Temperature-Sensitive Lethal Mutant of Era, a G Protein in *Escherichia coli*

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The *era* gene of *Escherichia coli* encodes a GTP-binding protein which has similarities to elongation factor Tu and the *Saccharomyces cerevisiae* RAS protein. To investigate its function, mutations affecting *era* were isolated. A mini-Tn10 insertion, which truncated 22 amino acids from the COOH end of Era, did not affect cell growth. By using this mini-Tn10 insert as a coselectable marker, a temperature-sensitive lethal *era* mutant was isolated by localized mutagenesis using P1 phage transduction. A single-base G to A change was found at position 23, causing a tyrosine residue to be substituted for the cysteine residue at position 8 (*era*-770), in addition to the COOH-terminal mini-Tn10 disruption. Both alterations were necessary for the temperature-sensitive phenotype. Purified Era-770 mutant protein exhibited reduced binding to GTP compared with that of the wild-type Era protein.

Guanine nucleotides play a crucial role in the regulation of gene expression and signal transduction by binding to special proteins called G proteins. One group of these G proteins includes the oncogene RAS protein family which act as transducers of signals across the cellular membrane (3, 9). Other GTP-binding proteins serve as initiation or elongation factors during translation (11, 14). All share a common polypeptide domain (G domain) for binding GTP and GDP.

In *Escherichia coli*, five GTP-binding proteins have been described. Three of them are involved in translational control; translation initiation factor IF2 binds fMet-tRNA to form the initiation complex, translational elongation factor EF-Tu binds aminoacyl-tRNA to the A site of the 30S ribosome, and elongation factor EF-G mediates the translocation step during translation (11, 14). The other two GTP-binding proteins were identified as proteins that had a consensus G-domain motif, as determined from their DNA sequences. These two genes, named *lepA* and *era*, map at 55.5 min of the *E. coli* chromosome (1, 17) and flank a 2.3-kilobase (kb) segment that contains two other genes, *lep* and *rnc* (16, 17, 21, 31). The *lep* gene encodes the leader peptidase and is in an operon with *lepA* (17). Both LepA and Lep are membrane-bound proteins (18, 31). The *era* gene is located in another operon with *rnc* and *recO* (25a). *rnc* encodes RNase III, an enzyme that processes specifically double-stranded RNA structures (1, 25a; J. Bardwell and P. Régner, personal communication), and *recO* is part of the *recF* recombination system (14a). The *era* gene encodes a 35,232-dalton protein with a G domain similar to those of other GTP-binding proteins (1; A. Barber, J. Maizel, and D. L. Court, unpublished data). The Era protein binds GTP and GDP and hydrolyzes GTP to GDP (19; S.-M. Chen, personal communication).

The cellular function of Era is unknown. It has been suggested that Era is essential to *E. coli* because of the failure to isolate a null mutation in *era* (19, 25a). To prove that Era is essential for *E. coli* growth and to facilitate

structural and functional analyses of Era, we aimed to isolate a conditionally lethal mutation in the *era* gene. In this report we describe the isolation of a temperature-sensitive (Ts) lethal defect in the *era* gene and the properties of the altered Era protein.

MATERIALS AND METHODS

Strains, plasmids, and media. The *E. coli* K-12 strains, λ phages, and the plasmids used in this study are listed in Table 1. The plasmid pIT7 was a derivative of the pUC19 plasmid which contained a 3.8-kb *EcoRI* fragment encoding *rnc*-70 *era*-770 *era*-13. pIT7 was isolated from an *EcoRI* DNA library of the Ts mutant (IT17) chromosome by screening it with the wild-type *era* probe. L broth was as described by Miller (20).

Construction of λ phages carrying different *era* mutations. A *NruI* site in *recO* of pACS3 (Fig. 1) (25a) was converted to an *EcoRI* site by *NruI* digestion and religation with an *EcoRI* linker. Then, the 2.5-kb *EcoRI*-*EcoRI* (*NruI*) fragment carrying *rnc*⁺, *era*⁺, and part of *recO* was isolated and inserted at a unique *EcoRI* site of pPP'106 to form pIT34 (*era*⁺) (Fig. 2). The plasmid pPP'106 was a kind gift from M. Imai (Kyoto University, Kyoto, Japan), which was constructed to develop a convenient system to transfer cloned fragments from plasmids to λ Charon 25 phage by in vivo recombination, as illustrated in Fig. 2. The plasmid pIT32 (*era*-770 *era*-13) was constructed by replacing the 1.4-kb *ClaI*-*NruI* (*era*⁺) portion of pIT34 with the 1.7-kb *ClaI*-*NruI* (*era*-770 *era*-13) fragment from the pIT7 plasmid, which was isolated from a Ts mutant DNA library as discussed above (Fig. 2). The *NruI* site used in pIT7 is located in the mini-Tn10 (Δ Tn10) transposon. The 0.44-kb *ClaI*-*EcoRV* internal fragment (containing *era*-770) of pIT32 was replaced with that from the wild-type DNA, thus generating pIT39 (*era*-13). The 0.14-kb internal *ClaI*-*BamHI* fragment of pIT34 (*era*⁺) was replaced by the same fragment carrying the *era*-770 mutation from pIT7, giving rise to the plasmid pIT31 (*era*-770). These pPP'106 derivatives were transformed into the cells (CSH22) that were lysogenic for λ Charon 25 phage. Note that the *rnc era* insert

