR factor does not influence the translation of amber codon (amino acid substitution) which takes place to a small extent in the absence of amber suppressor tRNA. This is understandable in view of the finding that RR factor does not influence the ribosome with a peptidyl tRNA bound to it. The substrate of RR factor is a complex of ribosome, mRNA, and deacylated tRNA on the donor (or P) site of ribosome (19).

Amino Acid Sequence of the NH_2-Terminal Region of the "Reinitiated" Polypeptide. The polypeptide Cd containing ^{14}C-labeled amino acids was purified by NaDodSO$_4$/polyacrylamide gel electrophoresis and subjected to Edman degradation. In parallel, coat protein synthesized under the direction of wild-type R17 RNA and polypeptides with a molecular weight of 13,800 produced in the absence of mRNA were also subjected to the first cycle of the Edman degradation. The NH_2 termini of all three samples consisted of two major amino acids, leucine and phenylalanine (Fig. 3). However, the intensity of phenylalanine was much higher in the CPP than in the two controls, suggesting that the NH_2 terminus of the CPP is phenylalanine. The presence of >PhNCS phenylalanine and >PhNCS leucine in the product made in the absence of mRNA is due to the "soluble" amino acid incorporation system (refs. 20–22; for review, see ref. 23). This system has been known to transfer leucine, phenylalanine, or tryptophan from the cognate aminoacyl tRNA to the NH_2 termini of certain acceptor proteins. In Fig. 3, most of the coat protein synthesized under the direction of wild-type R17 RNA has blocked NH_2 termini, presumably with

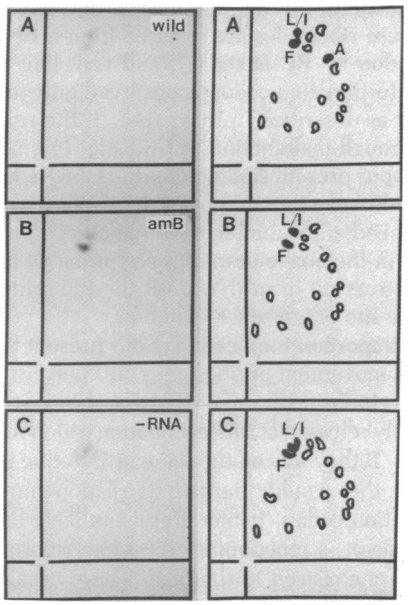

FIG. 3. NH_2-Terminal analysis of coat protein and CPP by the Edman degradation. The polypeptides were synthesized in the RR factor-free translation system containing ^{14}C-labeled amino acid mixture and isolated by NaDodSO$_4$/polyacrylamide gel electrophoresis. Only the first cycle of the Edman degradation was carried out and the resulting >PhNCS amino acids were separated by two-dimensional chromatography on a polyamide sheet together with >PhNCS amino acid standards as internal markers. The markers were located under the UV lamp (*Right*), and the NH_2 termini of the products were detected by autoradiography (7-day exposure) (*Left*). (*A*) Coat protein synthesized under the direction of wild-type R17 RNA. (*B*) CPP synthesized under the direction of amB$_2$ R17 RNA. (*C*) Molecular weight 13,800 polypeptides produced without mRNA. Only the relevant spots are indicated. A, alanine; F, phenylalanine; L/I, leucine or isoleucine. The UV spots corresponding to radioactive ones are shown as solid. The reaction mixtures and processing of the products for analysis were identical for *A*, *B*, and *C* except for the mRNA.

Table 2. Determination of NH_2-termini of CPP by the fluorodinitrobenzene method

DNP amino acid	Radioactivity, cpm × 10^{-1}	DNP amino acid	Radioactivity, cpm × 10^{-1}
Lys (di-DNP)	30	Gly	20
Trp	20	Met (sulfoxide)	10
Phe	870	Thr	50
Leu or Ileu	270	Ser	30
Val	10	Glu	10
Ala	20	Asp	20
Pro	0		

DNP amino acids derived from CPP containing ^{14}C-labeled amino acids were separated by two-dimensional thin-layer chromatography. The spot corresponding to each DNP amino acid was scraped from the thin-layer plate and the radioactivity was determined by assay for 10 min in scintillation fluid. The background radioactivity (15 cpm) was subtracted.

the formyl group. Prolonged autoradiography revealed alanine instead of methionine as a minor NH_2 terminus, which suggests that the NH_2-terminal methionine was immediately cleaved off once it was deformylated. Because the Edman analysis often fails to detect serine and threonine, a similar analysis was carried out by the fluorodinitrobenzene method (Table 2). The results confirmed the conclusion of the Edman analysis.

