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Localization of the Ribosome-Releasing Factor Gene in the *Escherichia coli* Chromosome

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The ribosome-releasing factor (RRF) gene was localized at a position between 2 and 6 min on the *Escherichia coli* chromosome by measuring the gene-dosage-dependent production of RRF in various *E. coli* F′ merozygotes. This position was confirmed and refined by using a nucleotide probe corresponding to a 16-amino-acid sequence in RRF. It was found that the RRF gene was contained in pLC 6-32 of the Clark-Carbon Gene Bank. Restriction enzyme mapping of *E. coli* genomic DNA with the above probe led us to conclude that the RRF gene is situated in the 4-min region, somewhere downstream (clockwise) of the elongation factor Ts gene, *tsf*. A pLC 6-32-derived DNA fragment which carries the RRF gene was found to contain a partial sequence of *tsf*. The exact location of the translational initiation site of the RRF gene was determined to be 1.1 kilobases downstream from the translational termination site of *tsf*. The RRF gene is designated *frr*.

Ribosome releasing factor (RRF) (15–17, 30, 34–37) functions to release ribosomes from mRNA at the termination step of translation after the nascent peptide has been released in response to termination factor (6). The amino acid sequence of purified RRF was used to clone its gene and effectively express it in *Escherichia coli* (S. Ichikawa and A. Kaji, submitted for publication). From the viewpoint of genetics, RRF is probably the least elucidated *E. coli* protein factor for protein synthesis. For example, the gene locations of initiation factor 1 (*infA*, 20 min [38]), initiation factor 2 (*infB*, 68.5 min [33]), initiation factor 3 (*infC*, 38 min [40]), elongation factor Tu, (EFTu) (*tufA*, 74 min, and *tufB*, 90 min [3, 20]), elongation factor Ts (EFTs) (*tsf*, 4 min [41]), elongation factor G (EFG) (*fusA*, 73 min [3, 23]), release factor 1 (*prfA*, 26.7 min [25]), and release factor 2 (*prfB*, 62.3 min [25]) have been established.

In the work described in this communication, a novel method of gene dose protein quantitation was used to localize the RRF gene to within 2 to 6 min on the *E. coli* chromosome. Southern analysis with a nucleotide probe and sequencing of a DNA fragment containing the RRF gene revealed the exact location of the RRF gene to be at 4 min, i.e., 1.1 kilobases (kb) clockwise from the translational termination site of *tsf*.

MATERIALS AND METHODS

Bacteria, plasmids, and enzyme preparation. The bacterial strains and plasmids used in this paper are listed in Table 1. The Clarke-Carbon Gene Bank and the F′ kit were a generous gift from B. Bachmann, Yale University. We use the *E. coli* Genetic Stock Center strain numbers in this paper. EFTu and RRF were purified to homogeneity as described previously (2, 36).

Preparation of *E. coli* cells labeled with [^{35}S]methionine. Cells were grown at 37°C overnight in TSBY medium (3 g of trypticase soy broth [BBL Microbiology Systems] and 5 ml of 10% yeast extract in 100 ml). They were then diluted to about 1.5×10^8 cells per ml and further grown in the same medium to 3×10^8 cells per ml. At this point, 1 ml of the culture was mixed with 10 μl (about 80 μCi) of [^{35}S]methionine. The cells were then grown at 37°C for an additional 1 h. A 10-μl portion was taken and placed on a Whatman 3MM filter disk for counting of the radioactivity. The rest of the culture was centrifuged in a Sorvall SS-34 rotor (12,000 rpm for 7 min at 4°C). The cell pellet was suspended in 1 ml of buffer S, containing 10 mM Tris hydrochloride (pH 7.4), 10 mM magnesium acetate, 50 mM ammonium chloride, and 6 mM 2-mercaptoethanol, and centrifuged under the same conditions. The cell pellet was stored at −80°C until the two-dimensional gel electrophoresis was carried out. A typical incorporation of [^{35}S]methionine was 50,000 cpm/10 μl of culture, as hot trichloroacetic acid-insoluble material. After the cells were harvested, there was no gain in nutritional requirement, indicating that there was no loss of F′.

Determination of radioactivity of polypeptides in the two-dimensional gel electrophoresis and activity assay of RRF. Two-dimensional gel electrophoresis was performed as described previously (29). Radioactive proteins separated by two-dimensional gel electrophoresis were quantitated as follows. The protein spot was cut out of the gel after fluorography. It was then soaked in 200 μl of 50 mM NH_4HCO_3 containing 200 μg of pronase (Calbiochem-Behring). Digestion was performed for 12 h at 37°C. The extract-containing pronase digests were placed on Whatman 3 MM filter disks. The remaining gel pieces were subjected to the same pronase digestion two more times. These two extracts were placed on the same filter disk as before. The filter disk was dried and placed in 5 ml of scintillation fluid for counting of the radioactivity. In some cases, stained spots were excised directly from the gel and counted without autoradiography. These gel pieces were dehydrated in methanol and subjected to pronase digestion. The RRF activity assay was carried out as described previously.