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TABLE 1. Bacterial strains, plasmids, and phages used in this study

Strain	Relevant genotype or phenotype ^a	Reference or source
<i>E. coli</i> K-12		
HT30	<i>era-13::ΔTn10 his relA1 ilv</i>	25a
KH5402	<i>supF</i> (Ts6)	22
IT17	KH5402 <i>era-770 era-13::ΔTn10 rnc-70</i>	This study
W3110		Laboratory stock
DC569	<i>glyA::Tn5 his ilv relA1 pglΔ8 rpsL</i>	25a
IT32	W3110 <i>era-770 era-13::ΔTn10 rnc-70</i>	This study
CSH22	$\Delta(lac\ pro)$	20
KK583	$\Delta(rnc-era)130::kan$ lysogenic for λ KKF2 (<i>rnc</i> ⁺ <i>era</i> ⁺ <i>recO::cat</i>)	K. Kawakami
IT136	W3110 $\Delta(rnc-era)130::kan$ (λ IT31)	This study
IT137	W3110 $\Delta(rnc-era)130::kan$ (λ IT32)	This study
IT139	W3110 $\Delta(rnc-era)130::kan$ (λ IT34)	This study
IT144	W3110 $\Delta(rnc-era)130::kan$ (λ IT39)	This study
TAP106	$[\Delta(int-cIII)BAM\ N::kan\ \lambda cI857\ \Delta(cro-bioA)]$	T. Patterson
Plasmids		
pTD101	<i>Amp</i> ^r <i>lepA</i> ⁺ <i>lep</i> ⁺ <i>rnc</i> ⁺	5
pIT3	<i>Kan</i> ^r <i>lep</i> ⁺	13
pIT7	<i>Amp</i> ^r <i>rnc-70 era-770 era-13</i>	This study
pACS1	<i>Amp</i> ^r <i>Tet</i> ^r <i>rnc</i> ⁺ <i>era</i> ⁺	25a
pACS3	<i>Amp</i> ^r <i>rnc</i> ⁺ <i>era</i> ⁺	25a
pACS21	<i>Amp</i> ^r <i>rnc</i> ⁺	25a
pIT12	<i>Amp</i> ^r <i>rnc</i> ⁺ <i>era</i> (<i>Bam</i> HI filled in with 4 base pairs)	This study
pIT13	<i>Amp</i> ^r <i>rnc</i> (<i>Bst</i> XI- Δ 4 bp) <i>era</i> ⁺	This study
pPP'106	<i>Amp</i> ^r <i>Eco</i> RI site cassette for in vivo transfer to λ Charon 25	M. Imai
pIT31	<i>Amp</i> ^r pPP'106 <i>rnc</i> ⁺ <i>era-770</i>	This study
pIT32	<i>Amp</i> ^r pPP'106 <i>rnc</i> ⁺ <i>era-770 era-13</i>	This study
pIT34	<i>Amp</i> ^r pPP'106 <i>rnc</i> ⁺ <i>era</i> ⁺	This study
pIT39	<i>Amp</i> ^r pPP'106 <i>rnc</i> ⁺ <i>era-13</i>	This study
pJL6	<i>Amp</i> ^r λ <i>p</i> _L promoter vector	25a
pCE31	<i>Amp</i> ^r pJL6 <i>era</i> ⁺	S.-M. Chen
pIT15	<i>Amp</i> ^r pJL6 <i>era-770</i>	This study
pIT19	<i>Amp</i> ^r pJL6 <i>era-770 era-13</i>	This study
pIT45	<i>Amp</i> ^r pJL6 <i>era-13</i>	This study
λ Phage		
Charon 25	<i>imm</i> ²¹ <i>attP</i> ⁺ <i>int</i> ⁺ <i>lac-5</i>	29
λ IT31	<i>Amp</i> ^r <i>rnc</i> ⁺ <i>era-770</i>	This study
λ IT32	<i>Amp</i> ^r <i>rnc</i> ⁺ <i>era-770 era-13</i>	This study
λ IT34	<i>Amp</i> ^r <i>rnc</i> ⁺ <i>era</i> ⁺	This study
λ IT39	<i>Amp</i> ^r <i>rnc</i> ⁺ <i>era-13</i>	This study

^a Genetic symbols are those described by Bachmann (2). All plasmids except pIT7 (a pUC19 derivative) were derived from pBR322. The λ phages were Charon 25 derivatives.

in the *Eco*RI site and the *bla* gene segment are both located in the λ DNA fragment of pPP'106 which is homologous to the λ Charon 25 genome. Homologous recombination between plasmid and phage DNAs can occur at each side of these inserts to transfer the inserts and *bla* to the phage. The lysogenic cells carrying the plasmids were irradiated with UV light to induce prophages and to stimulate recombination. The lysates were used to infect W3110, and ampicillin-resistant (*Amp*^r) colonies were selected. All these *Amp*^r colonies were lysogens with the immunity of λ Charon 25 (*imm*²¹). These prophages were recombinant phages since

they were Lac⁻, indicating that the *lac-5* segment of λ Charon 25 was replaced by an exogenous *rnc-era* segment.

