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Mechanism of Protein Chain Termination: Further Characterization of a Mutant Defective in a New Protein Synthesis Factor

(ribosomes/f2 bacteriophage/coding/peptidyl-tRNA/thermolabile proteins)

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ABSTRACT **Mutant N4316 is conditionally lethal at 43°. At 36° it suppresses the termination codons UGA and UAA, but not the UAG codon or a missense mutant of T4 bacteriophage. *In vitro*, a factor rescues protein synthesis from a temperature-dependent arrest when N4316 extracts are used with RNA from bacteriophage f2. Analyses of the substrate in the arrested synthesis and of the product of the rescue reaction indicate that the factor works at the level of coat protein termination, and that it also affects the synthesis of noncoat protein products. The rescue factor is different from the release factors RF-1, RF-2, and RF-3. Model systems previously used to study release fail to score for at least one vital function in protein chain termination.**

One of the principal assumptions about translation of the genetic code is that triplets are read in phase, one at a time, and that the response of the translation machinery to each codon is not affected by the neighboring triplets (1). Although this process may indeed be inevitable in protein chain elongation, recent data suggest that the proper recognition of AUG and GUG as initiator codons may depend on the sequence of the nucleotides surrounding them (2). In protein chain termination, similar considerations may be applicable. For example, there is evidence that suppression of nonsense codons may be drastically affected by the composition of the nucleotides adjacent to the termination triplets (3). This evidence invites speculation that the signal for termination of proteins that occurs in the intercistronic stretches (2, 4), and closely precedes initiation signals, may also be influenced by adjacent bases. In fact, the close proximity of the two markers may imply a coupling of the two biochemical events.

One of the *in vitro* experiments described here with a mutant (N4316) (5–7), reported to be temperature sensitive in polypeptide chain termination, suggests that under nonpermissive conditions when termination of f2 coat protein is impaired the reading of other cistrons may also be affected.

The *in vitro* defect in coat protein release is reversed by a factor isolated from the parental strain. This factor unlike RF-1, RF-2, and RF-3, is not required in the model *in vitro* termination assays that respond to the simple addition of the single nonsense codons UAA, UAG, or UGA (8). This protein also appears to be unrelated to the factors IF-1, IF-2, and IF-3 that are needed for proper initiation of the coat protein cistron *in vitro* (9).

Abbreviations: sus_3RNA, mutant of bacteriophage f2 with an amber codon at the 7th amino acid in its coat protein; su^+_{III} tRNA, suppressor tyrosine-accepting tRNA.

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RESULTS AND DISCUSSION

Certain Properties of the N4316 Mutation. The isolation and several properties of strain N4316 have been described (5–7). The parental strain of N4316 is *E. coli* strain D_{10} Met^- Thy^- $RNase_I^-$ (10, 11). The mutant, N4316, belongs to a class designated *sts* (starvation temperature sensitive) that is conditionally lethal at 43° (10). At 36°, strain N4316 suppresses UAA and UGA codons, but not UAG or a missense mutant of T4 bacteriophage (5). These two properties, thermolethality and suppression of nonsense codons, transduce together and revert to the parental phenotype together, suggesting that a single mutation is responsible for both effects (5).

In vitro, N4316 extracts programmed with f2 or R17 RNA are impaired in protein synthesis at nonpermissive temperatures (5–7). The extent of impairment is 2- to 3-fold compared to synthesis at permissive temperatures with N4316, or parental (D_{10}) extracts at either temperature. The defect in protein synthesis is not due to a mutation in any of the 20 aminoacid activating enzymes (6). When programmed with poly(U), (A), (AU, 3:1), (UG, 5:1), or (UC, 1:1), synthesis is the same at the higher or lower temperatures (ref. 7 and unpublished data). Furthermore, the formation of the initiation complex of R17 with fMet-tRNA is not affected (6).

Peptides accumulate on 70S ribosomes at nonpermissive temperatures (5, 6). This result, and the temperature-dependent suppression of nonsense codons *in vivo*, suggested that the strain might be defective in protein chain termination.

