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Cloning of the *Escherichia coli* Release Factor 2 Gene

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The protein release factor 2 (RF2) participates in *Escherichia coli* polypeptide chain termination with codon specificity (UAA or UGA). A colicin E1 recombinant identified in the Carbon and Clarke *E. coli* bank contains the protein release factor 2 gene. A 1.7-kilobase *E. coli* fragment has been subcloned into the plasmid pUC9 vector. Bacterial cells, containing the plasmid recombinant, produce elevated levels of protein release factor 2 as detected by an immune precipitation assay and in vitro measurement of UGA-directed peptide chain termination and [^{3}H]UGA codon recognition.

Peptide chain termination in *Escherichia coli* requires one of the two codon-specific protein release factors (RFs), RF1 (for UAA or UAG) or RF2 (for UAA or UGA) (10). Their ribosomal binding involves recognition of a termination codon (11). Since other codons are recognized by the tRNA anticodon, recognition of peptide chain termination codons involves a different mechanism. The molecular details of RF recognition of nucleic acid sequences have been investigated indirectly by using synthetic modified trinucleotide codons (19), ribosomal structural alterations (1), cross linkage of RF molecules to ribosomal proteins (14) and radioactive termination codons (8). The structure and functional domains of RF molecules have been difficult to identify because of the low number of molecules per cell (about 500 per cell) and the difficulties associated with purification of quantities required for physical and functional study.

In this article, we report the successful molecular cloning of the *E. coli* RF2 gene by recombinant DNA techniques. This new development should permit elucidation of the structural and functional aspects of RF2.

The screening of the Clarke and Carbon (4) *E. coli* recombinant library was performed by an immune precipitation assay with in vivo-labeled proteins. Colonies from the Clarke and Carbon colicin E1 recombinant library were stored frozen at −80°C in 96-well microtiter plates in 8% dimethyl sulfoxide. Colonies were replicated on Luria (6) agar plates and subsequently were used to inoculate 1.5 ml of M9 (6) minimal medium containing 40 μg of leucine, tryptophan, and threonine per ml. When colonies reached 8 to 9 h of growth (late-log-growth phase), chloramphenicol was added to a final concentration of 200 μg/ml, and growth was continued overnight. Overnight cultures containing amplified plasmid copies were pelleted for 10 min at 2,000 rpm, washed once in M9 medium, and resuspended in 750 μl of M9 medium with 10 to 12 μCi of [^{35}S]methionine. The isotope was incorporated for 20 min at 37°C. Cells were placed on ice, washed twice in cold M9 to remove the [^{35}S]methionine, and transferred to 1.5-ml Eppendorf tubes on ice. Cell pellets were suspended in 75 μl of 50 mM Tris (pH 8.1)–25% sucrose; 5 μl of 100 mM EDTA (pH 7.4) was added, and after 5 min at room temperature, 5 μl of 1% lysozyme was added, followed by an additional 5-min room temperature incubation. The bacteria were collected in a microfuge for 30 s, and the supernatant fluid was discarded. The cell pellet was lysed in 60 μl of 40 mM Tris-hydrochloride (pH 8.1); 6μl of 10% streptomycin sulfate was added, and after incubation on ice for 30 min for nucleic acid precipitation, the lysate was centrifuged. The precipitate was removed by microfuge for 3 min; the supernatant fraction was recovered and stored at −80°C. This technique is similar to that reported by Neidhardt et al. (7).

The immunoprecipitation was performed by combining 100 μl of bovine serum albumin (10 mg/ml) in PBSE-Nonidet P-40 (25 mM potassium phosphate, pH 7.6, 100 mM NaCl, 1 mM disodium EDTA, 0.5% Nonidet P-40); 5 μl of sheep anti-RF2 (8) serum and 800 μl of PBSE-Nonidet P-40 and 10^5 trichloroacetic acid-precipitable cpm of lysate in Eppendorf tubes (5). The reaction was mixed by vortexing and placed on ice for 1 h. One hundred microliters of 10% (grams per milliliter) formalin-fixed *Staphylococcus aureus* (Ig Gsorb) in PBSE-Nonidet P-40 was added to each tube and incubated 45 min on ice with vortexing every 5 to 10 min. The precipitate was collected by centrifugation for 1 min in a microfuge, and the supernatant was discarded. The immune precipitate was resuspended in sample buffer (100 mM Tris, pH 6.8, 1.6% sodium dodecyl sulfate, 40% sucrose, 0.01% bromphenol blue, 5% β-mercaptoethanol) and heated at 85°C for 5 min. Samples were run on 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Gels were treated with En^{3}hance, dried, and exposed for 3 to 4 days to Kodak XAR-5 film.

