Era GTPase of *Escherichia coli*: binding to 16S rRNA and modulation of GTPase activity by RNA and carbohydrates

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Era, an essential GTPase, appears to play an important role in the regulation of the cell cycle and protein synthesis of bacteria and mycoplasmas. In this study, native Era, His-tagged Era (His-Era) and glutathione S-transferase (GST)-fusion Era (GST-Era) proteins from *Escherichia coli* were expressed and purified. It was shown that the GST-Era and His-Era proteins purified by 1-step affinity column chromatographic methods were associated with RNA and exhibited a higher GTPase activity. However, the native Era protein purified by a 3-step column chromatographic method had a much lower GTPase activity and was not associated with RNA which had been removed during purification. Purified GST-Era protein was shown to be present as a high- and a low-molecular-mass forms. The high-molecular-mass form of GST-Era was associated with RNA and exhibited a much higher GTPase activity. Removal of the RNA associated with GST-Era resulted in a significant reduction in the GTPase activity. The RNA associated with GST-Era was shown to be primarily 16S rRNA. A purified native Era protein preparation, when mixed with total cellular RNA, was found to bind to some of the RNA. The native Era protein isolated directly from the cells of a wild-type *E. coli* strain was also present as a high-molecular-mass form complexed with RNA and RNase treatment converted the high-molecular-mass form into a 32 kDa low-molecular-mass form, a monomer of Era. Furthermore, a C-terminally truncated Era protein, when expressed in *E. coli*, did not bind RNA. Finally, the GTPase activity of the Era protein free of RNA, but not the Era protein associated with the RNA, was stimulated by acetate and 3-phosphoglycerate. These carbohydrates, however, failed to activate the GTPase activity of the C-terminally truncated Era protein. Thus, the results of this study establish that the C-terminus of Era is essential for the RNA-binding activity and that the RNA and carbohydrates modulate the GTPase activity of Era possibly through a similar mechanism.

Keywords: Era, GTPase, RNA-binding activity, GTPase activation, carboxylic acids

INTRODUCTION

Era is an essential GTPase that binds GTP and GDP, and hydrolyses GTP to GDP (Ahnn *et al.*, 1986; Britton *et al.*, 1997, 1998; Chen *et al.*, 1990; Gollop & March, 1991b; Inada *et al.*, 1989; Kawabata *et al.*, 1997; March *et al.*, 1988; Sato *et al.*, 1998; Takiff *et al.*, 1989; Wu *et al.*, 1995; Zhao *et al.*, 1999). The *era* gene was first identified in *Escherichia coli* and thought to encode a

member of the Ras GTPase superfamily based on its sequence similarity to the GTP/GDP binding motifs of Ras (Ahnn *et al.*, 1986). Homologues of Era have now been identified in all bacterial genomes sequenced to date (Fleischmann *et al.*, 1995; Fraser *et al.*, 1995; Kawabata *et al.*, 1997; Yamashita *et al.*, 1993; Zhao *et al.*, 1999; Zuber *et al.*, 1990, 1997). Era appears to be highly conserved functionally, since *era* genes cloned from diverse bacterial species are able to complement *E. coli* mutants deficient in *era* expression (Pillutla *et al.*, 1995; Zhao *et al.*, 1999; Zuber *et al.*, 1990, 1997). Recently, homologues of Era have also been identified in

Abbreviation: GST, glutathione S-transferase.

human and plant cells (Britton *et al.*, 1998; Ingram *et al.*, 1998). The human homologue of Era is distinct from Ras (Britton *et al.*, 1998), suggesting that Era is not a member of the Ras family (Britton *et al.*, 1998).

Several lines of evidence suggest that Era is involved in cell cycle regulation and ribosome assembly and that the GTP-binding and hydrolysis activities of Era are essential for its biological function (Britton *et al.*, 1997, 1998; Gollop & March, 1991b; Lerner *et al.*, 1992, 1995; Nashimoto, 1993; Nashimoto *et al.*, 1985; Nashimoto & Uchida, 1985). Mutations affecting the cellular level or function of Era led to a severe alteration in ribosome assembly, a drastic change in cell morphology, a significant reduction in cell viability and an apparent suppression of temperature-sensitive mutations in a number of the genes that are involved in DNA replication and chromosome partitioning (Britton *et al.*, 1997, 1998; Gollop & March, 1991b; Nashimoto, 1993; Nashimoto *et al.*, 1985; Nashimoto & Uchida, 1985). Data also suggest that Era might be involved in the regulation of energy metabolism, since the decrease of the cellular amounts of Era altered the ability of *E. coli* to utilize carbohydrates in a minimal medium (Lerner & Inouye, 1991; Shimamoto & Inouye, 1996). Together, these results suggest that Era is a multifunctional protein that is involved in the regulation of the cell cycle, protein synthesis and possibly energy metabolism. The mechanisms by which Era regulates these cellular processes, however, remain to be determined.

Era consists of two distinct domains (Chen *et al.*, 1999; Zuber *et al.*, 1997). The N-terminal domain of the protein contains the three GTP/GDP-binding motifs that are shared by many different families of GTPases (March, 1992; Pillutla *et al.*, 1995; Zuber *et al.*, 1997). The C-terminal domain of Era, in contrast, is highly conserved only among Era proteins and thereby is unique to the Era family (March, 1992; Pillutla *et al.*, 1995; Zuber *et al.*, 1997). Thus, the C-terminal domain of Era, although its function is unknown, most likely plays a direct role in the regulation of the cell cycle and protein synthesis. Consistent with this view, the C-terminal domain of the *Streptococcus pneumoniae* Era protein appears to be essential for its biological function in the cell (Zhao *et al.*, 1999). Recently, we have shown that the *S. pneumoniae* Era protein is primarily associated with the *E. coli* 16S rRNA when expressed in *E. coli* (Meier *et al.*, 1999). The binding of 16S rRNA to Era appears to stimulate its GTPase activity (Meier *et al.*, 1999). The part of Era which is responsible for this RNA-binding activity and the mechanisms by which the RNA stimulates the Era GTPase activity, however, were unknown.