Oligonucleotide probe. On the basis of the partial amino acid sequence of RRF (submitted for publication), a 47-mer oligonucleotide probe (5′-AT-GTC-AGA-ACC-CGC-ACC-GTT-CGG-GTT-CAG-ACC-CAG-GTC-AGA-CGC-CAT-3′) was designed by the best-guess method (21) from the most frequent *E. coli* codon usage (11). The oligonucleotide was labeled at the 5′ end with ^{32}P as described previously (27).

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TABLE 1. *E. coli* strains

Strain[a]	Genotype	Source or reference
KL110 (CGSC 4271)	F⁻ *leuB hisG recA thyA argG metB lacY gal xyl mtl malA rpsL strA tonA tsx* λ^r λ^- *supE*	K. B. Low
KL181 (CGSC 4275)	F⁻ *thi pyrD his trp recA mtl xyl malA galK strA rpsL* λ^r λ^-	K. B. Low
AB2463 (CGSC 2463)	F⁻ *thr leuB thi argE his proA recA lacY galK mtl xyl ara strA tsx* λ^- *supE*	18
JC 1553 (CGSC 4205)	F⁻ *argG metB hisG leuB recA mtl xyl malA gal lacY strA rpsL tonA tsx* λ^r λ^- *supE*	7
KL 251 (CGSC 4227)	F⁻ *thi metE trpE purE proC leuB recA mtl xyl ara lacZ azi strA rpsL tonA tsx* λ^- *supE*	K. B. Low
KLF22/KL110 (CGSC 4257)	KL110 (F′ *argG*⁺-*metC*⁺-*cysC*⁺)	K. B. Low
MAF1/JC1553 (CGSC 4289)	JC1553 (F′ *mtl*⁺-*argG*⁺)	26
KLF11/JC1553 (CGSC 4258)	JC1553 (F′ *pyrE*⁺-*malB*⁺)	26
KLF12/JC1553 (CGSC 4260)	JC1553 (F′ *metB*⁺-*pyrB*⁺-*uxuA*⁺)	26
KL723 (KLF4/AB2463) (CGSC 4251)	AB2463 (F′ *thr*⁺-*argF*⁺)	26
KLF1/AB2463 (CGSC 4250)	AB2463 (F′ *thr*⁺-*leu*⁺)	K. B. Low
E5014 (CGSC 4288)	F′ *proA*⁺*B*⁺-*lac*⁺/Δ(*proB-lac*) *thi relA*(?) *mal rpsE spcA* λ^- *supE*	E. Singer
CA100[b]	AB2463 (F′ *proA*⁺*B*⁺-*lac*⁺)	This work
ORF4/KL251 (CGSC 4282)	KL251 (F′ *lac*⁺-*lip*⁺)	5
KLF26/KL181 (CGSC 4253)	KL181 (F′ *rac*⁺-*nadA*⁺)	26
pLC6-32/JA200	*dapC*⁺ *garB*⁺ *mrcB*⁺ *fhuA*⁺*C*⁺*D*⁺*B*⁺ *popC*⁺ *optA*⁺ *dapD*⁺ *glnD*⁺ *cds*⁺ *rpsB*⁺ *tsf*⁺/F⁺ *thr leuB trpE recA ara lacY galK galT xyl mtl* λ^- *supE*	8, 33

[a] Numbers in parentheses indicate the *E. coli* Genetic Stock Center number.
[b] F′ *pro-lac* was transferred from E5014 to AB2463 by using *proA* and *lacY* markers.

***E. coli* DNA preparation.** *E. coli* W3110 was grown overnight at 37°C in 200 ml of L broth, centrifuged, and suspended in 20 ml of TE (10 mM Tris hydrochloride [pH 8.0], 1 mM disodium EDTA) containing 2 mg of sodium dodecyl sulfate (SDS). The mixture was shaken gently for 20 h at room temperature. DNA was extracted with 20 ml of phenol-chloroform-isoamyl alcohol (25:24:1) (27). The resulting DNA solution (20 ml) was subjected to ethanol precipitation in the absence of added salt. The precipitate was suspended in 15 ml of 2× SSC (1× SSC is 0.15 M NaCl plus 15 mM sodium citrate [pH 7.0]). RNase was added to a final concentration of 50 μg/ml, and the reaction mixture was incubated for 30 min at 37°C. Proteinase K was then added to 50 μg/ml, and the reaction mixture was further incubated for 3 h at 37°C. The mixture was extracted with 15 ml of phenol-chloroform-isoamyl alcohol (25:24:1), 15 ml of chloroform-isoamyl alcohol (24:1), and 15 ml of water-saturated ether and was then precipitated with 2 volumes of ethanol in the absence of added salt. DNA was dissolved in 10 ml of STE (0.1 M NaCl, 10 mM Tris hydrochloride [pH 7.6], 1 mM EDTA). Because DNase activity was still present in this preparation, proteinase K treatment was repeated in the following way. The DNA solution (5 ml) was mixed with proteinase K (100 μg) and incubated at 42°C for 2 h. The mixture was subjected to two extractions with phenol-chloroform-isoamyl alcohol followed by one extraction with chloroform-isoamyl alcohol. The DNA was precipitated with ethanol and redissolved in 5 ml of TE. The final concentration was 334 μg of DNA per ml.