The colicin E1 clone pLC42-13 was mapped by using restriction endonuclease digestion and was found to contain a 3.3-kilobase (kb) *E. coli* DNA insert. A 3.4-kb *Pvu*II restriction endonuclease fragment containing 1.7-kb *E. coli* and 1.7-kb ColE1 inserts was purified and isolated from 1% low-melting-point agarose gel and subcloned into the *Hinc*II restriction endonuclease site of pUC9. This subclone, designated pRF2, was identified to produce RF2 by an immune precipitation assay.

Samples of the strains with plasmids pRF2 and the control ColE1 were plated, and colonies were selected for ampicillin resistance and tetracycline sensitivty. Cultures (1 1) seeded with 10 to 20 ml of an overnight culture were grown in Luria broth (6) to an optical density at 650 nm of 0.8 in the presence of ampicillin (20 μg/ml) and chloramphenicol (25 μg/ml). After incubation at 37°C overnight, the cells were harvested, washed free of chloramphenicol, and suspended in fresh medium. The cultures were harvested at the late-log-phase of growth with yields of about 4 g. The cells were then

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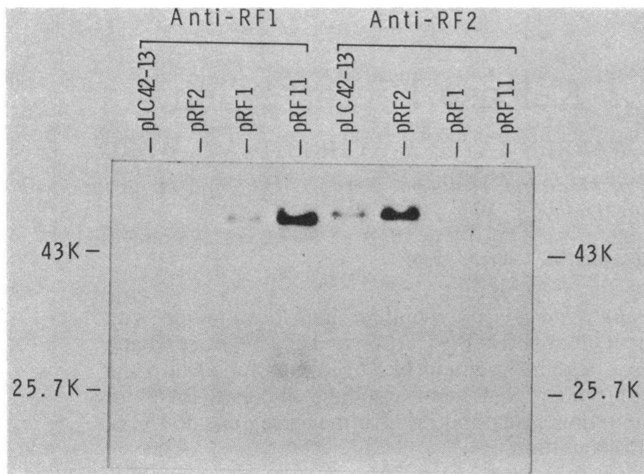

FIG. 1. Immune precipitation specificity for RF1 and RF2. Plasmids pLC42-13, pRF2, pRF1, and pRF11 are described in the text. An estimated 80,000 cpm of total trichloroacetic acid ^{35}S was precipitated with RF antisera and Staph A before sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The recombinant plasmids were amplified in the presence of chloramphenicol before in vivo radiolabeling and immune precipitation.

ground with alumina (8 g) in 8 ml of buffer (3), and a supernatant fraction was isolated after extract centrifugation at 30,000 × *g* for 1 h. After removal of ribosomes by centrifuging at 100,000 × *g* for 3 h, 0 to 80% ammonium sulphate precipitate was prepared from the S100. This fraction was resuspended in a minimum volume, dialyzed against 50 mM Tris-chloride (ph 8.0)–50 mM KCl–3 mM dithiothreitol, and chromatographed on a column (100 by 0.8 cm) of Sephadex G-100 in the same buffer (3). Samples were analyzed for RF1- and RF2-dependent in vitro termination activity, RF1- and RF2-dependent binding of UA[^{3}H]G or UG[^{3}H]A, and for overproduction of RF protein by immunodiffusion against anti-RF1 or anti-RF2 antiserum or by radial immunodiffusion with ani-RF2 antiserum together with a known amount of highly purified RF2.

Fractions were analyzed for factor activity in an assay of 50 μl containing 3 to 4 pmol of f[^{3}H]Met-tRNA AUG ribosome substrate (4,000 cpm/pmol), 0.02 to 0.08 absorbance units (at 260 nm) of UAG or UGA, and various amounts of the extracts as appropriate in a buffer of 50 mM Tris-chloride (pH 7.4)–75 mM NH_4Cl–30 mM $MgCl_2$. Reaction mixtures were incubated for 30 min at 20°C and analyzed for formyl[^{3}H]methionine extracted into ethyl acetate at pH 1 (2).

Fractions were analyzed for RF-dependent binding of termination codon in an assay of 50 μl containing two absorbance units (at 280 nm) of 70S ribosomes, 400 pmol of UA[^{3}H]G or UG[^{3}H]A (100 cpm/pmol) in a buffer of 20 mM Tris-chloride (pH 7.2)–100 mM NH_4Cl–20 mM $MgCl_2$, containing 10% (vol/vol) ethanol. Reaction mixtures were incubated for 20 min at 4°C, and the formation of an RF · [^{3}H]UGA or UAG · ribosome complex was analyzed on glass fibre filters (11).