Table 1 shows our attempt to detect temperature sensitivity with *N*-acetylmethionine release as a model system (8). This assay responds to the release factor RF-1 with UAA or UAG, or to RF-2 when UAA or UGA are included (8). Treatment of parental or mutant extracts at 43° reduced synthesis with phage f2 RNA irreversibly, but it did not affect the nonsense codon-dependent release of *N*-acetylmethionine stimulated by UAA, UAG, or UGA.

Since release of *N*-acetylmethionine is stable to heat, another termination assay was tried, namely, the release of polypeptides from ribosomal-bound polypeptidyl-tRNA. Fig 1 shows that the percent free peptides released with poly(A_3U), a message that contains a high frequency of UAA codons (12,

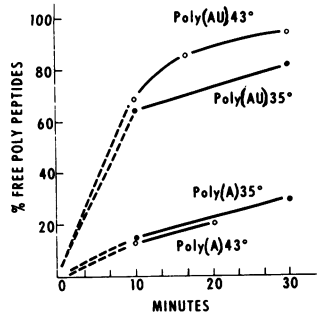

FIG. 1. Free polypeptides synthesized by N4316 ribosomes and soluble proteins. Analysis of total and free peptides was as described (12, 13) except that incubations (0.155 ml) containing 150 μg of ribosomes and 6.3 μg of soluble protein were heated for 5 min at 43° (where indicated). The reaction was run at 35° for 15 min. Counts incorporated without added polynucleotides (less than 10% of the incorporation) were subtracted from each value. With parental extracts, the incorporation with poly(A) or poly(AU) was slightly higher after 43° treatment, but the percent release was identical at all temperatures.

13), is normal in a thermally inactivated extract of N4316. We conclude that the mutation does not affect any of the soluble or ribosomal factors needed in release that are scored by this assay (14). In addition, all other attempts to show thermolability of release in these assays by variation of reaction conditions failed. Taken together, these results suggested that the defect with f2 RNA observed *in vitro* is not scored by the model systems commonly used to study release.

TABLE 1.

Extract	Prior treatment at 43°	*N*-acetyl-[^{3}H]methionine released, pmol		
		UAA	UAG	UGA
D_{10}	−	1.27	0.19	2.60
D_{10}	+	1.02	0.19	2.15
N4316	−	1.10	0.16	1.37
N4316	+	0.98	0.19	2.20

Trinucleotide-dependent release was assayed as described by Caskey *et al.* (28), except that *N*-acetylmethionyl-tRNA was used (18) instead of *N*-formylmethionyl-tRNA. Reactions (45 μl) contained 2.6 pmol of *N*-acetyl-[^{3}H]methionyl-tRNA$_f^{Met}$–AUG ribosome complex, 5 nmol of UAG, UAA, or UGA, and 5 μl of crude extract from N4316 or D_{10}. Since this assay cannot be performed at 43° (25), portions of each extract were heated for 5 min at 43° before assay at 24°. Reactions containing UAA or UAG were incubated for 15 min, those containing UGA for 60 min. Counts released without added triplets (0.15 pmol for 15 min and about 0.3 pmol for 60 min) were subtracted. Values are the average of triplicates, which were within 10% of each other. Purification of the extracts did not elevate the activity of RF-1. Unheated parental extracts incorporated 85,000 cpm of [^{3}H]lysine (see Fig. 2 for description of assay) in response to f2 RNA, and 75,000 cpm after heat treatment. Mutant extracts incorporated 78,000 cpm before heat treatment and 39,000 cpm of [^{3}H]lysine after heat treatment. After they were heated at 43°, extracts were exposed to 24° for 60 min without change in the incorporation values.

Analysis of Products Synthesized under Nonpermissive Conditions. We have analyzed the products of synthesis after separation of peptidyl-tRNA from free protein by means of sucrose density gradients containing dodecyl sulfate (13). The separated peptidyl-tRNA and free peptides were concentrated by ethanol precipitation, and were further analyzed by electrophoresis in polyacrylamide gels containing Na dodecyl sulfate.