Southern blot analysis of restriction enzyme digests of *E. coli* W3110 chromosomal DNA. *E. coli* chromosomal DNA (10 μg), prepared as described above, was precipitated with ethanol. For digestion with 100 U of *Bam*HI, *Pst*I, *Eco*RI, *Pst*I-*Hin*dIII, or *Eco*RI-*Bgl*II, 20-μl portions of buffers recommended by Bethesda Research Laboratories, Inc., were added to the DNA precipitate, and the mixture was incubated with restriction enzyme at 37°C for 10 h in a total volume of 200 μl. For digestion with *Hin*dIII-*Eco*RI or *Hin*dIII-*Bgl*II, 100 U of *Hin*dIII was mixed with 20 μl of buffer (Bethesda Research Laboratories, Inc.). This mixture and 10 μg of DNA were incubated together for 10 h at 37°C in the same reaction volume as above. The sodium chloride concentration was then raised from 50 to 100 mM. The second restriction enzyme (100 U) was added, and the mixture was incubated for 10 h at 37°C. For *Pst*I-*Eco*RI digestion, *Pst*I digestion was carried out first, followed by *Eco*RI digestion. The reactions were stopped by the addition of 10 μl of 500 mM EDTA (pH 8.0) followed by precipitation with 2 volumes of ethanol in the presence of 0.3 M sodium acetate (pH 5.0). The precipitates were redissolved in 20 μl of TE, and 10-μl aliquots were subjected to 1% agarose (with 1 μg of ethidium bromide per ml) gel electrophoresis in TAE (Tris-acetate-EDTA) at 5 v/cm for 4 h at room temperature. The gel was pretreated as described previously (27) and transblotted onto a Zeta Probe Membrane (Bio-Rad Laboratories). The membrane was incubated in 10× Denhardt solution–6× SSPE (1× SSPE is 0.18 M NaCl, 10 mM sodium phosphate [pH 7.7], plus 1 mM EDTA)–1% SDS–20 μg of yeast tRNA per ml–50 μg of calf thymus DNA per ml for 1 h at 42°C. The blot was hybridized to the 47-mer probe end labeled for 30 h at 42°C with 10^8 cpm (10 pmol) of ^{32}P in 2 ml of 6× SSPE–1% SDS. The membrane was then washed three times at 25°C for 20 min each with 6× SSPE–1% SDS and then once at 60°C for 20 min with 1× SSPE–1% SDS.

Nucleotide sequence determination. The DNA sequence was determined by M13 dideoxy sequencing (39) with adenine 5′-[α-^{35}S]thiotriphosphate (>5,000 Ci/mmol; Amersham Corp.) and the Sequenase sequence kit (U.S. Biochemical Corp.). M13 clones for sequencing were made by subcloning restriction enzyme digests into M13mp18 or M13mp19. Restriction enzymes and T4 DNA ligase were obtained from Bethesda Research Laboratories, Inc., and used as specified by the manufacturer.

RESULTS

Localization of the RRF gene to the 2- to 6-min region of the *E. coli* chromosome. To determine the general location of the RRF gene in the *E. coli* genome, merozygotes which carry various portions of the *E. coli* genome in diploid were tested for their content of RRF. It was reasoned that a diploid RRF gene would produce twice as much RRF as the haploid RRF