In this study, we purified and characterized the *E. coli* Era protein. The results show that, like the *S. pneumoniae* Era protein, the *E. coli* Era protein is also associated primarily with 16S rRNA. The Era-RNA complex exhibited a GTPase activity higher than that of the Era protein free of RNA. Era was also shown to bind RNA *in vitro*. In addition, this study shows that a C-terminally truncated Era protein could not bind 16S

rRNA when expressed *in vivo*. The GTPase activity of the Era protein free of RNA, but not the Era-RNA complex and the C-terminally truncated Era protein, was stimulated by acetate and 3-phosphoglycerate. Together, our studies demonstrate that the C terminus of the Era protein is essential for the RNA-binding activity and that the GTPase activity of Era appears to be modulated by the RNA and carbohydrates, possibly by a similar mechanism.

METHODS

Materials. Sodium acetate, glyceraldehyde 3-phosphate, 3-phosphoglycerate, 2-phosphoglycerate, glycerate, lactate and pyruvate were purchased from Sigma. The remaining materials used in this study were the same as those described previously (Meier *et al.*, 1999).

Bacterial strains and culture conditions. The following *E. coli* strains were used in this study: K-12, a wild-type strain (Zhao & Winkler, 1995), BL21(DE3) pLysS (Cm^r) (Stratagene), XL1-Blue (Stratagene), LY66 [XL1-Blue/pRBP-13 (GST-*era*⁺, Ap^r)] (this study), LY68 [BL21(DE3) pLysS/pRBP-11 (*era*⁺, Ap^r)] (this study), LY70 [BL21(DE3) pLysS/pRBP-12 (His-*era*⁺, Ap^r)] (this study), LY52 [XL1-Blue/pLY52 (GST-*Δera*⁺, Ap^r)] (Zhao *et al.*, 1999), LY41 [XL1-Blue/pLY41 (GST-*era*⁺, Ap^r)] (Zhao *et al.*, 1999) and LY160 [XL1-Blue/pGEX-2T (GST⁺, Ap^r)] (Pharmacia).

For expression of *era*, all *E. coli* strains were grown, induced and collected as described previously (Meier *et al.*, 1999). LY12 (a wild-type *E. coli* K-12 strain; Zhao & Winkler, 1995) was grown overnight in LB at 35 °C with vigorous shaking. The overnight culture (9 ml) was inoculated into 250 ml fresh LB medium. The culture was harvested at an OD₆₀₀ of 0.8–1.00 by centrifugation as described above.

Cloning of the *E. coli era* gene. The *E. coli era* gene was cloned directly by PCR amplification of chromosomal DNA (Zhao *et al.*, 1999) by using two primers based on the published sequence (Ahnn *et al.*, 1986; Britton *et al.*, 1997, 1998). The 5' PCR primer (5'-CCGGAATTCAGATCTCATATGAGCA-TCGATAAAAGTTAC-3') was designed to begin at the ATG start codon of *era* and contained added *EcoRI*, *BglII* and *NdeI* sites for cloning purposes. The 3' PCR primer (5'-CCGG-AATTCAGATCTTTAAAGATCGTCAACGTAACCGAG-3') was designed to end at the stop codon of *era* and contained added *EcoRI* and *BglII* sites after the stop codon. These primers were then used to amplify the *era* gene from *E. coli* as described previously (Zhao *et al.*, 1999) under the following conditions: denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s and polymerization at 72 °C for 30 s for 25 cycles. Five PCR reaction products were combined and a portion of the pooled PCR products was digested with *EcoRI*. The *EcoRI*-digested PCR fragment was cloned into pBCKS+ (Stratagene) previously digested with *EcoRI* and dephosphorylated with calf intestinal alkaline phosphatase (Gibco-BRL). *era* from several pBCKS+ clones was sequenced and a clone containing the consensus *era* gene sequence (pRBP-10) was used to construct the following expression plasmids. pRBP-10 was partially digested with *NdeI* and then *BglII*. The *NdeI*-*BglII* DNA fragment containing *era* was subcloned into pET-11a and pET-15b at the *NdeI* and *BamHI* sites (Novagen). The resulting constructs were designated pRBP-11 and pRBP-12, respectively. pRBP-10 was also digested with *BglII* and the *BglII* fragment of *era* was subcloned into pGEX-2T at the *BamHI* site. The resulting construct was designated pRBP-13.

Purification of native Era, His-Era, GST-Era, GST-ΔEra and GST proteins. The glutathione *S*-transferase (GST)-Era fusion protein (GST-Era) of *E. coli* was purified from cells of LY66 by glutathione-Sepharose affinity column chromatography (Pharmacia) as described previously (Zhao *et al.*, 1999). The purified GST-Era was dialysed in dialysis tubing with a molecular mass cut-off of 50 kDa (Sigma) against 4 l 20 mM Tris/HCl, pH 8.0, 5 mM MgCl₂ (buffer A) at 4 °C overnight. After dialysis, glycerol was added to the Era preparation at a final concentration of 16% (v/v) and the resulting preparation was stored in aliquots at -70 °C. Protein concentration was determined by using a Bradford Protein Assay Kit (Bio-Rad) with BSA as standard (Bradford, 1976).

The native Era (non-tagged) protein of *E. coli* was purified from cells of *E. coli* LY68 by using a 3-step column chromatographic method (Zhao *et al.*, 1999), dialysed by using dialysis tubing with a molecular mass cut-off of 25 kDa (Sigma) against 4 l buffer A overnight at 4 °C and stored in small aliquots at -70 °C as described above.

GST, and the GST-ΔEra and GST-Era proteins of *S. pneumoniae* were purified from LY160, LY52 and LY41, respectively, by using a glutathione-Sepharose affinity column as described for the GST-Era protein.

Histidine-tagged Era (His-Era) was purified from LY70 by using an affinity nickel column as described previously (Zhao *et al.*, 1999).

Determination of RNA association with the Era protein of *E. coli*. To examine the association of RNA with Era, an affinity-purified GST-Era preparation was subjected to anion-exchange column chromatography (Mono-Q column, Pharmacia) as described by Meier *et al.* (1999). Fractions (1 ml each) were collected and analysed by using SDS-PAGE (10% gel), agarose gel electrophoresis and spectrophotometric scanning (200–600 nm) on a Bio-Spec 1601 spectrophotometer (Shimadzu) as described by Meier *et al.* (1999).

Affinity-purified GST protein preparations were also subjected to analyses similar to those for the purified GST-Era protein as described above.