Fig. 2 shows the separation in sucrose gradients of peptidyl-tRNA (peak I) and free protein (peak II) made at nonpermissive temperatures with either strain D_{10} or strain N4316. 90% of the total peptides made by the parental extract were released at 47°, whereas the mutant always released significantly less of the protein it synthesized, e.g., 70% in the experiment of Fig. 2 and as much as 50% in other experi-

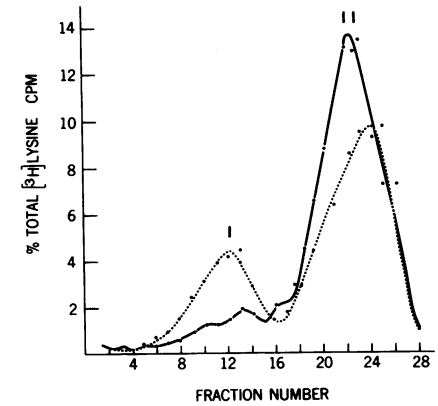

FIG. 2. Dodecyl sulfate–sucrose density gradient centrifugation of f2 RNA products made with N4316 or D_{10} extracts at 47°. Cells were grown at 33° to a density of 5×10^8 viable cells per ml. Thermostable revertants were routinely less than 0.1%. Before analysis, cell extracts were prepared (26) and assayed for incorporation of amino acids into protein (18) at several temperatures; the temperature at which maximum relative inactivation of N4316 extracts occurred varied and had to be determined for each new pair of mutant and wild-type extracts made. Routinely, D_{10} extracts lost less than 10% activity at the higher temperatures (here, 47°). The standard assay (60 μl) contained: 10 μl of incubated S-30 extract, 10 mM $MgCl_2$, 30 mM NH_4Cl, 30 mM Tris·HCl (pH 7.4), 5.3 mM P-enolpyruvate, 3.3 mM ATP, 0.4 mM GTP, 8 mM dithiothreitol, 2.5 μCi [^{3}H]lysine, 0.1 mM (each) of a mixture of 19 [^{12}C]aminoacids lacking lysine, 7.5 μg of calcium leucovorin, 5 μg of pyruvate kinase, and 10 μg of f2 RNA. Extracts of D_{10} and N4316 were incubated for 10 min at the permissive or nonpermissive temperatures. f2 RNA was added, and incubation was continued for 15 min at 47°. The first incubation destroyed completely the activity of a partially purified rescue factor (see below) isolated from N4316. An aliquot (60 μl) of the incubation mixture was layered on a sucrose gradient (5–12% sucrose, 3 ml total volume) containing 0.5% dodecyl sulfate and 0.1 M LiCl (13). Sedimentation was at 12° for 17 hr at 40,000 rpm in an SW56 Beckman rotor. Fractions were collected, precipitated with 15% Cl_3CCOOH, heated for 15 min at 90°, and filtered on Millipore filters (18, 26). Recoveries of radioactivity from the gradients were 12,000 cpm (D_{10} reaction) or 6800 cpm (N4316 reaction); 80–90% recovery of the total acid-insoluble counts analyzed. Parallel gradients of reaction mixtures without f2 RNA did not contain significant radioactivity in the region of peak I, but 2900 cpm or 3200 cpm were recovered in peak II when D_{10} or N4316 were analyzed. These counts were subtracted from the experimental values.

ments. The smaller extent of release observed with the mutant must be related to the temperature lesion, since less than 5% of the products synthesized at permissive temperatures were found attached to tRNA with extracts of either D_{10} or N4316.

Electrophoretic analysis of the free peptides made at nonpermissive temperatures with D_{10} or N4316 are shown in Fig. 3*a–c*. The major product released at 47° with D_{10} or N4316 corresponds in size to coat protein. The conclusion that the major peak observed in Fig. 3*a–c* is coat protein rests on the position of this product on polyacrylamide gels. In addition, we show in Fig. 3*c* that this major product is eliminated when synthesis is programmed by an amber mutant (sus_3RNA) (15) that cannot make coat protein. Under conditions shown to eliminate polarity effects of this mutation *in vitro* (16), addition of a purified suppressor tRNA (17, 18) restores synthesis of this major product.

The lack of extensive release of noncoat products (Fig. 3*d–f*) occurred at permissive temperatures with both D_{10} and N4316, indicating that this effect is unrelated to the mutation.