The identification of plasmids which carry RF2 genes was made from the *E. coli* ColE1 recombinant bank of Clark and Carbon (4). Each clone was grown overnight in the presence of chloramphenicol for plasmid amplification, and chloramphenicol was removed before in vivo incorporation of [^{35}S]methionine into protein. We employed an immunoprecipitation test for RF2 with sheep anti-RF2 and Staph A protein. The identification of two RF2-containing plasmids was made after screening 1,000 colonies. The two colonies recovered from adjacent microtiter wells had similar protein profiles and identical restriction endonuclease maps and are considered duplicates. The RF2-containing plasmid is designated pLC42-13 according to the accepted designation of the Clarke and Carbon *E. coli* plasmid bank. An RF1-containing plasmid isolated by Weiss et al. and reported in a companion paper (17) was compared in Fig. 1 by immunoprecipitation to our RF2-containing plasmid. The pRF1 and pLC42-13 refer to the original plasmid isolates. The pRF11 and pRF2 plasmids are recombinants containing the RF inserts in the plasmid pUC9. Antisera to RF1 and RF2 (Fig. 1) provided specific immunoprecipitant tests for the respective RFs (48,000 molecular weight). The data suggests higher plasmid-directed RF synthesis in the pUC9 plasmid vector than with the original ColE1 (4) and YRp7 (11) plasmids.

The restriction map of the colicin plasmid pRF2 and plasmid pUC9 is shown in Fig. 2. A *Pvu*II fragment (3.4 kb) containing a portion of the ColE1 plasmid (1.7 kb) and ca. 0.5 of the original *E. coli* insert (1.7 kb) was found to encode and express RF2 when inserted into the pUC9 *Hin*cII site. A *Hin*dIII-to-*Eco*RI fragment from pRF2, when inserted into the corresponding pUC9 restriction sites, was found to produce an immunoprecipitable RF2 polypeptide fragment of 42,000 molecular weight (data not shown). We predict on

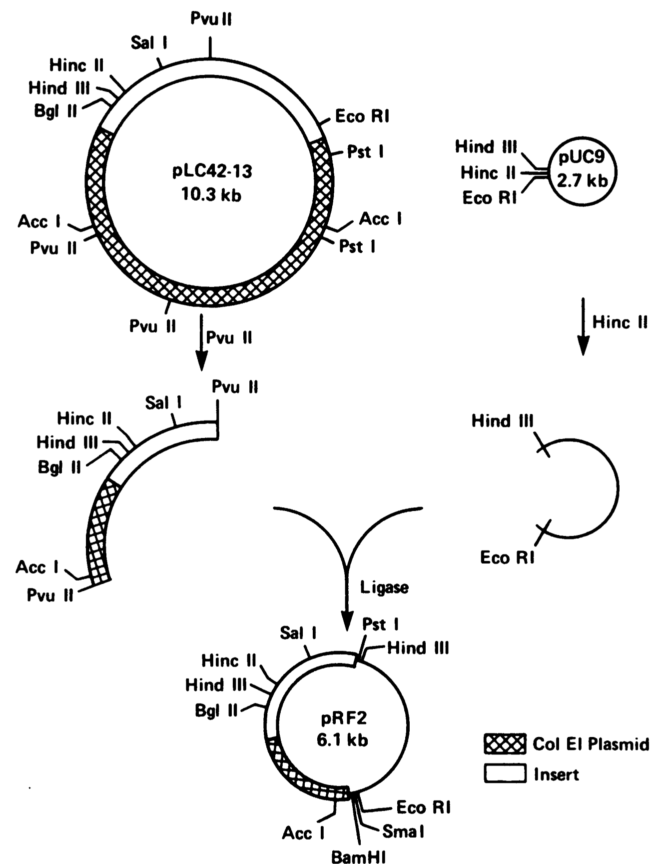

FIG. 2. Construction of pRF2. The *Pvu*II restriction endonuclease fragment (3.4 kb) of pLC42-13 was purified from low-melting-point agarose before ligation with T4 ligase into the *Hin*cII site of pUC9. The newly formed recombinant is designated pRF2.

the basis of this data that the *Hin*dIII endonuclease has interupted the RF2 coding sequence in its carboxyl terminal sequence.

The functional level of RF2 has been examined by two independent methods. By using partially purified extacts prepared from a strain with pRF2 and control cells (Fig. 3), a fivefold enhanced level of RF2(UGA)- but not RF1(UAG)-dependent release of formyl[^{3}H]methionine was detected by in vitro peptide chain termination. The formation of the RF · ^{3}H-termination codon · ribosome complex was determined with extracts from pRF2 and control plasmid (Fig. 4). A 10- to 15-fold increase in levels of binding to [^{3}H]UGA but not [^{3}H]UAG was observed with pRF2 extracts. Studies reported in the companion paper (17) indicate that the pRF2 plasmid reduced in vivo tRNA UGA suppression efficiency. Thus, on the basis of immunological, in vitro biochemical, and in vivo suppression studies, plasmid pRF2 encodes and expresses the bacterial peptide chain terminating factor RF2.