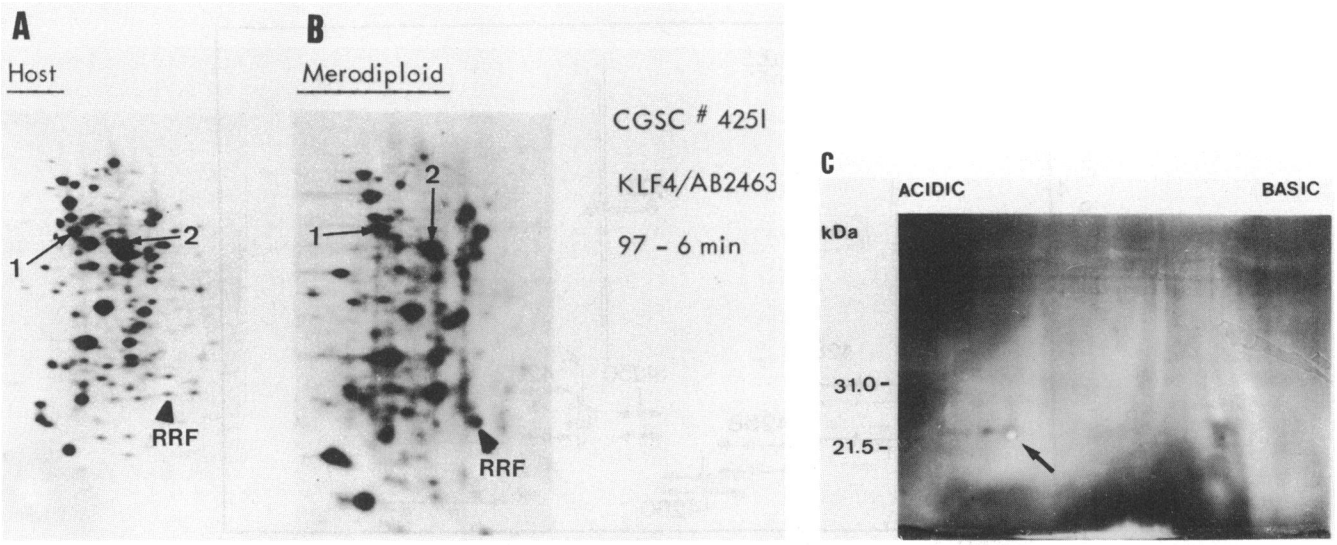

FIG. 1. Two-dimensional gel electrophoresis of total proteins from *E. coli* merodiploid strains. (A and B) The total cellular proteins were solubilized, and a portion containing 1.3×10^6 cpm of [^{35}S]methionine-labeled proteins was subjected to two-dimensional gel electrophoresis followed by fluorography. The exposure time for fluorography was adjusted (one to several days) so that the intensities of the spots look nearly the same for this pair. The arrowhead indicates the location of RRF. Spots 1 and 2 were used for references. Only the relevant portion of the fluorograms is shown. (C) Purified RRF (230 ng) was subjected to two-dimensional gel electrophoresis and stained with the silver staining kit from Bio-Rad. The arrow indicates RRF, which is stained negatively (white spot) by the silver staining method.

gene. Total cell proteins labeled with [^{35}S]methionine were prepared from each pair of an F′-carrying strain and its F$^-$ parent. Since each F′ factor is carried by a genetically different host, it was necessary to compare the quantity of RRF within each pair. It was also important to compare cells which were grown under identical conditions. Thus, all the strains carrying different nutritional requirement markers were grown in the same rich medium. F′ factors were fairly stable, and practically all the cells were found to maintain their F′ factor even after growth without selection. Logarithmic-phase cultures were labeled with [^{35}S]methionine for 1 h and harvested. Total cell proteins were then subjected to two-dimensional gel electrophoresis (29). Figures 1 A and B show autoradiograms of two-dimensional gel electrophoresis of *E. coli* proteins with and without F′ factor. Since the intensity of a spot depends upon the amount of sample applied, the efficiency of fluorography, and the exposure time, a comparison between two strains must be made by comparing the intensity of the RRF spot with that of other proteins on the same gel. Spots 1 and 2 were arbitrarily chosen for this purpose. It can be seen that the pairs CGSC 4251 (F′) and AB2463 (F$^-$) showed that the F′ factor had a significant effect on the intensity of RRF. In this manner, the radioactivities of the RRF spots and the reference proteins were determined, and the ratio of RRF and each reference spot was calculated to give the relative amount of RRF. The relative amount was then used to compare an F′-carrying strain with its F$^-$ counterpart to give the normalized ratio. The normalized ratio is defined as (RRF/spot 1 or 2 in the F′ strain)/(RRF/spot 1 or 2 in the F$^-$ strain). Only the F′ factor in CGSC 4251 stimulates the production of RRF (Fig. 2), in agreement with the visual observation of the autoradiogram (Fig. 1A and B). It is noted that the F′ factor in CGSC 4250, which carries part of the F′ factor in CGSC 4251 (Fig. 2), does not increase the amount of RRF. Thus, we conclude that the gene for RRF is located at 2 to 6 min on the *E. coli* genetic map.

Since the method described here depends upon only a two- to fourfold difference in the amount of the proteins, we attempted to map the EFTu gene by the same method, to examine the validity of our approach. This factor is genetically well characterized, and its purification is relatively easy. Although there are two genes for EFTu in *E. coli* (*tufA* at 74 min and *tufB* at 90 min) (3, 10, 20), more than 70% of this protein is produced by the *tufA* gene (31). This unbalanced expression makes this method applicable to EFTu; otherwise, the gene dosage effect would increase the quantity of EFTu by only 50%. EFTu was purified to electrophoretic homogeneity (2). It was then subjected to two-dimensional gel electrophoresis together with total cell proteins of CGSC 4251. It was found that only one spot was stained more heavily than the corresponding spot of CGSC 4251 extract without added EFTu. We thus determined that this spot was EFTu (data not shown). This position corresponded well to the two-dimensional gel location of EFTu described in the literature (32). Two-dimensional gel analysis of [^{35}S]methionine-labeled proteins was carried out as had been done with RRF. Figure 3 summarizes the results. As expected, GCSC 4289, which has an F′ factor carrying the *tufA* gene, was found to contain more EFTu than the others did. This supports the validity of our mapping method. CGSC 4258, which has an F′ factor covering the *tufB* gene (90 min), does not have an elevated level of EFTu (Fig. 3). This observation is consistent with the above notion that the *tufB* gene is not as active as the *tufA* gene. Hayward and Fyfe (13) also reported that the F′ factor, KLF10, covering the *tufB* gene increases the amount of EFTu by only 10%. The reason why CGSC 4288 has a higher normalized ratio than others remains obscure.