Since no qualitative differences could be observed in the peptides released by N4316 and D_{10}, we examined the size distribution of the nascent peptides made by both strains. Fig. 3*d* and *e* show electropherograms of the nascent peptides

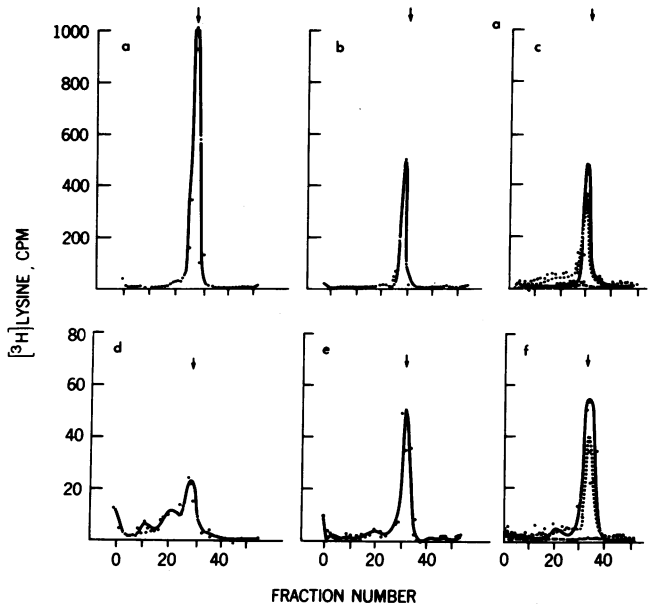

FIG. 3. Released peptides (*a–c*) or nascent peptides (*d–f*) synthesized with D_{10} (*a*, *d*) and N4316 (*b*, *c*, *e*, and *f*) at 47°. Incorporation of labeled lysine (3×10^3 Ci/mol) was described in Fig. 2. Conditions for suppression of coat protein and purification of su^+_{III} tRNA were described (18). Incubations (0.10 ml) were layered and centrifuged on sucrose containing dodecyl sulfate and LiCl (see Fig. 2). After addition of carrier S-100 and two volumes of ethanol, fractions I and II were concentrated by centrifugation. The pellets were suspended in 0.01 M Tris·HCl (pH 7.4) and were treated with 10 μg of RNase at 35° for 10 min. Each sample was made 1% in dodecyl SO_4, 50% in glycerol and 0.1 M in 2-mercaptoethanol. Polyacrylamide gel electrophoresis was for 3.5 hr at 8 V/cm, and 4 A per gel (27). Fractions were cut, digested, and counted for 50 min (18). Recovery of added radioactivity was 80–100%. Each gel contained, as markers, f2 coat and maturation proteins (26). Peptides of small size are not seen because they are soluble in ethanol.

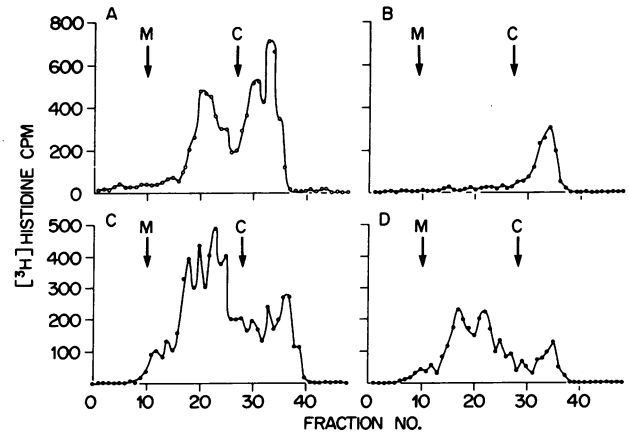

FIG. 4. Nascent peptides containing [^{3}H]histidine (4×10^4 Ci/mol) made with D_{10} extracts at 46° (*A*) or at 35° (*C*) or with N4316 extracts at 46° (*B*) or at 35° (*D*). Conditions of synthesis and analysis of products as in Figs. 2 and 3. The position of the coat (C) and maturation proteins (M) are indicated by the *arrows*.

made by D_{10} or N4316 at 47°. N4316 makes only one major product attached to tRNA; it corresponds in molecular weight to coat protein. In contrast, D_{10} makes nascent peptides larger than coat protein, as well as some peptides that correspond in size to the coat. The major product made by N4316 is eliminated when synthesis is programmed by sus_3-RNA (Fig. 3*f*). Addition of a purified suppressor tRNA also restores synthesis of this major product. The data in Fig. 3*d–f* show that release of coat protein is affected in the mutant strain. Furthermore, at permissive temperatures, both D_{10} and the mutant made and released the same amount and type of peptides. At nonpermissive temperatures, however, synthesis by N4316 is lowered, more nascent coat protein molecules accumulate on ribosomes, and fewer larger nascent peptides are observed.