To examine if Era isolated directly from LY12 was also associated with RNA, a crude extract of LY12 grown to an OD₆₀₀ of 0.5 was prepared in buffer A, treated or not treated with RNaseA (1 mg ml⁻¹) at room temperature for 3 h and subjected to gel filtration column chromatography as described by Meier *et al.* (1999). Fractions (2 ml each) were collected and 15 µl of each of the fractions was subjected to SDS-PAGE (Laemmli, 1970). The resolved Era protein was transferred to a PVDF membrane and detected by Western blotting analysis as described previously (Zhao *et al.*, 1999) using polyclonal antibodies against SDS-denatured native Era protein of *E. coli*, which were prepared (Robert Sargent, Inc.) as described previously (Zhao *et al.*, 1999; Zhao & Winkler, 1995).

Analysis of RNA associated with Era. Purified GST-Era, native Era and GST proteins (4 mg each) were extracted with equal volumes of phenol/chloroform/isoamyl alcohol and the resulting material was collected by ethanol precipitation (Sambrook *et al.*, 1989). The pellets (RNA) collected were air-dried, resuspended in 200 µl diethyl-pyrocyanate-treated water and 10 µl of each was analysed by agarose gel electrophoresis (1.5% agarose containing 0.5 µg ethidium bromide ml⁻¹; Sambrook *et al.*, 1989). *E. coli* rRNAs were isolated from LY12 by the same method as described by Sambrook *et al.* (1989).

To directly detect RNA associated with the protein, purified Era proteins (10 µg each) were run on an agarose gel as described above. To determine whether RNA was associated with Era proteins, purified Era proteins and phenol/chloroform-extracted material were treated with 1 mg RNaseA ml⁻¹ (Boehringer Mannheim) or 0.2 mg DNaseI ml⁻¹ (Gibco-BRL) for 15 min at room temperature and then run on an agarose gel as described above.

Determination of Era GTPase activity. The GTPase activity of Era was assayed by HPLC as described previously (Zhao *et al.*, 1999). Reaction mixtures (300 µl each) containing 50 mM Tris/HCl, pH 7.5, 5 mM MgCl₂ and 5–10 µM Era were incubated at 23 °C. Reactions were initiated by the addition of GTP at concentrations ranging from 0 to 2 mM. Reaction mixtures (50 µl each) were injected into an HPLC column and separated under the conditions described by Zhao *et al.* (1999). The GDP produced was quantified by comparing its peak areas with those of GDP standards.

Identification and quantitation of the RNA associated with the purified GST-Era of *E. coli*. To determine the identity of the RNA associated with the purified GST-Era protein, an affinity-purified GST-Era preparation was subjected to gel filtration column chromatography as described above. A void-volume fraction from the column, which contained the GST-Era protein associated with RNA, was used as a source of RNA. Total RNA was extracted from 1 ml of the void fraction by using an RNeasy Midi Prep Kit (Qiagen) and resuspended in 150 µl H₂O.

Standards for 16S and 23S rRNA, for use in RT-PCR, were prepared as follows. A 500 bp DNA fragment internal to the *E. coli* 16S or 23S rRNA gene was cloned from *E. coli* genomic DNA by PCR as described by Sambrook *et al.* (1989) and Zhao *et al.* (1999) by using the following primers: 5'-CGCGGATCCTGACGTTACCCGACAGAAGAAG-3' and 5'-CCATCGATAAGGTTCTTCGCGTTGCATCG-3' (for 16S rRNA), and 5'-CGCGGATCCTGACCGATAGTGAAC-CAGTAC-3' and 5'-CCATCGATTCTCCCGTGATAACA-TTCTCC-3' (for 23S rRNA) based on the published sequences (Brosius *et al.*, 1978, 1980). Both 5' primers contained a *Bam*HI site and the 3' primers contained a *Cla*I site for cloning purposes. The amplified DNA fragments were digested with *Bam*HI and *Cla*I and cloned into pBlueScriptII KS+ (Stratagene). The resulting clones, containing a DNA fragment corresponding to a segment of either the 16S or 23S rRNA gene, were digested to completion with *Xho*I and 1 µg of each digested clone was used for *in vitro* transcription by using a MEGascript T7 kit according to the instructions of the manufacturer (Ambion). After transcription, the resulting RNA was purified by using an RNeasy Midi Kit (Qiagen). Under these conditions, 123 and 137 µg RNA was obtained from the clones containing fragments of 16S and 23S rRNA, respectively.

For RT-PCR, all RNA samples were treated with DNaseI (10 µg RNA, 20 units DNaseI, final volume 200 µl) at 25 °C for 15 min. The reaction mixtures were then mixed with 20 µl 25 mM EDTA and heated to 65 °C for 10 min. The final concentration of each RNA preparation was adjusted to 45.5 ng µl⁻¹. RT-PCR was performed by using a GeneAmp EZ *rTth* RNA PCR Kit (Perkin Elmer) with the following primers: 5'-TTAACGCGTTAGCTCCGGAAG-3' and 5'-GCACGC-AGGCGTTTGTTAAG-3' (for 16S rRNA), and 5'-ACGA-GGCGCTACCTAAATAGC-3' and 5'-CATGCTTAGGCG-TGTGACTGC-3' (for 23S rRNA) based on the published sequences (Brosius *et al.*, 1978, 1980). As a control, amplification reactions were done without any reverse transcription

process. For quantitation purposes, amplification reactions were performed under the conditions in which the formation of amplified products increased exponentially using sample RNA and standard RNA preparations. We found that the exponential amplification of products was achieved within the first eight cycles of reaction. Therefore, all amplification reactions were performed for seven cycles. Reaction mixtures (50 μ l each) contained 91 ng RNA, 1 $\times$ EZ *rTth* RNA PCR buffer (Perkin Elmer), 0.3 mM each nucleotide (dATP, dCTP, dGTP and dTTP), 2.5 units *rTth* DNA polymerase, 2.5 mM manganese acetate, 0.5 μ l [α -³²P]dCTP [Easy Tides dCTP, 2000–4000 Ci mmol⁻¹, 10 mCi ml⁻¹ (1 Ci = 37 GBq); NEN Life Sciences Products] and 0.15 pM each primer. Reverse transcription reactions were carried out at 60 °C for 30 min. PCR amplification reactions were carried out in triplicates for seven cycles under the following conditions: denaturation at 94 °C for 15 s and annealing and extension at 60 °C for 30 s. After seven cycles, 10 μ l of each of the reaction mixtures was subjected to PAGE (4–20% gradient gel) in TBE buffer (Sambrook *et al.*, 1989). The resulting gels were exposed to a phosphorimager plate (Molecular Dynamics) for 2–3 h and then scanned. The amounts of cDNA produced were quantified by measuring the intensity of each band using Imagequant (Molecular Dynamics).