In support of the conclusion drawn from the two-dimensional gel electrophoresis, two additional biochemical results were provided. First, the RRF activity of strain 4251 was approximately 60% higher than that of other strains (data not shown). Second, we measured the RRF protein quantity by an additional method. Using radial immunodiffusion (14), we quantitated the content of RRF in the cell extract of CGSC 4251 and AB2463. Comparison of the precipitation areas indicated that CGSC 4251 contains 60% more RRF than

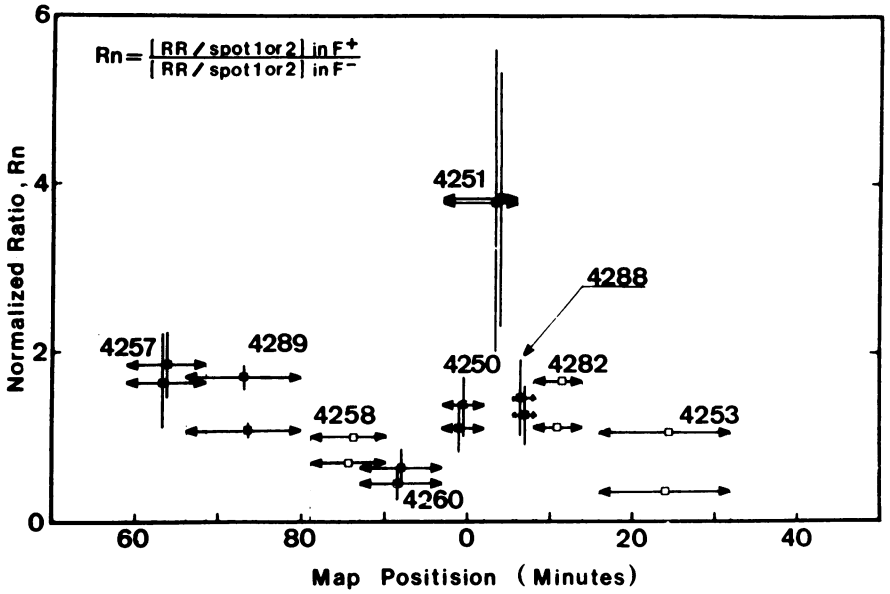

FIG. 2. Quantity of RRF in various merodiploid strains relative to the corresponding haploid strains. Horizontal arrows indicate the chromosomal segments carried by the F′ factors. The distance of each horizontal arrow from the *x*-axis represents the averaged value of the normalized ratio for two to four experiments. The standard deviations of the data are indicated by vertical bars. There are two values of the normalized ratio, derived from the two reference spots 1 and 2. The open squares on some of the arrows indicate that the data were obtained from a single experiment. The data for strain 4288 were obtained by using an equivalent strain, CA100 (Table 1).

strain AB2463 does. This is in close agreement with the results of the activity assay. The differences between the RRF content measured by two-dimensional gel electrophoresis analysis and the RRF content measured by other methods remains unexplained. However, all three methods of detection point out the fact that the level of RRF is significantly higher in CGSC 4251.

Exact localization of RRF. On the basis of the 2- to 6-min position of the RRF gene as indicated above, we found the RRF gene in pLC 6-32 (4-min region) of the Clarke-Carbon Gene Bank and concluded that a 2.2-kb *Eco*RI fragment derived from this plasmid contained the RRF gene (Ichikawa and Kaji, submitted for publication). During these studies, it came to our attention that Bendiak and Friesen had reported