Further Edman analysis was carried out only with the CPP up to the fourth residue from the NH_2 terminus (Fig. 4). It clearly demonstrated the existence of a discrete sequence Phe-Val-Leu (or Ileu)-Val which exactly matches the 8th to 11th residues of R17 coat protein. This sequence does not occur in any other place in the R17 coat protein (16).

Evidence that the Reinitiated Polypeptide is not Produced by Proteolytic Degradation. Because the reinitiated polypep-

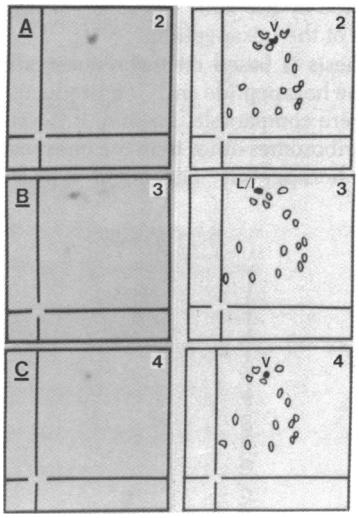

FIG. 4. Amino acid sequence of the NH_2-terminal region of the CPP. (*Left*) Autoradiography. (*Right*) UV fluorescence. Manual Edman degradation was carried out up to the fourth cycle with the purified CPP (4800 cpm) containing ^{14}C-labeled amino acids. Ninety percent of >PhNCS amino acids obtained in each cycle of degradation was subjected to the two-dimensional chromatography. The result of cycle 1 was basically the same as in Fig. 3. (*A*) Cycle 2; note radioactive >PhNCS valine (V). (*B*) Cycle 3; note predominantly radioactive >PhNCS leucine (L) [or possibly isoleucine (I)]. The two faint spots observed next to distinct >PhNCS leucine correspond to >PhNCS valine and >PhNCS proline (the former is a trace of carryover from the preceding cycle). >PhNCS proline and one additional faint spot in the middle of the plate were occasionally observed. (*C*) Cycle 4; note radioactive >PhNCS valine.

tide we observed had the discrete sequence Phe-Val-Leu (or Ileu)-Val and no such polypeptide was observed in the *in vitro* product programmed by wild-type R17 RNA, we concluded that it was not produced by proteolytic degradation of the complete coat protein.

In order to rule out further the possibility that polypeptide Cd may be produced by degradation of complete coat protein, we carried out an experiment to detect degradation. In this experiment, [^{35}S]methionine incorporation programmed by amB_2 R17 RNA was interrupted by the addition of chloramphenicol, and decrease of radioactivity in the CPP was monitored. As seen in the autoradiogram, the decrease was not detected during 20-min incubation after the chloramphenicol addition (Fig. 5). It should be pointed out that coat protein synthesized *in vitro* has two internal methionines and one formylmethionine. Therefore, if the degradation took place in the NH_2-terminal region, resulting in the removal of the first seven amino acids including formylmethionine, nearly 30% of the radioactivity in the band of CPP should have been lost after the addition of chloramphenicol.

DISCUSSION

In the absence of RR factor, a CPP of R17 phage was synthesized without suppressor tRNA under the direction of RNA of a mutant R17 phage carrying an amber mutation at codon 7 of the coat cistron. The data presented in this paper have established that this CPP shares most of the coat protein sequence except for the missing first seven amino acids fMet-Ala-Ser-Asn-Phe-Thr-Gln. Very little protein having the entire coat protein sequence (except for the amino acid substitution at codon 7) was found. Our interpretation of this observation is that, in the absence of RR factor, the ribosome that has released the NH_2-terminal hexapeptide at the amber codon is able to "reinitiate" translation immediately after the amber codon. This reinitiation is thus performed by the same ribosomes that have completed the translation of the hexapeptide.

This hypothesis is based on three observations. First, the quantities of the hexapeptide and CPP produced in the absence of RR factor were comparable. Second, if the reinitiation were performed by ribosomes other than the ones that translated the NH_2-terminal hexapeptide, one might expect that the reinitiated protein may have formylmethionine at the NH_2-terminus. On the contrary, most of the coat-like protein had no formylmethionine at the N-terminal. Deformylation and subsequent demethionylation of nascent polypeptides do not take place efficiently *in vitro* (24–26). As a matter of fact, we observed that most of the coat protein synthesized under the direction of wild-type R17 RNA retained formylmethionine. It therefore is unlikely that the CPP observed in the absence of RR factor was derived from formyl polypeptides. Third, the possibility that the CPP might be produced by proteolytic degradation of the complete coat protein was ruled out (Fig. 5).