Construction of overproducing plasmids. Plasmid pCE31 was constructed by inserting the *era*-containing *Bst*XI-to-*Nru*I fragment from pACS1 into pJL6 (λ *p*_L promoter expression vector; 14b, 25a) and then removing all of the *rnc* sequence to a *Cl*aI site in the third codon of *era* and replacing it with a synthetic oligonucleotide containing a strong ribosomal binding site (S.-M. Chen, unpublished data). A 120-base-pair (bp) *Cl*aI-*Bam*HI piece of *era* in pCE31 was replaced by that from pIT7, thus giving rise to pIT15 (*era-770*). The pIT19 (*era-770 era-13*) plasmid was constructed by replacing the *Cl*aI-*Nru*I fragment of *era* in pCE31 with that from pIT7. The *Cl*aI-*Eco*RV fragment of pIT19 was replaced by that of the wild type, generating plasmid pIT45 (*era-13*).

Purification of Era. Cells (TAP106) carrying a defective prophage genome encoding the Ts repressor gene *cI857* were transformed with the Era-overproducing plasmids pCE31 (*era*⁺), pIT15 (*era-770*), pIT19 (*era-770 era-13*), and pIT45 (*era-13*) at 32°C. Plasmid-bearing cells were grown at 32°C in 20 ml of broth containing 50 μ g of ampicillin per ml and shifted to 42°C at the mid-log phase for 2 h. Portions of 1 ml of culture were centrifuged with an Eppendorf microcentrifuge (15,000 rpm, 10 min) at 4°C. The cell precipitates were suspended in 35 μ l of Tris hydrochloride (50 mM, pH 8.0)–sucrose (25%) buffer and lysed with 35 μ l of lysozyme (1 mg/ml) at 0°C for 5 min. Then, 5 μ l of MgCl₂ (0.2 M), 2.5 μ l of DNase I (10 U/ μ l), and 20 μ l of 5 $\times$ lysis buffer (5% Nonidet P-40, 2.5% sodium deoxycholate, 0.5% NaCl, 50 mM Tris hydrochloride [pH 7.2], and 5 mM EDTA) were added and incubated at 0°C for 30 min. Era protein was separated from other cellular components of the crude lysate as precipitates by centrifugation with the microcentrifuge (15,000 rpm, 10 min). These Era aggregates were suspended and washed in 1 $\times$ lysis buffer, and the pellet was collected and washed for three additional cycles. The washed pellet was suspended in ground buffer (50 mM Tris hydrochloride [pH 8.0], 1 mM EDTA, and 1 mM dithiothreitol) and washed in three more cycles with ground buffer. The final Era aggregates that were suspended in ground buffer were contaminated with only minor proteins, if any, as judged by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (see Fig. 6B).

GTP-binding assay. Era (5 μ l, 1.75 μ g) was mixed with 5 μ l of nucleotide-binding solution containing 100 mM Tris hydrochloride (pH 7.5), 10 mM MgCl₂, 0.2% Nonidet P-40, 0.2 mg of bovine serum albumin per ml, 20 μ M nonradioactive nucleotide, and 0.2 μ Ci of α -³²P-labeled nucleotides per μ l (400 Ci/mmol; Amersham Corp., Arlington Heights, Ill.). After incubation at 32°C for 30 min, 8 μ l was spotted onto a nitrocellulose filter and washed gently three times with a 10-ml solution containing 50 mM Tris hydrochloride (pH 7.5), 5 mM MgCl₂, and 1 mM dithiothreitol at 20°C. The filter was dried and exposed to X-ray films (Eastman Kodak Co., Rochester, N.Y.) for autoradiography, and the radioactivity was counted.

Other procedures. The DNA sequence was determined by the dideoxy chain-termination method (24) with a 15-nucleotide *lacZ* primer and [α -³²P]dCTP from subclones in M13. DNA hybridization analysis was conducted as described previously (15) except that GeneScreen Plus membrane (E. I. du Pont de Nemours & Co., Inc., Wilmington, Del.) was used, and DNA was transferred electrophoretically.

FIG. 1. Chromosomal structure of the *rnc-era* region and the DNA segments that were cloned on plasmids. Bold bars indicate bacterial DNA, and arrowheads indicate the location and orientation of the genes. The triangle represents a $\Delta Tn10$ insertion. Chimeric plasmids indicated do not carry the $\Delta Tn10$ insert. $\Delta(rnc-era)130::kan$ represents a deletion of a 2.2-kb *PstI*-*NruI* fragment which was replaced by a *kan* gene. pIT12 and pIT13 are *BamHI*- and *BstXI*-blunted derivatives of pACS3, respectively. The scale is in kilobases.