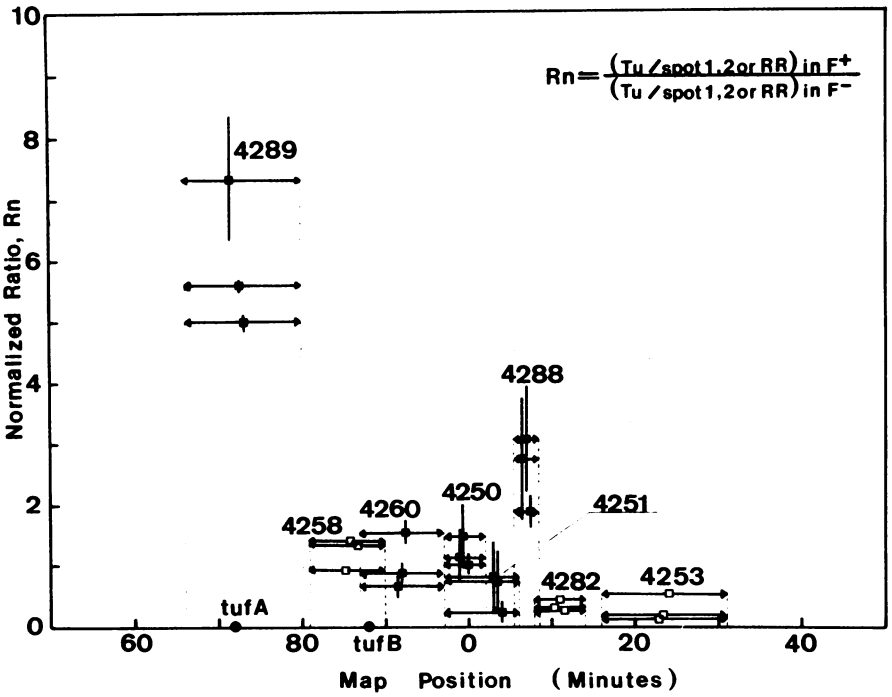

FIG. 3. Quantity of EFTu in various merodiploid strains. The same analysis as described for Fig. 2 was performed for EFTu. Three other spots (RRF, spot 1, and spot 2) were used as references. Thus, each chromosomal segment has three horizontal arrows. The distance or each arrow from the *x*-axis represents the normalized ratio derived from each of the three reference spots. RRF is referred to as RR in this figure.