In vitro binding of Era to RNA. To test if purified native Era protein binds to RNA, a purified native Era preparation (16 μ g) was mixed with or without 300 μ g total RNA prepared from cells of *E. coli* LY12 by phenol/chloroform extraction (Sambrook *et al.*, 1989) in 200 μ l 25 mM Tris/HCl, pH 7.5, 150 mM NaCl and 2.5 mM MgCl₂. The reaction mixtures were incubated at 4 °C for 30 min and then subjected to gel filtration column chromatography as described above. Fractions (2 ml each) were collected and the presence of Era in the fractions was detected by Western blotting analysis (Zhao *et al.*, 1999).

RESULTS

GTPase activities of the purified GST-Era and native Era proteins of *E. coli*

To further understand the physiological function of Era, we expressed the native Era and GST-Era proteins in *E. coli* and purified both proteins (see Figs 2 and 4). The GST-Era protein was purified in a single step by using an affinity glutathione-Sepharose column, but the native Era protein (non-tagged) was purified to homogeneity in three steps by using a different column chromatographic method (Zhao *et al.*, 1999). We then determined their GTPase activities and found that the purified GST-Era protein exhibited a specific activity (51 mmol min⁻¹ mol⁻¹) that was threefold higher than the specific activity (17 mmol min⁻¹ mol⁻¹) of the native Era protein. We also purified His-Era in a single step by using an affinity nickel column (Zhao *et al.*, 1999) and found that this preparation was only 80–85% pure (data not shown). Nevertheless, the partially purified His-Era protein also showed a GTPase activity significantly higher than that of the native Era protein (data not shown). The specific activity determined for the native Era protein was very similar to those reported for the *E. coli*, *Streptococcus mutans* and *S. pneumoniae* Era proteins (Chen *et al.*, 1990; Wu *et al.*, 1995; Zhao *et al.*, 1999). Thus, the GST-Era and His-Era proteins of *E.*

Fig. 1. Analysis of RNA association with the Era protein of *E. coli*. All samples were electrophoresed on a 1% agarose gel containing ethidium bromide. Lanes: 1, DNA standards (100 bp increments; Gibco-BRL); 2, total rRNA isolated from *E. coli* (see Methods); 3 and 5, phenol/chloroform-extracted material from a purified GST-Era protein preparation untreated and treated with RNaseA, respectively; 4 and 6, purified GST-Era protein preparation untreated and treated with RNaseA, respectively.

coli appear to be significantly more active than the native form of the enzyme and also those of *S. mutans* and *S. pneumoniae* (Wu *et al.*, 1995; Zhao *et al.*, 1999). In light of these results and those of our previous studies on the Era protein of *S. pneumoniae* (Meier *et al.*, 1999), it is predicted that RNA is probably also associated with the *E. coli* Era protein and might stimulate its GTPase activity.

RNA is co-purified with the Era protein of *E. coli*

To determine whether RNA is associated with the GST-Era protein and if this association might have been responsible for its high GTPase activity, we analysed the purified GST-Era protein and phenol/chloroform-extracted material from the protein by agarose gel electrophoresis. As shown in Fig. 1, the phenol/chloroform-extracted material appeared as one major, distinct band in the gel after staining with ethidium bromide (Fig. 1, lane 3). This band exhibited a mobility corresponding to that of *E. coli* 16S rRNA (Fig. 1, lanes 2 and 3), suggesting that the RNA associated with the Era protein was probably *E. coli* 16S rRNA. The purified GST-Era protein appeared as a broad band with a mobility slower than that of 16S rRNA (Fig. 1, lane 4), suggesting that the GST-Era protein is probably complexed with RNA. To confirm this further, we performed RNase and DNase treatments and found that treatment with RNase, but not DNase, of both the GST-Era protein preparation and its phenol/chloroform-extracted material eliminated the bands

Fig. 2. Analysis of GST and GST-Era proteins of *E. coli* by anion-exchange column chromatography. GST-Era of *E. coli* (a) and GST protein (b) were purified and subjected to anion-exchange chromatography as described in Methods. Fractions (1 ml each) were collected as indicated, analysed by SDS-PAGE (10% gels) and stained with Coomassie blue. Lanes M, molecular mass standards (Bio-Rad prestained SDS-PAGE standards (kDa): myosin, 209; β -galactosidase, 124; BSA, 80, ovalbumin, 49.1; carbonic anhydrase, 34.8; soybean trypsin inhibitor, 28.9).

(Fig. 1, lanes 5 and 6). Thus, these results demonstrated that RNA, but not DNA, was associated with the GST-Era protein. We then analysed the purified native Era protein and its phenol/chloroform-extracted material for the presence of RNA and found that RNA was absent in these preparations as judged by ethidium bromide staining (data not shown). These findings suggest that RNA was co-purified with GST-Era and that the RNA associated with the native Era protein was probably removed during purification.

To examine whether anion-exchange chromatography, a step used for the purification of the native Era protein, was able to remove the RNA associated with Era from the protein, we subjected an affinity-purified GST-Era protein preparation to Mono-Q column chromatography (see Methods). Two peaks were obtained, one eluted at a low salt (~ 150 mM KCl) concentration and one at a high salt concentration (~ 650 mM KCl) (Fig. 2a, top panel). SDS-PAGE analysis showed that the fractions of the low-salt peak contained GST-Era (Fig. 2a, bottom panel). The fractions of the high-salt peak contained RNA (Fig. 2a) because they exhibited a maximal absorption at 260 nm and a distinct band in an

agarose gel after staining with ethidium bromide, which had a mobility similar to that of 16S rRNA, and were sensitive to RNase digestion (data not shown). The GTP hydrolysis activity of the GST-Era protein eluted in the low-salt peak fractions was reduced to $3 \text{ mmol min}^{-1} \text{ mol}^{-1}$. Similar column profiles and specific activities were obtained for an affinity-purified His-Era protein preparation (data not shown). We then examined a purified GST protein preparation under identical conditions. Unlike the purified GST-Era protein, the purified GST protein was eluted as a single peak at a low salt concentration (Fig. 2b, top panel). In addition, spectrophotometric analysis of the purified GST protein preparation or its phenol/chloroform-extracted material showed no significant absorbance at 260 nm (data not shown). These results suggest that the RNA was specifically associated with Era rather than the GST- or His-tag portion of the protein and was required for the high GTPase activity of Era. In addition, these results suggest that the RNA associated with the native Era protein was removed during purification.