RESULTS

***Tn10* insertion mutation in *era*.** A collection of *E. coli* insertion mutants with mutations within the *rnc-era* region was constructed by random insertion of a $\Delta Tn10$ transposon (25a, 28). One mutant harbored the TD1-13:: $\Delta Tn10$ insertion near *era* and was used in the present study. This mutant was

FIG. 2. Strategy for the construction and structure of plasmids and λ phages carrying the mutant *era* gene. Experimental details are described in the text. *EcoRI*-*NruI* fragments encoding *rnc* and *era* were isolated from pACS3 (*era*⁺) and pIT7 (*era*-770 *era*-13:: $\Delta Tn10$), digested with the appropriate restriction enzymes, and ligated to generate chimeric DNA fragments. The *NruI* ends of these fragments were converted to an *EcoRI* end by linker ligation and inserted in the *EcoRI* site of pPPP'106. pPPP'106 carries only one *EcoRI* site at the junction of the *lac*-5 segment and the *bla* gene; two other *EcoRI* sites were deleted by blunt-end ligation when pPPP'106 was constructed. Bold bars indicate the λ genome, and boxes indicate bacterial DNAs. pBR322 DNAs are shown by coils. In the diagram at the bottom, the open boxes represent DNA from wild-type sources, the closed box represents the $\Delta Tn10$ insertion segment in *era*, and stippled regions are derived from the Ts mutant *era* DNA. Abbreviations: Kp, *KpnI*; RI, *EcoRI*; Ba, *BamHI*; Cl, *ClaI*; RV, *EcoRV*; Nr, *NruI*.

UV sensitive relative to the wild type. Mutants in *recO* were UV sensitive. Thus, $\Delta Tn10$ may affect *recO*.

DNA from the mutant was examined by Southern hybridization analysis by using a 1.8-kb *BglI*-*BglI* fragment encoding the entire *era* gene and its flanking sequences (Fig. 1). Two bands from the mutant hybridized to the probe after chromosomal digestion with *HindIII*, *BglI*, or *EcoRI*, although no *HindIII* or *EcoRI* sites were present within the 1.8-kb probe sequence (Fig. 3). Each hybridized band from the mutant was larger than the probe. These results suggest that the $\Delta Tn10$ insert, which contains *HindIII*, *BglI*, and *EcoRI* sites in its sequence, is located in the *BglI* fragment close to or within *era*.

One of the two unique *EcoRI* fragments (3.8 kb) from the mutant was cloned in the pUC19 plasmid by screening it with the *era* probe. This 3.8-kb fragment contained *era* and part of the $\Delta Tn10$ element; it was sequenced from the $\Delta Tn10$ end by the dideoxy chain-termination method. The sequence data

FIG. 3. Southern hybridization analysis of the $\Delta Tn10$ insert. The chromosomal DNA isolated from HT30 (*era*-13:: $\Delta Tn10$) was digested with *HindIII*, *BglI* or *EcoRI*, and subjected to electrophoresis in 1% agarose gels. DNAs were transferred to Gene-ScreenPlus Membrane and hybridized to the 1.8-kb *BglI*-*BglI* fragment labeled with [³²P]dCTP by nick translation. This probe DNA was isolated from pACS1. The *EcoRI* lane contains two close but separate bands.

FIG. 4. DNA sequence of the regions that contain the *era*- Δ Tn10 junction and the *era*-770 substitution mutation.

revealed that the Δ Tn10 inserted between positions 882 and 883 of the coding region of *era* (Fig. 4 and 5). The TD1-13 mutation in the *era* gene was redesignated *era*-13:: Δ Tn10. The insertion removed 22 codons from *era* and fused the *era* coding region to 25 nonspecific codons of the transposon to yield a predicted fusion protein of 36,033 daltons, which was slightly larger than the protein from the wild type, as confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis (see below). This *era*-13:: Δ Tn10 insertion mutant grew well under all culture conditions except under exposure to UV light, although *era* itself was essential to the growth of *E. coli*, as disclosed below. Sensitivity to UV light in the *era*-13:: Δ Tn10 insertion mutant was complemented by pACS1 but not by pACS3 (data not shown). Therefore, the UV sensitivity is not a consequence of the *era* defect but is probably due to the polarity of Tn10 on *recO* expression.