The cloning of the RF2 gene reported here and the RF1 gene reported in the companion paper by Weiss et al. (17) offer a new experimental approach to the study of RFs. Microbial cells with 20-fold elevated levels of RF1 and RF2 protein improve the availability of these proteins for functional and structural studies. The plasmid-encoded RF2 appears identical to the chromosomal gene product as measured in vitro peptide chain termination, codon-directed ribosomal binding, and peptidyl-tRNA hydrolysis (data not shown). In vitro studies with plasmid-encoded RF1 are in progress.

The anti codon, ribosomal binding sites (13), and peptidyl transferase (14) domains of RF molecules now appear amenable to study with RF molecules rendered mutant by in vitro mutagenesis methods (12). Earlier studies that established the codon specificity and ribosomal binding properties of *E. coli* RF1 and RF2 genes suggested that they recognize nucleic acid sequences with fidelity. It is noteworthy that a single mammalian RF binds to ribosomes in response to all three peptide chain termination codons. We predict that structural studies of *E. coli* RF1 and RF2 genes will enhance our knowledge of these quite interesting protein codon specificities. The amino acid sequences of RF1 and RF2 can be predicted from the DNA sequence of these RF-encoded plasmid recombinants. Our preliminary DNA analysis indicates that RF1 and RF2 genes have considerable homology.

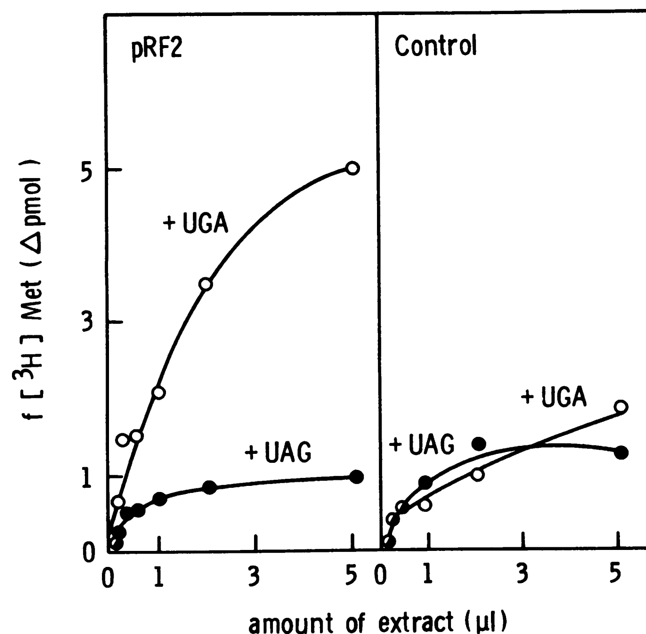

FIG. 3. Codon and RF-dependent formyl[^{3}H]methionine from f[^{3}H]met-tRNA · AUG · ribosome complexes. *E. coli* containing pRF2 and ColE1 control plasmids was used to prepare extracts as described in the text. RF1-dependent in vitro termination activity was assessed with UAG (●) and RF2-dependent activity was assessed with UGA (○) as described. A background of 0.26 pmol was subtracted from all values.

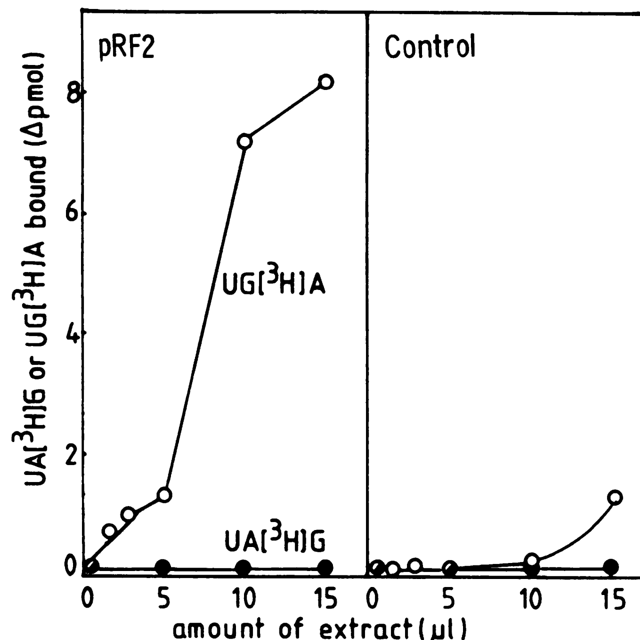

FIG. 4. Formation of RF · ^{3}H-termination codon · ribosome complexes. Fractions from the Sephadex G-100 column fractionation of extracts prepared from *E. coli* containing pRF2 and ColE1 control plasmids were examined for their ability to bind UA[^{3}H-]G · ribosome (●) and UG[^{3}H]A · ribosome (○) complexes as described earlier (11). Background values (1.2 to 1.3 pmol) were subtracted from each value.

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