To further establish that RNA is associated with Era, we subjected a crude extract of a wild-type strain of *E. coli*

Fig. 3. Analysis of Era–RNA complex formation in a crude extract of *E. coli* by gel filtration column chromatography. A crude extract of *E. coli* was prepared, treated with RNaseA (1 mg ml⁻¹) or untreated and subjected to gel filtration column chromatography as described in Methods. Fractions (2 ml each) were collected as indicated. The column was calibrated by using the molecular mass standards as described in Methods and the molecular mass values of the following fractions were determined (kDa): fraction 23, >600; fraction 30, 200; fraction 33, 150; fraction 37, 66; fraction 42, 29; fraction 44, 12. The presence of Era in these fractions was detected by Western blotting analysis (Zhao *et al.*, 1999) using polyclonal antibodies prepared against the native Era protein of *E. coli*. Lanes M, 10 ng purified *E. coli* Era protein.

(without overexpression of Era) to gel filtration column chromatography. We reasoned that if RNA is associated with Era, then the Era–RNA complex should be present as a higher molecular mass species. As shown in Fig. 3, the Era protein isolated directly from the *E. coli* cells exhibited two peaks; one located in the void-volume fractions from the column with an estimated molecular mass of 600 kDa and one eluted at a position with a molecular mass of about 32 kDa (monomer) (Fig. 3, top panel). However, when the crude extract was treated with RNaseA, the Era protein exhibited only one peak with a molecular mass of around 32 kDa (Fig. 3, bottom panel). In addition, when a crude extract of *E. coli* LY68 (a native Era overexpresser strain) was chromatographed under identical conditions, the native Era protein was also present in two peaks similar to those of the wild-type *E. coli* cells (data not shown). Together, these findings suggest that the Era protein is complexed with RNA in *E. coli* and that the RNA associated with the native Era protein purified from the Era overexpresser strain was removed during purification.

To examine whether the RNA associated with Era is required for its high GTPase activity, an affinity-purified GST–Era protein preparation was subjected to gel filtration column chromatography under identical conditions as described above. Two peaks were obtained; the major one eluted in the void-volume fractions from the column with an estimated molecular mass of 600 kDa and the minor one eluted at a position indicating a molecular mass of 120 kDa, a dimer of GST–Era (Fig. 4a, top panel). As judged by SDS-PAGE, about 40% of the total GST–Era protein was associated with RNA (Fig. 4a, bottom panel). The specific activity for the GST–Era protein was determined to be 50 mmol min⁻¹ mol⁻¹, but the specific activities for the GST–Era proteins eluted in the fractions of the high- and the low-molecular-mass peaks (Fig. 4a) were 120 and 9 mmol min⁻¹ mol⁻¹, respectively. Clearly, the GST–Era protein that was not associated with RNA exhibited a significantly lower GTPase activity as compared with the GST–Era protein that was associated with RNA. When the His–Era protein was chromatographed, similar column profiles and specific activities were obtained (data not shown). In contrast, when the

native Era protein purified by using different chromatographic columns was chromatographed under these conditions, one single peak was detected, which was eluted at a position corresponding to 32 kDa (data not shown). Furthermore, when an affinity-purified GST protein preparation was also chromatographed under identical conditions, only a single peak was observed, which corresponded to an estimated molecular mass of 50 kDa, a dimer of GST (Fig. 4b, top panel). Together, these results suggest that RNA is associated with Era and is required for its high level of GTPase activity.

Purified native Era protein can bind to RNA *in vitro*

To examine whether purified Era protein could bind to RNA *in vitro*, we mixed a purified native Era preparation with total cellular RNA isolated from *E. coli* cells, subjected the reaction mixtures to gel filtration column chromatography and examined column fractions for the presence of Era by Western blotting analysis. As shown in Fig. 5, two peaks were observed; one eluted in the void-volume fractions with an estimated molecular mass of 600 kDa and the other eluted in the fractions indicating a molecular mass of 32 kDa. The presence of a significant amount of Era in the void-volume fractions suggests that Era was bound to RNA. However, which species of RNA was bound to Era was not known and could not be determined, since a mixture of RNAs was used and each individual rRNA was not available. Nevertheless, these results have confirmed that Era is indeed an RNA-binding protein. We also tested the purified native Era protein preparation and the Era and RNA reaction mixtures for their GTPase activity. Apparently, in the presence of RNAs, the GTPase activity (19 mmol min⁻¹ mg⁻¹) of Era seemed to be higher than that (12 mmol min⁻¹ mg⁻¹) of Era free of RNA. This appears to suggest that the binding of RNA to Era stimulates its GTPase activity.

RNA is not associated with a C-terminally truncated Era protein of *S. pneumoniae*

We wanted to examine if RNA is associated with Era through its C-terminal domain, since the C-terminal domain of Era proteins is highly conserved across many

Fig. 4. Analysis of the *E. coli* GST-Era and GST proteins by gel filtration column chromatography. GST-Era (a) and GST (b) proteins were purified and subjected to gel filtration as described in Methods. The fractions (2 ml each) were collected as indicated and analysed by SDS-PAGE (10% gels) and stained with Coomassie blue. Lanes M, Bio-Rad prestained molecular mass standards (see legend to Fig. 2).