Isolation of a Ts lethal *era* mutant. The *era*-13:: Δ Tn10 insert was used as a tetracycline-resistant (Tet^r) selective

TABLE 2. Complementation test for Ts mutations

Class	No. of isolates	Growth of transformants at 42°C ^a						
		pACS1	pACS21	pACS3	pIT13	pIT12	pTD101	pIT3
I (<i>era</i>)	1	+	—	+	+	—	—	—
II (<i>lep</i>)	22	—	—	—	—	—	+	+
III	67	—	—	—	—	—	—	—

^a Plasmids were transformed into the 90 mutant cells; selection for kanamycin resistance for pIT3 or ampicillin resistance for the others was done at 32°C. These transformants were purified and scored for growth at 42°C after 24 h on L-agar plates containing the selective antibiotic.

marker in localized mutagenesis of the *lep-rnc-era* chromosomal region. P1 phage was first grown on strain HT30 (*era*-13:: Δ Tn10), and the phage lysate was mutagenized by treatment with hydroxylamine and was used to infect *E. coli* KH5402. Tet^r transductant colonies were selected at 32°C, replica plated, and incubated at 42°C to screen for Ts mutants. The rationale of selection was based on the close genetic linkage between Δ Tn10 and the target genes *lep*, *rnc*, and *era*, which enabled P1-mediated localized mutagenesis. A total of 90 Ts colonies that grew at 32°C but not at 42°C on either minimal or nutrient agar plates were detected from a set of 5×10^5 Tet^r colonies that were examined.

Plasmids containing different genes of the *lep-era* region were introduced into these Ts mutants for complementation tests (Fig. 1). Of 90 mutants, 22 grew at 42°C on transformation either with pTD101 or pIT3 plasmids (Table 2). The latter contained only the *lep* gene. These mutations therefore affected the leader peptidase (13). One other mutant, IT17 (Ts), grew at 42°C with plasmid pACS3, which contained only *rnc* and *era*. Plasmid pACS21, which encoded only *rnc*, did not allow IT17 to grow at 42°C. These results suggest that the IT17 mutant is affected in the *era* gene. An *rnc*-defective plasmid, pIT13, was constructed by deleting 4 bases at a unique *Bst*XI site in the coding region of *rnc* with T4 DNA polymerase (Fig. 1). This plasmid still complemented IT17, whereas an *era*-defective plasmid, pIT12, in which 4 bases were added at a unique *Bam*HI site in the proximal portion

FIG. 5. DNA coding sequence of the mutant *era* gene. Numbers noted at the amino acids or nucleotides are positions from the NH₂ terminus of *era*. *era*-770 is a G to A substitution at amino acid position 8, and *era*-13:: Δ Tn10 is an insertion between positions 882 and 883, which generated a fusion protein three amino acids larger than *Era*, as shown in the lower part of the figure. The entire region of the *era* gene from the mutant DNA was sequenced by the dideoxy chain-termination method, and no base alterations relative to the wild type were found, other than those indicated above. Three consensus regions composed of the G domain are noted by G-1, G-2, and G-3 (1). bp, Base pairs.

TABLE 3. Phage P1 transductional mapping of the *era* Ts mutation

Donor (markers)	Recipient (markers)	Selected marker	Distribution of unselected markers ^a		
			Class	No.	% Recombinants
IT17 (Tet ^r Ts)	W3110 (Tet ^s Ts ⁺)	Tet ^r	Ts	731	
			Ts ⁺	20	2.7 (Ts/Tet)
DC569 (Kan ^r Tet ^s <i>era</i> ⁺)	IT32 (Kan ^s Tet ^r Ts)	Kan ^r	Tet ^s Ts ⁺	145	
			Tet ^s Ts	0	0.0 (Ts/Tet)
			Tet ^r Ts ⁺	0	
			Tet ^r Ts	152	51.2 (Ts/Kan)

^a Recipient cells were infected with phage P1 *vir* grown on donor cells, incubated in L broth for 1 h, and plated onto L-agar plates containing 25 µg of tetracycline or kanamycin per ml. The antibiotic-resistant colonies were picked, purified, and scored for unselected markers.

of *era*, did not allow the mutant to grow at 42°C (Table 2). No lethal Ts *rnc* mutant was found in this isolation, consistent with the fact that *rnc* is not essential to *E. coli* (25a).

Mapping of mutations in the Ts mutant by P1 transduction and sequencing. A cross was conducted between the mutant (IT17; Tet^r Ts), which was used as a donor in P1 transduction, and a wild-type strain (W3110; Tet^s [tetracycline-sensitive] *era*⁺), which was used as a recipient. Tet^r transductants were selected at 32°C and scored for growth at 42°C. The Ts phenotype was 97.3% (731 of 751 transductants) linked with Tet^r (Table 3). Next, the Ts mutation was mapped in a three-point cross by using a *glyA::Tn5* strain (DC569; Kan^r [kanamycin-resistant] Tet^s *era*⁺) as the donor, and the mutant (IT32; Kan^s Tet^r Ts) was used as the recipient. Kan^r transductants were selected at 32°C and scored for the unselected markers of tetracycline and Ts growth. The results in Table 3 indicate that strains with the Ts and Tet^r phenotypes did not segregate from each other under these selection conditions; i.e., there were no Tet^r Ts⁺ or Tet^s Ts recombinants. This was in contrast to the previous cross, in which the Ts allele was separable from Tet^r in 2.7% of the recombinants.