To gain some insight into the nature of the nascent peptides that were larger than the coat, the products were labeled with histidine, an amino acid that does not occur in the coat protein (19). Fig. 4 shows analyses of nascent peptides labeled with histidin At permissive temperatures, the bulk of thee. histidine-containing nascent peptides in both D_{10} and N4316 are larger than the coat protein. A striking lack of synthesis of histidine-labeled larger noncoat products occurs only in N4316, and not in the parental strain; this lack may be due to faulty initiation of other cistrons.

At nonpermissive temperatures, both D_{10} and the mutant make more peptides that are slightly smaller than coat protein. These peptides do not appear to be precursors of the larger products, because their mobility was unaltered in pulse–chase experiments of the sort reported in Fig. 5.

Site of Action and Characteristics of the Rescue Factor. When thermally inactivated extracts are included at saturating levels, addition of increasing amounts of similarly heated parental extracts increased the rate of protein synthesis by about 3-fold (data not shown). There is a similar dependence on the amount of parental "rescue" factor added that can be used as an assay to purify the relevant factor [previously designated Z (6, 7)]. Our partially purified factor gives three bands on dodecyl sulfate–polyacrylamide electrophoresis (manuscript in preparation).

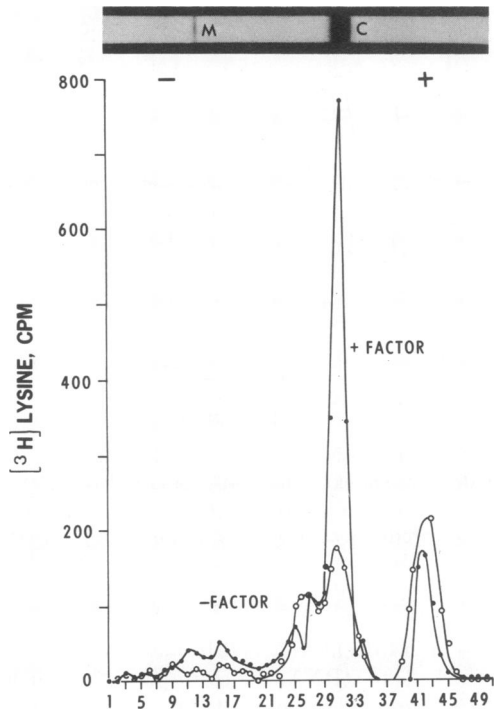

FIG. 5. Effect of factor on release of coat protein. Incorporation of amino acids into protein was as described in Fig. 2 and refs. 18 and 26: crude ribosomes and soluble protein from heat-inactivated N4316 extracts were used. Incubation was for 5 min at 35°. Then 17 mM [^{12}C]lysine was added and incubation was continued for 10 min at 35°. Products were isolated on sucrose gradients (26), and the ribosome-free fraction was concentrated by addition of cold 5% Cl_3CCOOH. The pellets were suspended in buffer, extracted with ether, and analyzed on dodecyl SO_4–polyacrylamide gels (see Fig. 3). The coat (*C*) and maturation protein (*M*) used as internal markers are shown. Reactions without f2 RNA were also electrophoresed, and the small amounts of radioactivity observed in the regions of small peptides were subtracted from the above profiles. Complete system minus rescue factor (O——O); complete system, with 5 μl of partially purified rescue factor (●——●). The presence of small peptides in this experiment may be due to the fact that the products were concentrated with Cl_3CCOOH rather than ethanol.

The component that reverses the N4316 lesion purifies as a protein; its activity can be destroyed by trypsin, elevated temperatures, or reagents that block sulfhydryl groups (6, 7), but treatment with pancreatic RNase is ineffective. The factor has a molecular weight of about 96,000, as determined by both sucrose density gradient analysis and chromatography on Sephadex columns. Thus, we clearly are not dealing with a temperature-sensitive suppressor tRNA of the sort reported by Smith *et al.* (20).