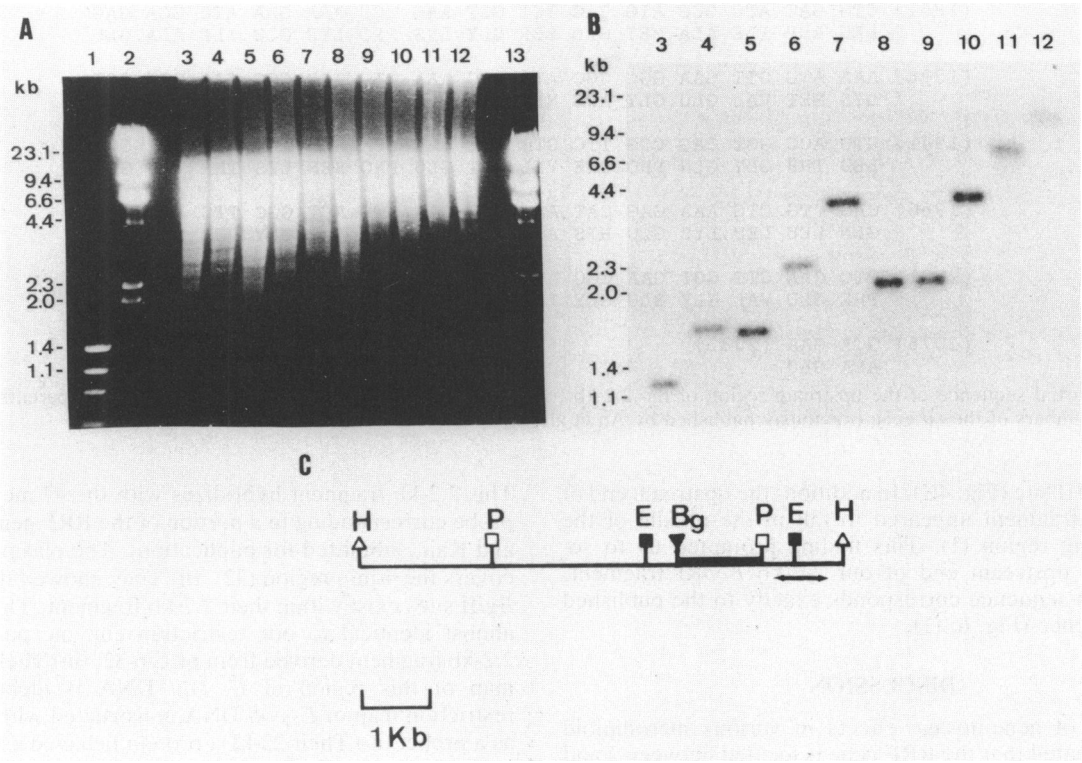

FIG. 4. Southern blot analysis of restriction enzyme digests of *E. coli* W3110 chromosomal DNA. (A) Ethidium bromide staining of the various restriction enzyme digests of *E. coli* DNA. *E. coli* chromosomal DNA was prepared as described in Materials and Methods. Lanes: 1, φX174-*Hae*III marker; 2, λ-*Hin*dIII marker; 3, *Bgl*II-*Pst*I; 4, *Eco*RI-*Bgl*II; 5, *Eco*RI-*Pst*I; 6, *Bgl*II-*Hin*dIII; 7, *Pst*I-*Hin*dIII; 8, *Eco*RI-*Hin*dIII; 9, *Eco*RI; 10, *Pst*I; 11, *Hin*dIII; 12, *Bam*HI; 13, λ-*Hin*dIII marker. (B) Southern blot analysis of panel A. The gel was pretreated as described previously (27), transblotted onto a Zeta Probe Membrane, and hybridized with 10^8 cpm (10 pmol, 2ml) of the 47-mer probe 5′ end labeled with ^{32}P as described in Materials and Methods. The blot was exposed to X-ray film for 17 h with an intensifying screen at −70°C. (C) Restriction fragment map of *E. coli* genomic DNA containing the RRF gene deduced from panel B. Abbreviations: H, *Hin*dIII (△); P, *Pst*I (□); E, *Eco*RI (■); Bg, *Bgl*II (▼). Symbols are identical to those used by Bendiak and Friesen (4). ▬, the smallest fragment which hybridizes with the 47-mer probe; ⟷, coding region of EFTs (1).

cloning the 4-min region of the *E. coli* genome (4). Since this region contains at least two genes coding for proteins related to translation (*tsf* and *rpsB* [41]), we examined their paper more closely and found that the behavior of their 22-kilodalton (kDa) protein in two-dimensional gel electrophoresis was similar to that of RRF (Fig. 1C). Since the exact experimental conditions used by Bendiak and Friesen are not available, direct comparison of the behavior of RRF and the 22-kDa protein is not possible. However, purified RRF moved like the 22-kDa protein under similar experimental conditions (Fig. 1C).

Since Bendiak and Friesen (4) used the restriction enzymes *Pst*I, *Eco*RI *Hin*dIII, *Bgl*II, and *Bam*HI for their analysis of the 4-min region, we digested *E. coli* chromosomal DNA with these restriction enzymes and probed the digests with the 47-mer nucleotide probe (see Materials and Methods) (Fig. 4). From these results, a restriction enzyme map was constructed. This map was identical to the map presented by Bendiak and Friesen (4). The restriction map derived from genomic Southern analysis (Fig. 4) was further supported by restriction enzyme analysis of the 2.2-kb *Eco*RI fragment from pLC 6-32 of the Clarke-Carbon Gene Bank (Fig. 5). Restriction enzyme digestion of this 2.2-kb fragment yielded 0.5- and 1.7-kb (*Bgl*II; Fig. 5, lane 1) and 1.65- and 0.55-kb (*Pst*I; lane 2) fragments. Further examination of previous work revealed that the *Eco*RI 2.2-kb fragment we cloned partially overlaps the *Pst*I 3.3-kb fragment of An et al. (1), who found another *Pst*I site 1.9 kb upstream

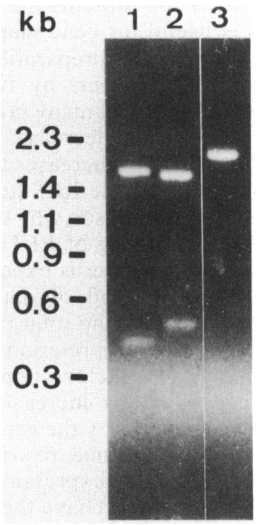

FIG. 5. Digestion of the *Eco*RI 2.2-kb fragment by various restriction enzymes. The *Eco*RI 2.2-kb fragment (800 μg) was digested with 10 U of *Pst*I (lane 2), or *Bgl*II (lane 1) at 37°C for 3 h in a reaction mixture of 10 μl. One-fourth of each restriction enzyme digest was subjected to 1.5% agarose gel electrophoresis. Lane 3 contains 100 ng of the undigested 2.2-kb fragment.

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(1851) CTG GAT ATC GCG ATG CAG TCT GGT AAG CCG AAA GAA ATC GCA GAG
       LEU ASP ILE ALA MET GLN SER GLY LYS PRO LYS GLU ILE ALA GLU

(1896) AAA ATG GTT GAA GGC CGC ATG AAG AAA TTC ACC GGC GAA GTT TCT
       LYS MET VAL GLU GLY ARG MET LYS LYS PHE THR GLY GLU VAL SER

(1941) CTG ACC GGT CAG CCG TTC GTT ATG GAA CCA AGC AAA ACT GTT GGT
       LEU THR GLY GLN PRO PHE VAL MET GLU PRO SER LYS THR VAL GLY

(1986) CAG CTG CTG AAA GAG CAT AAC GCT GAA GTG ACT GGC TTC ATC CGC
       GLN LEU LEU LYS GLU HIS ASN ALA GLU VAL THR GLY PHE ILE ARG

(2031) TTC GAA GTG GGT GAA GGC ATC GAG AAA GTT GAG ACT GAC TTT GCA
       PHE GLU VAL GLY GLU GLY ILE GLU LYS VAL GLU THR ASP PHE ALA

(2076) GCA GAA (2081)
       ALA GLU
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FIG. 6. Partial sequence of the upstream region of the 2.2-kb fragment isolated from pLC 6-32. The numbers in parentheses indicate nucleotide numbers of the *tsf* gene previously published by An et al. (1).

of the *Hin*dIII site (Fig. 4C). In addition, the upstream end of our *Eco*RI fragment appeared to fall in the middle of the EFTs coding region (1). This finding prompted us to sequence the upstream end of our 2.2-kb *Eco*RI fragment. Indeed, this sequence corresponds exactly to the published EFTs sequence (Fig. 6) (1).