The 3.8-kb *EcoRI* fragment from the IT17 chromosomal DNA was cloned in pUC19 by screening it with the *era* probe (see above). The mutant *era* DNA from pIT7 was sequenced by the dideoxy chain-termination method. A single-base substitution of A for G was found at position 23 of the coding region of *era* (Fig. 4 and 5). The wild-type DNA which was sequenced as a control in parallel with the mutant DNA contained a guanine residue at position 23. Therefore, the substitution was designated as the *era*-770 mutation. It caused a cysteine to tyrosine change at position 8 in the peptide sequence, close to the G-domain consensus sequence. The presence of the Δ Tn10 insert in the mutant *era* DNA was also confirmed. Although no other changes in the *era* gene were found relative to the wild-type gene, sequences further upstream of *era* revealed an additional single-base change in the *rnc* gene. However, this base substitution in *rnc* was not involved in the Ts phenotype, as determined by complementation or by reconstitution of the Ts mutant from individual mutations (see below).

Reconstitution of the *era* Ts mutant. The Ts mutant contained two alterations in the *era* gene, namely, a single-base substitution (*era*-770) and an insertion (*era*-13:: Δ Tn10). One interpretation for our inability to isolate Ts Tet^s recombinants in the three-factor cross was that the Δ Tn10 alteration participated in the Ts phenotype. To examine this, the *era*-770 mutation was separated from Δ Tn10 by *in vitro* manipulation (Fig. 2; see above) and reexamined for its effect on the host. The chimeric fragments thus reconstructed were cloned into plasmid pPP'106. This plasmid contained a segment of the λ genome and a unique *EcoRI*

site within this segment. It also contained the β -lactamase gene (*bla*) for Amp^r (Fig. 2). The hybrid plasmids pIT34 (*era*⁺), pIT39 (*era*-13), pIT31 (*era*-770), and pIT32 (*era*-770 *era*-13) were transformed into *E. coli* CSH22, which carried a λ Charon 25 prophage. By homologous recombination between the λ DNA insert in the pPP'106 derivatives and the λ Charon 25 phage, the cloned *era* fragments and the *bla* gene were transferred from the plasmid to the λ Charon 25 genome. These rare recombinant phages were readily selected as Amp^r transductants from phage lysates that were prepared by induction (see above and Fig. 2) (M. Imai, unpublished data). The transducing phages were purified from the Amp^r transduced cells and named λ IT34 (*era*⁺), λ IT39 (*era*-13), λ IT31 (*era*-770), and λ IT32 (*era*-770 *era*-13).

The λ Charon 25 derivatives described above carried a wild-type *imm*²¹ repressor, an intact attachment site (*attP*), and the *int* gene, which allowed the phage to lysogenize *E. coli* without a helper phage. These phages were directly lysogenized in the Ts mutant IT32 (*rnc*-70 *era*-770 *era*-13:: Δ Tn10) by infecting and selecting for Amp^r at 32°C. The results (Table 4) indicate that phage λ IT34, λ IT39, or λ IT31 is able to complement the Ts lethal growth of IT32 at 42°C, whereas the λ IT32 phage carrying the *era*-770-*era*-13 double-mutant gene did not complement this growth. These results suggest that both mutations are necessary for the Ts phenotype.

To firmly establish that only the double *era* mutant was Ts, growth of cells lysogenic for these transducing phages were examined under conditions in which the chromosomal copy of the *era* gene was entirely deleted. First, the chimeric phages were lysogenized in the wild-type strain W3110. Next, a chromosomal substitution mutation, Δ (*rnc-era*)130::kan, was transduced into these lysogens by phage P1 by

TABLE 4. Complementation activity of single or double *era* mutations

Relevant genotype of host chromosome	Growth of lysogens at 42°C			
	λ IT34 (<i>era</i> ⁺)	λ IT39 (<i>era</i> -13)	λ IT31 (<i>era</i> -770)	λ IT32 (<i>era</i> -13 <i>era</i> -770)
<i>era</i> ⁺ (W3110) ^a	+	+	+	+
<i>era</i> -770 <i>era</i> -13 (IT32) ^a	+	+	+	—
Δ (<i>rnc-era</i>)130::kan ^b	+	+	+	—

^a Cells were infected with the indicated phages at 32°C, and lysogens were selected as resistant to ampicillin. These Amp^r colonies were scored for immunity to λ *imm*²¹ phage and growth at 42°C.

^b W3110 cells lysogenic for the λ IT phages were infected with P1 *vir* grown on KK583 containing the chromosomal *era* deletion Δ (*rnc-era*)130::kan. Kan^r transductants were selected at 32°C, purified, and examined for the chromosomal structure of the *rnc-era* region by Southern hybridization analysis. Those in which the genomic *rnc-era* region was replaced with Δ (*rnc-era*)130::kan were scored for growth at 42°C in the presence of ampicillin.