Fig. 5. *In vitro* RNA-binding to purified Era. Native Era protein was purified as described by Zhao *et al.* (1999) and mixed with (+RNA) or without (–RNA) total RNAs isolated from *E. coli* cells. The mixtures were subjected to gel filtration column chromatography (see Methods) and fractions were collected as indicated. The presence of Era in the fractions was detected by Western blotting analysis as described by Zhao *et al.* (1999). The column was calibrated with molecular mass standards as described in the legend to Fig. 3. Lanes M: 5 ng purified *E. coli* native Era protein.

species of bacteria (Pillutla *et al.*, 1995; Zuber *et al.*, 1997) and is similar to the KH domain of the pre-mRNA-binding K protein that is known to bind RNA

(Chen *et al.*, 1999; Siomi *et al.*, 1993; Zhao *et al.*, 1999). We therefore examined an available *S. pneumoniae* GST-Era protein whose C-terminal domain (67 aa) was

Fig. 6. Analysis of C-terminally truncated GST-Era for association of RNA by gel filtration and anion-exchange chromatography. The C-terminally truncated GST-Era (GST- Δ Era) protein of *S. pneumoniae* was purified (Zhao *et al.*, 1999) and subjected to anion-exchange (a) and gel filtration (b) chromatography (see Methods). The fractions (1 ml each for Mono-Q and 2 ml each for gel filtration) were collected as indicated, analysed by SDS-PAGE and stained with Coomassie blue. Lanes M, Bio-Rad prestained molecular mass standards (see legend to Fig. 2).

deleted (designated GST- Δ Era) (Zhao *et al.*, 1999) for the presence of associated RNA. It should be noted that this truncated Era protein still retains all the GTP/GDP-binding motifs except the C-terminal domain (Zhao *et al.*, 1999). We found no evidence that RNA is associated with this GST- Δ Era protein (Fig. 6). First, two peaks that were eluted at a low salt concentration were observed when affinity-purified GST- Δ Era protein preparations were subjected to anion-exchange chromatography (Fig. 6a, top panel). The affinity-purified GST- Δ Era preparations were known to contain GST. These two peaks observed were shown to correspond to GST and GST- Δ Era by SDS-PAGE analysis (Fig. 6a, bottom panel). Second, two peaks containing GST and GST- Δ Era were again observed when the affinity-purified GST- Δ Era protein preparation was subjected to gel filtration column chromatography (Fig. 6b, top panel). These two peaks were eluted at positions corresponding to the molecular mass values of a dimer of GST- Δ Era and GST (Fig. 6b, top panel). Third, spectrophotometric and agarose gel electrophoresis analyses of the protein and its phenol/chloroform-extracted material showed no detectable RNA present

in these preparations (data not shown). Together, these results suggest that RNA is not associated with the C-terminally truncated Era protein.

The RNA associated with the Era protein of *E. coli* is 16S rRNA

To determine the identity of the RNA associated with the Era protein of *E. coli*, we extracted the RNA associated with GST-Era by phenol/chloroform treatment. The extracted RNA was used for quantitative PCR. Since the RNA associated with GST-Era appeared to be predominately 16S rRNA (>99%), with a possible trace amount of 23S rRNA (<1%) as judged by agarose gel electrophoresis (Fig. 1), we designed two primers complementary to the sequence of the *E. coli* 16S or 23S rRNA gene and used these primers for detection and quantitation of the RNA associated with GST-Era (see Methods). When the same amounts of the 16S rRNA standard and the RNA associated with GST-Era (~91 ng each) were used as templates for amplification (seven cycles), the amount of the products formed for the 16S rRNA standard was three times (35915/11950 $\approx$

Table 1. Identification and quantitation of the RNA bound to the GST-Era protein of *E. coli*

The GST-Era protein preparation of *E. coli* was purified and subjected to gel filtration column chromatography as described in Methods. A void-volume fraction of the GST-Era protein that was associated with RNA was collected and used as a source of RNA for RT-PCR.

RNA associated with Era	RNA standards	Primer set (rRNA)	Intensity of DNA band*	Relative molar concentration of DNA produced†
+	—	16S	11950	1
—	+	16S	35915	3
+	—	23S	46	1
—	+	23S	109697	2400

* After RT-PCR, all samples were subjected to PAGE. The gel was scanned by using a phosphorimager as described in Methods and the area of each DNA band was quantified by using the phosphorimager software, according to the manufacturer's instructions (Molecular Dynamics).

† The relative molar concentration of the DNA produced was calculated as follows. The intensity of each band was divided by the corresponding molecular mass values of each DNA fragment. The values obtained for the 16S and 23S rRNA standards were divided by those obtained for the RNA associated with Era by using 16S and 23S rRNA primers, respectively.

Table 2. Effects of carbohydrates on the GTPase activities of *E. coli* Era proteins

The native Era protein was purified as described by Zhao *et al.* (1999). The GST-Era and GST proteins were affinity-purified as described by Zhao *et al.* (1999). The GST-Era-RNA complex was obtained by subjecting an affinity-purified GST-Era protein preparation to gel filtration column chromatography. The void-volume fractions from the column containing GST-Era associated with RNA were collected and designated GST-Era-RNA complex and the peak fractions with a molecular mass of 120 kDa (GST-Era dimer) were collected and designated GST-Era (free of RNA).

Test compounds	Native Era		GST-Era		GST-Era-RNA complex	
	GTPase activity* (mmol min mol ⁻¹)	-Fold induction	GTPase activity* (mmol min mol ⁻¹)	-Fold induction	GTPase activity* (mmol min mol ⁻¹)	-Fold induction
Control (no compound)	17	1	4	1	61	1
Sodium acetate	50	3	39	10	84	1.3
Glyceraldehyde 3-phosphate	5	0.3	<1	0	3	0.05
3-Phosphoglycerate	37	2.2	50	12.5	86	1.4
2-Phosphoglycerate	21	1.2	4	1	58	1
Glycerate	12	0.7	4	1	48	0.8
Lactate	16	1	1	0.3	62	1
Pyruvate	16	1	<1	0	68	1.1

* GTPase activities of the Era proteins and RNA were assayed by measuring their ability to hydrolyse GTP to GDP in the presence or absence of 10 mM of each test compound by using the HPLC method (Zhao *et al.*, 1999).

3) more than that of the RNA associated with GST-Era (Table 1). The 16S rRNA standard used was 503 bases long with a molecular mass of ~171 020 Da (Brosius *et al.*, 1978). If the RNA associated with GST-Era is 16S rRNA, the RNA should be 1542 bases in length with an estimated molecular mass of 523 940 Da (Brosius *et al.*, 1978). Then, the molar concentration of the 16S rRNA standard used for amplification should be approximately three times ($523\,940/171\,020 \approx 3$) higher than that of the RNA associated with GST-Era, since 91 ng

of each RNA template was used. As a result, the amount of the product formed for the 16S rRNA standard should be three times more than that of the RNA associated with GST-Era, which is in total agreement with the results obtained (Table 1). Thus, the results of this study establish that the RNA associated with GST-Era was virtually all 16S rRNA (>99%). The results of this experiment also show that the amount of 23S rRNA present in the RNA associated with GST-Era was minimal under the conditions used (Table 1).