DISCUSSION

Analysis of gene-dosage effects in various merodiploid strains indicated that the RRF gene is located between 2 and 6 min on the *E. coli* genetic map (3). This mapping method depends upon the direct quantitation of RRF after separation of total cell proteins by two-dimensional gel electrophoresis (29). Although the method itself has been used by several investigators, the quantitation has been performed solely by immunological means (12, 28). In mapping the tRNA genes, Ikemura and Ozeki (19) used two-dimensional gel electrophoresis for separation and subsequent quantitation of each tRNA species. Two-dimensional gel electrophoresis has allowed us to use the same approach for mapping the RRF gene. It should be emphasized that our method can be applied to many other proteins in *E. coli*, since the migration positions of more than 200 proteins (32) are already identified. This method is useful for gene mapping because purification of protein for antibody preparation is difficult. When we compared F′ and F⁻ strains by two-dimensional gel electrophoresis, we noticed that many proteins, for instance, spots 1 and 2, appeared to decrease in intensity in the presence of the F′ factor. This increases the normalized ratio to more than 1, even though the RRF gene is not on the F′ factor. This seems to be the reason why the normalized ratio even reached 7 during mapping of EFTu (Fig. 3), although only a two- to threefold increase is expected from the gene dosage effect. Another method of data processing (i.e., normalization with respect to the total radioactivity of proteins) would also cause misinterpretation, but in the opposite direction; in this case, F′ factors appear to reduce the relative gene dosage effect by increasing the amounts of many other proteins coded for by the genes on the F′ factor.

Friesen and co-workers cloned restriction fragments of λ*polC-9* (4, 9) and studied gene expression in these clones to show that the *rpsB* and *tsf* genes have the same transcription unit. During their work, they showed that, adjacent to this transcription unit, a gene coding for a 22-kDa protein exists. We believe that this 22-kDa protein is identical to RRF for the following reasons. (i) Their restriction map of this region contains the 2.2-kb *Eco*RI fragment which was found in *Eco*RI digests of pLC 6-32 of the Clarke-Carbon Gene Bank. This 2.2-kb fragment hybridizes with the 47-mer nucleotide probe corresponding to a portion of the RRF gene (Ichikawa and Kaji, submitted for publication). The plasmid pLC 6-32 covers the 4-min region (32). (ii) They showed that *Pst*I and *Bgl*II sites exist within their 2.2-kb fragment. The results are almost identical to our restriction enzyme pattern of the 2.2-kb fragment derived from pLC 6-32. (iii) Their restriction map of this region of *E. coli* DNA is identical to the restriction map of *E. coli* DNA constructed with the 47-mer as a probe. (iv) Their 22-kDa protein behaved as a relatively basic protein in two-dimensional gel electrophoresis, similar to the behavior of RRF (Fig. 1C). The molecular mass of RRF is 23.5 kDa (36), which is very close to the 22-kDa protein reported by them. (v) The DNA sequence of the upstream end of the 2.2-kb fragment corresponds to the EFTs sequence (Fig. 6). These considerations lead us to conclude that a portion of the RRF gene corresponding to the 47-mer probe is situated within 1.4 kb downstream from the translational termination site of *tsf*. The RRF gene consists of 558 base pairs and ends approximately 0.3 kb from the downstream end of the 2.2-kb fragment (Ichikawa and Kaji, submitted for publication). The EFTs sequence found in the upstream end of the 2.2-kb fragment from pLC 6-32 shows that the translation termination site of *tsf* is located approximately 0.3 kb downstream from the upstream end of the 2.2-kb fragment. Therefore, we calculated that the translation initiation site of the RRF gene is located approximately 1.1 kb downstream from the translation termination site of *tsf*. The next genes downstream are *cds* (18a) and *firA* (3, 24). This conclusion was also supported by the restriction enzyme map of this region reported by Kohara et al. (22). According to this restriction enzyme map of the entire *E. coli* genome, the RRF gene is situated 205 kb from the 0-min position.

The genetic loci of EFG, EFTu, EFTs, initiation factor 1, initiation factor 2, initiation factor 3, release factor 1, and release factor 2 are already known. The present studies have provided the genetic locus of one more soluble factor in the *E. coli* protein synthesis machinery. We have named the RRF gene *frr* (factor for ribosome release) because the term *rrf* already is used to indicate the 5S ribosomal RNA gene (3).

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