FIG. 6. Overproduction and purification of the wild-type and mutant *era* proteins. Experimental procedures and conditions were as described in Materials and Methods. (A) TAP106 cells carrying Era-overproducing plasmids were grown at 32°C (lanes 1, 3, 5, and 7) and shifted to 42°C for 2 h (lanes 2, 4, 6, and 8). Total proteins were subjected to SDS-PAGE and stained with Coomassie brilliant blue. Lanes: 1 and 2, pCE31 (*era*⁺); 3 and 4, pIT15 (*era*-770); 5 and 6, pIT19 (*era*-770 *era*-13); 7 and 8, pIT45 (*era*-13). (B) Purified Era proteins. Lanes: 1, *era*⁺; 2, *era*-770; 3, *era*-770 *era*-13; 4, *era*-13. Note that the two *era*-13 products are slightly larger than their counterparts as judged by mobility.

selecting for Kan^r at 32°C. The mutation $\Delta(rnc-*era*)130$ deleted the 2.2-kb *Pst*I-*Nru*I fragment encoding *rnc*, *era*, and part of *recO*; this segment is substituted by a *kan* gene that confers resistant to kanamycin (K. Kawakami, unpublished data) (Fig. 1). The resulting lysogenic strains (Table 1) containing the substitution were confirmed by Southern hybridization analysis (data not shown). Of the four strains, only the IT137 lysogen (λ IT32; *era*-770 *era*-13) failed to grow at 42°C (Table 4). These results led us to conclude that both mutations, *era*-770 and the truncation caused by *era*-13, are necessary for the Ts lethal phenotype. These are the only alterations carried by phage λ IT32, which has part of the Δ Tn10 and the distal end of *era* deleted in its construct. This was consistent with the three-factor cross mapping data described above. No Tet^s Ts recombinants were found because the Tet^r insertion was part of the phenotype, and no Tet^r Ts⁺ recombinants were found because this recombinant resulted from a rare double crossover based on the order of markers used in the P1 transduction experiments (Table 3).

Reduced GTP-binding of the Era-770 protein. The Era protein has a consensus sequence for the GTP-binding domain in the NH₂-terminal region that is composed of three regions, numbered 1 through 3 (1) (Fig. 5). The *era*-770 substitution was located only 6 amino acid residues from region 1, suggesting that it has a possible effect on GTP binding. In view of this possibility, the *era*-770, *era*-13, and double-mutant proteins were purified and examined for GTP-binding capacity in comparison with that of the wild-type Era protein. The TAP106 strain, containing a Ts λ repressor (cI857) for the *p*_L promoter and either plasmid pCE31 (*era*⁺), pIT15 (*era*-770), pIT19 (*era*-770 *era*-13), or pIT45 (*era*-13), was grown at 32°C and shifted to 42°C to induce overproduction of Era via *p*_L transcription. After 2 h at 42°C, both the wild-type and the mutant Era proteins accumulated up to 70% of total proteins, as judged by sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis (Fig. 6A). The cells were lysed by sucrose-lysozyme

TABLE 5. Binding of Era protein with different nucleotides^a

α - ³² P-labeled nucleoside triphosphate	Radioactivity (cpm) bound to:		
	Bovine serum albumin	Era	Era-770
dGTP	3	23,731	2,098
CTP	0	832	119
UTP	0	14	23
ATP	9	1,605	260
GTP	0	44,596	1,421

^a Experimental procedures and conditions were as described in the text. α -³²P-labeled nucleotides (20 μ M) were mixed with the purified protein (50 pmol/10 μ l); the complexes that formed were trapped on the nitrocellulose filter and their radioactivities were measured.

treatment, and the Era protein was purified. The overproduced Era protein formed insoluble aggregates in the lysate; these aggregates were readily separated from other cellular components (see above). The final products were nearly homogeneous as judged by SDS-PAGE (Fig. 6B). The Era-13-derived proteins migrated more slowly in the gel, indicating their slightly greater sizes. The Era proteins were mixed with α -³²P-labeled nucleotides, and the complexes that formed at 32°C were examined by a nitrocellulose filter binding assay. The results (Table 5) (Chen et al., unpublished data) indicated that the wild-type Era protein, like other G proteins, binds to GTP, GDP, and dGTP but not to other nucleotides, including cyclic GMP. These results are essentially the same as those deduced with the wild-type Era protein purified in soluble form (Chen et al., unpublished data). Under these conditions, the mutant Era-770 protein binds GTP, GDP, and dGTP at 5% of the level of the wild-type Era protein, suggesting that the Era-770 protein is defective in G-domain activity. Dose-response experiments (Fig. 7) indicated that the *era*-13 mutant protein binds GTP as efficiently as does the wild-type protein, whereas the *era*-770 *era*-13 double-mutant protein binds less efficiently than Era770 at 32°C. Addition of Era-770 and the double mutant protein to the wild-type and the truncated protein solutions did not reduce the GTP binding, showing that no inhibitory contaminants participated in the activity of the reduced protein (data not shown). When GTP binding was assayed at 42°C, the mutant and even the wild-type Era proteins were less active than at 32°C, indicating that Era is naturally Ts in vitro (data not shown).