Fig. 7. Activation of GTPase activity of the *E. coli* native Era protein by acetate and 3-phosphoglycerate. The native Era protein of *E. coli* was purified by using a three-step purification method as described by Zhao *et al.* (1999) and its activity was assayed in the presence of various amounts of acetate or 3-phosphoglycerate by using HPLC (see Methods).

Table 3. Acetate activation of the GTPase activity of Era proteins

Enzyme*	K_m (μ M)		V_{max} (mmol min mol ⁻¹)	
	Acetate (50 mM)		Acetate (50 mM)	
	+	-	+	-
Native Era protein of <i>E. coli</i>	288	724	115	44
GST- Δ Era of <i>S. pneumoniae</i>	108	132	16	11
GST-Era of <i>S. pneumoniae</i>	102	491	336	24
GST-Era-RNA complex of <i>S. pneumoniae</i>	293	271	544	601

* The native Era protein of *E. coli* was purified by using three different columns as described previously (Zhao *et al.*, 1999). The GST- Δ Era and GST-Era proteins of *S. pneumoniae* were purified first by glutathione affinity column chromatography as described by Zhao *et al.* (1999) and then by Mono-Q column chromatography as described in Methods. The GST-Era-RNA complex of *S. pneumoniae* was obtained by subjecting an affinity-purified GST-Era protein preparation to gel filtration column chromatography. The void-volume fractions from the column containing GST-Era associated with RNA were collected and designated GST-Era-RNA complex. All purified Era protein preparations were assayed for GTPase activity in the presence or absence of 50 mM acetate as described (Methods).

Effects of carbohydrates on the GTPase activity of Era proteins

To understand the biochemical basis of the GTPase activation by the bound RNA, we examined the effect of acetate on Era proteins associated and not associated with RNA. Acetate, a natural metabolite and a small carboxylic acid (Fraenkel, 1996; Holms, 1996), is known to activate mutant enzymes that require a carboxylic acid for efficient catalysis (Drueckes & Schinzel, 1996). As shown in Table 2, acetate caused a threefold activation of the GTPase activity of the purified native Era. An even more pronounced activation (10-fold) was observed with the GST-Era protein that was not associated with RNA (Table 2). However, this activation effect of acetate on the GTPase activity of Era almost diminished when the GST-Era-RNA complex (void-volume fractions of GST-Era associated with RNA) was tested (Table 2), suggesting that when Era is

bound with RNA, the GTPase activity of the Era-RNA complex could not be further activated by acetate. We then examined a number of phosphorylated and non-phosphorylated carbohydrates with structures similar to that of acetate. Among those tested, 3-phosphoglycerate was also found to stimulate the GTPase activity of the Era protein free of RNA, but failed to stimulate the GTPase activity of the Era protein bound with RNA (Table 2). Both acetate and 3-phosphoglycerate exhibited a dose-dependent stimulation of the GTPase activity of the native Era protein (Fig. 7). Interestingly, glyceraldehyde 3-phosphate appeared to be a potent inhibitor of Era GTPase activity (Table 2).

To further investigate the mechanism by which the RNA and carbohydrates modulate the GTPase activity, we examined the effects of these carbohydrates on the GTPase activity of the C-terminally truncated Era and the full-length Era proteins of *S. pneumoniae*. As shown

in Table 3, acetate was able to activate the full-length Era protein, but failed to stimulate the GTPase activity of the C-terminally truncated Era protein. Similar results were obtained with 3-phosphoglycerate (data not shown). The kinetic analyses of the acetate activation revealed that acetate increased the $V_{\max}$ values, but decreased the K_m values of Era (Table 3). Thus, these results clearly suggest that the carbohydrates and RNA modulate the GTPase activity of Era through a similar mechanism and further confirm that the RNA associated with Era does stimulate its GTPase activity.

DISCUSSION

In this study, we have shown that like the Era protein of *S. pneumoniae* (Meier *et al.*, 1999), the Era protein of *E. coli* is also associated primarily with 16S rRNA *in vivo* and appears to require the RNA for efficient catalysis. In addition, we have shown that the C-terminal domain of Era from *S. pneumoniae* is responsible for the binding of 16S rRNA to the protein and that the GTPase activity of Era is modulated by acetate, 3-phosphoglycerate, glyceraldehyde 3-phosphate and RNA, possibly through a mechanism that requires a carboxylic-acid-like residue for efficient catalysis.

There are several lines of evidence showing that 16S rRNA is associated with the *E. coli* Era protein. The native Era protein was found to be present as two species of 600 and 32 kDa in a wild-type *E. coli* strain. The Era–16S rRNA complex is predicted to be around 600 kDa, since the *E. coli* 16S rRNA has an estimated molecular mass of 510 kDa (Brosius *et al.*, 1978). RNase treatment of a crude extract of *E. coli* converted the 600 kDa species into a 32 kDa low-molecular-mass species, a monomeric form of Era. Similarly, the affinity-purified GST–Era protein was also present as high-molecular-mass species complexed with RNA. The results of agarose gel electrophoresis established that the RNA was physically associated with the Era protein and exhibited a mobility slower than that of free 16S rRNA. When associated with RNA, the Era protein of *E. coli* exhibited a GTPase activity significantly higher than that of the GST–Era protein that was free of RNA. A purified native Era protein preparation was also shown to bind to RNA *in vitro*. In the presence of total cellular RNAs, the GTPase activity of Era appeared to be higher than that of Era free of RNA. The RNA associated with Era was identified primarily as 16S rRNA. Finally, Johnstone *et al.* (1999) have also shown that the *E. coli* Era protein was able to bind RNA *in vitro*. In light of these findings, we conclude that RNA is indeed associated with the Era protein of *E. coli* and might be required for its high GTPase activity. Therefore, the results of this study have confirmed our previous finding that the Era protein of *S. pneumoniae* is primarily associated with *E. coli* 16S rRNA (Meier *et al.*, 1999) and have further established that the binding of 16S rRNA to Era is an intrinsic property of Era proteins.