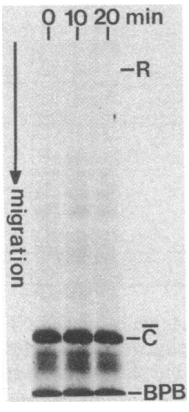

FIG. 5. Lack of degradation of the CPP. [^{35}S]Methionine-labeled CPP was synthesized in the RR factor-free translation system (70 μl). After 15 min at 30°C, chloramphenicol was added to 1 mM to stop further translation. The incubation was continued and aliquots were taken at 0, 10, and 20 min after the addition. The CPP was separated by NaDodSO$_4$/polyacrylamide gel electrophoresis, and the radioactivity in the CPP (C̄) was determined. 0 min, 1.9×10^4 cpm; 10 min, 2.1×10^4 cpm; 20 min, 1.9×10^4 cpm. Band R corresponds to replicase. BPB, bromphenol blue.

The findings described in this paper provide a good basis for understanding of the reinitiation of protein synthesis distal to the site of nonsense mutation as shown with T4 *rIIB* (27), *E. coli trpD* (28), *Salmonella typhimurium trpB* (29), and *E. coli lacI* and *lacZ* (30–33). In every case, COOH-terminal fragments were detected by their partial enzymatic activity or immunological crossreactivity. For example, Files *et al.* (30) isolated the COOH-terminal fragments of *lac* repressor protein immunologically and found that the reinitiation of translation occurs from a codon next to a methionine, valine, or leucine codon located far from the site of nonsense mutation. It was suggested that the COOH-terminal fragments were originally initiated with formylmethionine which was subsequently cleaved off. However, it was not clear whether the ribosomes that translated the NH_2-terminal portion would reinitiate the COOH-terminal fragment. We propose that, in these cases, ribosomes are released from the mRNA at the nonsense codon by RR factor, and the reinitiation from the distal initiation codon takes place by different ribosomes through the normal initiation mechanism. This is because ribosomes are released from mRNA at the termination codon by RR factor (7), and traveling of ribosomes beyond the termination codon is observed only in the absence of RR factor as described in this paper. If the reinitiation had occurred through malfunction of RR factor *in vivo*, one would expect from our present finding that the COOH-terminal polypeptide would have initiated from the triplet right next to the termination codon. The internal initiation codons used for the reinitiation in the above examples are probably hidden by the secondary structure of mRNA until the translating ribosomes travel to the site of nonsense mutation.

Another important implication of our present finding is concerned with movement of ribosomes on a polycistronic mRNA. The present studies with amB_2 R17 RNA showed that ribosomes are efficiently released at the termination codon in the presence of RR factor. If they stay on the same mRNA due to the absence of RR factor, they would reinitiate translation immediately after the termination codon. It therefore is unlikely that, in cells in which RR factor is functioning, the same ribosome continues to translate one cistron to the next. There usually exist intercistronic regions that would be translated if the same ribosome stayed on the mRNA, as shown in this paper. This would lead either to "out-of-phase" reading of the distal cistron or to the synthesis of a protein with an intercistronic peptide attached to its NH_2 terminus. The same argument would apply to the cases in which two adjacent cistrons overlap each other (34, 35). It has been suggested that ribosomes can glide on the intercistronic region of mRNA to the next initiation codon without polypeptide synthesis ("phaseless" and "sterile" travel) (4, 36–44). The data presented here do not support such movement of ribosomes. If we assume that mRNA exposes the initiation region of the distal cistron when ribosomes have translated the proximal cistron (45), we do not need to postulate the sterile travel. Perhaps the "scanning" of 40S ribosomal subunit from the 5′ cap to the first initiation codon of eukaryotic mRNA may be

unique to eukaryotes and does not apply to this discussion (46–48).

Kinetic studies by Zalkin *et al.* (49) indicated that the translation of *trp* operon is carried out by ribosomes independently bound to each cistron. Grubman and Nakada (50) demonstrated that the ribosome that has translated the coat cistron of MS2 phage does not translate further the distal replicase gene at a detectable level. These results, together with our present findings regarding the function of RR factor, strongly suggest that ribosomes are released from mRNA at each termination codon in the polycistronic mRNA to ensure proper translation of the next cistron.

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