DISCUSSION

Successful isolation of a Ts lethal mutant of Era indicated that Era is essential to *E. coli*. The same conclusion was reached because of the failure to isolate a null mutation in *era* (19, 25a). Two structural changes of Era are involved in the Ts lethality. One is a single-amino-acid substitution at position 8 (*era*-770), and the other is a disruption in the COOH-terminal region (*era*-13:: Δ Tn10). Purified protein analysis revealed that the single-substitution mutant protein Era-770 is much less active than wild-type Era in GTP and GDP binding. However, strains with this single mutation appear to have no major disadvantage in cell growth. This fact suggests that the COOH-terminal disruption affects protein stability or protein activity by changing the conformation of the mutant protein; therefore, it is lethal in combination with *era*-770. The following observation is consistent with this assumption. λ IT32 phage carrying double *era* mutations did not restore the growth of the Ts strain (IT32; *era*-770 *era*-13:: Δ Tn10) or the strain containing the

FIG. 7. Filter-binding assay of the wild-type and mutant *era* proteins. Experimental procedures and conditions were as described in Materials and Methods and Table 5, except that the final Era aggregates suspended in ground buffer were sonicated by 0.1-s pulse up to 6 s and that a mixture of [α - 32 P]GTP and Era was passed through a nitrocellulose filter.

rnc-era deletion in the chromosome at 42°C. However, a multicopy plasmid, pIT32, containing these double mutations restored the growth of the Ts strain or the *rnc-era* deletion mutant at 42°C (data not shown). This result can be interpreted as indicating that high-level expression of the double-mutant protein from a multicopy plasmid can overcome the reduced stability or activity of the protein. Thus, while Era may retain adequate activity for cell growth with either mutation alone, the combination of the reduced G-protein-binding activity and a loss of stability adds up to a lethal combination at high temperatures. The naturally Ts activity of the protein observed in vitro may partly assist in the mutant Ts phenotype. Precise conclusions about the activities of the singly and doubly mutant proteins may await purification of Era in soluble forms which are synthesized at nonrestrictive temperatures.

The original mutant (IT17) sustained a base alteration in the *rnc* gene (*rnc-70*) other than those in *era*. A single *rnc-70* mutant has properties similar to those of the *rnc-105* strain, which has been well characterized as an RNase III mutant (unpublished data). The *rnc-70* mutation is not lethal or conditionally lethal and is not necessary to the Ts phenotype of the *era-770 era-13* mutant discussed here. However, the *rnc-70* mutant formed smaller colonies than the wild type did at any temperature tested. This phenotype of *rnc-70* for slow growth seemed to participate in reducing some leaky growth of the *era* Ts mutant at 42°C. Occurrence of a change in *rnc* in addition to that in *era* might be rare. This was consistent with the fact that we screened up to 5.0×10^5 Tet^r transductant colonies to find one Ts mutant of Era.

The Era protein was named because of the similarity of its proposed amino acid sequence in the NH₂-terminal portion to that of the *Saccharomyces cerevisiae* RAS protein (1). However, in the COOH-terminal portion, there are no amino acid sequence similarities between Era and *S. cerevisiae* RAS1 and RAS2, except that a cysteine residue that is conserved in all RAS proteins in the fourth position from the

COOH end of *S. cerevisiae* RAS1 and RAS2 is found in the third position of Era (1, 3). This cysteine residue of the RAS family has been found to be necessary for it to function, i.e., focal transformation of NIH 3T3 cells by the Harvey murine sarcoma virus p21 *ras* protein (30) or activation of adenylate cyclase by the yeast RAS2 protein (27). In either case, the cysteine residue is necessary for membrane localization and the binding of lipid to the protein (7, 26, 30). Results of the present study suggest that the analogous cysteine residue of Era is dispensable because the *era-13::ΔTn10* disruption removes several amino acids, including the cysteine residue, yet it does not lead to a major defect in cell growth by itself. Moreover, while the *S. cerevisiae* RAS protein participates in regulation of adenylate cyclase (27), cellular cyclic AMP levels in the Ts *era* mutant were not altered (data not shown). The results are consistent with extended computer analyses that reveal that Era does not have any significant similarity to non-*S. cerevisiae* Ras proteins (A. Barber, J. Maizel, and D. L. Court, unpublished data). Based on these observations, we do not consider Era to be a Ras or Ras-like protein, although it is a member of the GTP-binding protein superfamily (Chen et al., unpublished data).

Three facts show that the *era* gene is in an operon with *rnc*. First, the translation termination codon for *rnc* overlaps with the initiation codon of *era* (1). Second, cloning of a DNA fragment containing the two genes under control of the *lpp*^{P-5} promoter or the λ p_L promoter allows coexpression of both RNase III and Era (1; Chen et al., unpublished data). Third, an ~2-kb transcript encoding both *rnc* and *era* has been detected by Northern and S1 nuclease mapping experiments (J. Bardwell and P. Régnier, personal communication). Genetic association in an *E. coli* operon often indicates a relationship. RNase III is a classical RNA-processing enzyme that has been shown to affect the translation of several genes by altering the availability of the ribosome-binding site, the stability of the messages, or both (4, 6, 8, 10, 12, 23, 25). Its possible relationship to a G protein such as Era may reveal important regulatory circuits. The Ts lethal *era* mutant isolated in this study will facilitate advances in the study of the Era protein.

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