The rescue factor is separable from the termination factors RF-1, RF-2, and RF-3 (8) by partition on DEAE-Sephadex and alumina C_γ. In Table 2 we show that the rescue factor preparation does not contain any of the other known release factors. We also find (data not shown) that the rescue factor is free of the initiation proteins IF-1, IF-2, and IF-3. The factor that rescues synthesis, seemingly unlike the initiation factors, is found in ribosome-free cytoplasm.

The assay for rescue of synthesis from the thermal defect of N4316 extracts by parental extracts is nonspecific, since it only measures incorporation of amino acids into acid-insoluble precipitates. We can obtain some idea regarding the mechanism of this rescue by examining the products of the rescue assay. Fig. 5 shows an analysis by dodecyl sulfate–polyacrylamide gel electrophoresis of the peptides released from ribosomes before and after addition of rescue factor. In these experiments, synthesis with f2 RNA was performed for 5 min with thermally inactivated extracts of the mutant; then, further incorporation of amino acids into protein was blocked by addition of a 1000-fold excess of [^{12}C]lysine. After 30 sec, rescue factor was added and synthesis was allowed to continue for another 10 min. As shown in Fig. 5, some release of coat protein does occur without added rescue factor. Addition of the factor results in an about doubled release of coat protein free of tRNA, leading to the disappearance of ribosomal-bound coat protein tRNA (data not shown). The experiment strongly suggests that the rescue factor participates in release when a natural messenger RNA programs synthesis. If labeled histidine is added at the same time as the rescue factor, nascent peptides larger than coat protein (see Fig. 4*A–D*) are observed, supporting the notion that this protein affects the initiation of noncoat protein products.

The Possible Role of the Rescue Factor. This factor rescues protein synthesis *in vitro* from a temperature-dependent arrest when N4316 extracts are used with f2 RNA as messenger. Analysis of the substrate in the arrested synthesis and of the products of the rescue reaction indicate that the factor works at the level of chain termination when a natural messenger RNA programs synthesis. This factor is different from the known termination proteins RF-1, RF-2, and RF-3 that stimulate release in response to single nonsense codons.

RF-1 and RF-2, which participate at least indirectly in recognition of single nonsense codons (21–23), are required for release of the coat protein and the replicase of R17 bac-

TABLE 2.

Factor added	*N*-Acetyl-[^{3}H]methionine released, pmol			
	Exp. 1			Exp. 2
	UAA	UAG	UGA	UAA
None	1.35	1.35	1.35	0.96
RF-1 + RF-2	5.37	7.50	1.93	3.00
Rescue	1.05	0.88	1.15	1.02
RF-1 + RF-2 + Rescue	3.69	4.86	2.05	2.24
RF-1 + RF-2 + RF-3	—	—	—	9.77

Release of formylmethionine was assayed (28) with fraction 3 (21) as a source of RF-1 and RF-2. In Exp. 1, reaction mixtures (45 μl) contained 22 pmol of [^{3}H]acetylmethionyl-tRNA$_f^{Met}$–AUG–ribosome complex (18) and, where indicated, 6.3 nmol of UAG, 2.5 nmol of UAA, 4.0 nmol of UGA, 80 μg of RF-1 and RF-2 or 2 μg of rescue factor (fraction 5). Incubation was for 30 min at 24°. In Exp. 2, reaction mixtures contained 2.5 nmol of UAA and, where indicated, 5 μl of an ammonium sulfate fraction of RF-3 (25) or 2 μg of rescue factor (fraction 5). The inhibition of release observed with rescue factor added to RF-1 and RF-2 occurred also when a purified rescue factor from N4316 was thermally inactivated. Variation of triplet concentration, inclusion of 0.1 mM GTP, increase in rescue factor levels, or alteration of salt concentration did not alter the lack of effect of rescue factor in this assay; 0.1–2 μg of the rescue factor restored synthesis completely in the rescue assay.

teriophage (24). However, participation of the rescue protein in recognition of the normal signal for release is not excluded by these observations.

Since we find that the mutation also affects the synthesis of noncoat products, we do not know whether the rescue protein affects termination directly or indirectly. The lack of synthesis of noncoat products could, for example, occur if arrested ribosomes, bearing coat-protein tRNA, prevented initiation of the contiguous cistron. By the same token, the impaired termination could result from a fault in the initiation of the replicase cistron.

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