GTPases play a variety of regulatory roles in the cell, in such processes as translation, cell differentiation and cell

cycle regulation (Barbacid, 1987; Bourne *et al.*, 1990, 1991; March, 1992). Small GTPases, in particular, generally function as regulatory switches that can be converted from an active state to an inactive state by hydrolysis of GTP to GDP to regulate their effector enzymes (Barbacid, 1987; Bourne *et al.*, 1990, 1991; March, 1992). Era appears to play a role in cell cycle control and translation (Britton *et al.*, 1997, 1998; Gollop & March, 1991b). Presently, it is not known whether the RNA-bound or RNA-free form of Era is responsible for this regulation, nor is the mechanism of this regulation known. One possibility is that the RNA-bound form of Era, which has a higher GTPase activity, is in an active state that signals cell division and possibly translation (Meier *et al.*, 1999). The RNA-free form of Era may be in an inactive state that signals a termination of cell division and translation.

Sequence analysis revealed that the C-terminal domain of Era is highly conserved within, and unique to, the Era family. Era also appears to bind to the cytoplasmic membrane and to partition between the cytoplasm and the cytoplasmic membrane of the bacterial cell (Gollop & March, 1991a; Lin *et al.*, 1994; Wu *et al.*, 1995; Zhao *et al.*, 1999). The C-terminal domain of Era seems to possess some characteristics of the KH domain of the pre-mRNA-binding K protein that is known to bind RNA (Chen *et al.*, 1999; Johnstone *et al.*, 1999; Siomi *et al.*, 1993). In this study, we demonstrated that a C-terminally truncated Era protein of *S. pneumoniae* was unable to bind to RNA *in vivo* when expressed in *E. coli*. We have shown previously that the intact *era* gene of *S. pneumoniae* complemented an *E. coli* mutant deficient in the production of Era, without overexpression (Zhao *et al.*, 1999). This truncated *era* gene, however, failed to complement the same *E. coli* mutant when a sufficient level of the protein was produced (Zhao *et al.*, 1999). Consistent with these results, mutations in the C-terminal domain of the *E. coli* Era protein abolished the RNA-binding activity of the protein and these mutant *era* genes failed to complement the *E. coli* mutant strain deficient in the production of Era (Johnstone *et al.*, 1999). Together, these results suggest that the C-terminal domain of Era is essential for the binding of 16S rRNA to the protein and that this RNA-binding activity of Era is required for the biological function of the Era protein. Previously, our studies suggested that the C-terminal domain of Era is required for the membrane-binding activity of the protein (Zhao *et al.*, 1999). In light of the new findings that RNA is bound to the C terminus of Era, it is quite possible that this membrane-binding activity is due to its binding to ribosomes. Clearly, this hypothesis warrants further study.

The mechanism by which RNA stimulates the GTPase activity of Era remains to be elucidated. This study has, however, provided several lines of evidence suggesting that the mechanism by which the small carbohydrates and RNA modulate the GTPase activity of Era might be similar. First, acetate and 3-phosphoglycerate stimulated the GTPase activity of the Era protein free of RNA, but failed to stimulate the GTPase activity of the

Era protein bound with RNA. These results clearly validate the finding that the RNA associated with Era stimulates the GTPase activity of the enzyme. Second, the GTPase activity of the C-terminally truncated Era protein could not be stimulated by acetate and 3-phosphoglycerate, indicating that the carbohydrates must bind to the C-terminal domain of Era to function. Third, the RNA is bound to the C-terminal domain of Era. Together, these results also suggest that the small carbohydrates and RNA modulate the GTPase activity of Era probably through their direct binding to and interaction with the C-terminal domain of the protein.

Acetate, a small carboxylic acid (Fraenkel, 1996; Holms, 1996), has been shown to stimulate the activities of some mutant enzymes that require a carboxylic acid for efficient catalysis (Drueckes & Schinzel, 1996). 3-Phosphoglycerate is also a small carboxylic acid (Fraenkel, 1996; Holms, 1996). On the basis of their structural similarities and their ability to stimulate the GTPase activity of Era, it appears that Era also requires a small carboxylic-acid-like molecule for efficient catalysis. Presently, we do not know why pyruvate, glycerate, lactate, 2-phosphoglycerate and phosphoenolpyruvate, also small carboxylic acids, failed to exert significant effects on the GTPase activity of Era. However, it is possible that their side chains, located at the C-2 position, might interfere with their interaction with Era. If Era requires a carboxylic-acid-like molecule for efficient catalysis, it is clear that this molecule must bind to the C-terminal domain of Era to function, since these carbohydrates did not modulate the GTPase activity of the C-terminally truncated Era protein. Thus, it is likely that the binding of this carboxylic-acid-like molecule to Era causes a conformational change that allows efficient catalysis to occur. It is also possible that the RNA, like the carbohydrates tested, is a negatively charged molecule and causes a similar conformational change upon binding to the C terminus of Era which allows the catalysis of the enzyme to proceed more efficiently. It should be noted that we cannot rule out the possibility that other mechanisms might be involved in the modulation of the GTPase activity of Era in the cell.

The physiological role of carbohydrates in the modulation of the Era GTPase activity *in vivo* is unknown. There are two observations that appear to suggest that Era might be involved in the regulation of bacterial energy metabolism. First, the utilization of carbohydrate intermediates such as pyruvate was increased in the cells in which the level of Era was severely reduced (Lerner & Inouye, 1991). Second, when a dominant negative mutant of Era was overexpressed in cells of a wild-type *E. coli* strain, the growth of this strain was inhibited in a minimal medium supplemented with TCA cycle intermediates (Shimamoto & Inouye, 1996). In this study, we showed that 3-phosphoglycerate and glyceraldehyde 3-phosphate, key intermediates in glycolysis (Fraenkel, 1996; Holms, 1996), modulated the GTPase activity of Era although pyruvate did not. The physiological concentrations of 3-phosphoglycerate and glyceraldehyde 3-phosphate in the cell are not known, but

probably are similar (Fraenkel, 1996; Holms, 1996). The concentration of acetate in the cell varies significantly depending on growth conditions and carbon sources (Holms, 1996). The finding that acetate, 3-phosphoglycerate and glyceraldehyde 3-phosphate appear to exert similar levels of effects on the GTPase activity of Era suggests that their overall effects on the GTPase activity of Era in the cell might be minimal. Therefore, the RNA might play an important role in the regulation of the Era GTPase activity in the cell.

ACKNOWLEDGEMENTS

We thank J. M. Colacino, D. LeBlanc and X. Ye for their suggestions and critical reading of the manuscript and also thank P. L. Skatrud for his support.

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Received 11 August 1999; revised 16 December 1999; accepted 19 January 2000.