Expression and Characterization of a Recombinant Yeast Isoleucyl-tRNA Synthetase*

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We describe the heterologous expression of a recombinant Saccharomyces cerevisiae isoleucyl-tRNA synthetase (IRS) gene in Escherichia coli, as well as the purification and characterization of the recombinant gene product. High level expression of the yeast isoleucyl-tRNA synthetase gene was facilitated by site-specific mutagenesis. The putative ribosome-binding site of the yeast IRS gene was made to be the consensus of many highly expressed genes of E. coli. Mutagenesis simultaneously created a unique Bcll restriction site such that the gene coding region could be conveniently subcloned as a “cassette.” The variant gene was cloned into the expression vector pKK223-3 (Brosius, J., and Holy, A. (1984) Proc. Natl. Acad. Sci. U. S. A. 81, 6929–6933) thereby creating the plasmid pKR4 in which yeast IRS expression is under the control of the isopropyl-thio-β-galactopyranoside (IPTG)-inducible tac promoter. Recombinant yeast IRS, on the order of 10 mg/liter of cell culture, was purified from pKR4-infected and IPTG-induced E. coli strain TG2. Yeast IRS was purified to homogeneity by a combination of anion-exchange and hydroxypapitate gel chromatography.

Inhibition of yeast IRS activity by the antibiotic pseudomonic acid A was tested. The yeast IRS enzyme was found to be 104 times less sensitive to inhibition by pseudomonic acid A (Kᵢ = 1.5 × 10⁻⁴ M) than the E. coli enzyme. E. coli strain TG2 infected with pKR4, and induced with IPTG, had a plating efficiency of 100% at inhibitor concentrations in excess of 25 μg/ml. At the same concentration of pseudomonic acid A, E. coli strain TG2 infected with pKK223-3 had a plating efficiency <1%. The ability of yeast IRS to rescue E. coli from pseudomonic acid A suggests that the eukaryotic synthetase has full activity in its prokaryotic host and has specificity for E. coli tRNA.

The aminocacyl-tRNA synthetases catalyze the ligation of amino acid and transfer RNA in a two-step, ATP-dependent, reaction (1). The synthetases fall into at least two distinct classes based upon sequence homologies (2). The class I enzymes, of which the isoleucyl-tRNA synthetase (IRS) is a member, all contain an 11-amino-acid signature sequence ending in the tetrapeptide sequence His-Xaa-Gly-His (HXGH), where Xaa is a hydrophobic amino acid. Crystal diffraction studies of three class I enzymes indicated that the signature sequence is a component of a Rossmann nucleotide binding fold (3–5). All members of the class I family of enzymes are predicted to have a Rossmann nucleotide binding fold (6). Five enzymes, the methionyl-, valyl-, leucyl-, isoleucyl-, and cysteinyl-tRNA synthetases, share more extensive sequence homologies than other members of the class I enzymes and may, therefore, be considered as a subclass of enzymes (7).

Ultimately, the aminocacyl-tRNA synthetases are responsible for maintaining the high fidelity of protein biosynthesis, ensuring that each amino acid is faithfully coupled to its corresponding or cognate tRNA. In the case of the isoleucyl-tRNA synthetase, high fidelity has necessitated the evolution of an editing mechanism (1). Editing has also been recognized in the valyl- (9) and methionyl-tRNA synthetases (10). Although the kinetics of editing have been studied intensively (11,12), nothing is known of relationships between the proofreading activities and the structure/organization of the proteins.

In this paper, we describe the heterologous expression of a cytoplasmic Saccharomyces cerevisiae isoleucyl-tRNA synthetase gene in Escherichia coli and subsequent purification of the gene product to homogeneity. We have developed a strategy, employing the antibiotic pseudomonic acid A, for distinguishing yeast and E. coli isoleucyl-tRNA synthetase activities in vivo. The expression of the yeast IRS in E. coli will make it possible for us to probe relationships between the structure and activity of the enzyme by a combination of in vivo and in vitro mutagenesis methods.

For the sake of clarity in distinguishing host IRS and recombinant IRS enzymes, the yeast (S. cerevisiae) isoleucyl-tRNA synthetase is abbreviated yIRS and the bacterial (E. coli) isoleucyl-tRNA synthetase is abbreviated bIRS.

EXPERIMENTAL PROCEDURES

Materials—T4 DNA ligase, T4 DNA polymerase, E. coli strain DH5α, and all restriction enzymes (excluding SnaBI) were purchased from Bethesda Research Limited. SnaBI was obtained from New England Biolabs, and T7 DNA polymerase (Sequenase Version 2.0) was purchased from United States Biochemicals. Baker's yeast tRNA, E. coli MRE600 tRNA, and calf intestinal phosphatase were purchased from Boehringer Mannheim. The radiolabels [α-³²P]ATP (800

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CI/mmol) and [1-14C]isoleucine (324 CI/mmol) were purchased from Amersham Corp. Inorganic pyrophosphatase was purchased from Sigma and pseudomonadic acid A was kindly donated by SmithKline Beecham Pharmaceuticals (Brockham Park, Betchworth, Surrey). Sodium pseudomonadic was prepared by dissolving pseudomonadic acid A in 20% (v/v) methanol and adjusting the pH to 7.0 with dilute NaOH (13). A stock solution of 150 mg/ml pseudomonadic acid in 60% (v/v) methanol was used in the preparation of pseudomonadic acid-containing plates.

Cloning and Mutagenesis—The isolation of the isoleucyl-tRNA synthetase gene from S. cerevisiae (yIRS) and its cloning in the vector pZG100 was described previously (14).

Assembly of expression vectors is illustrated in Fig. 1. The expression plasmid pKR2 was constructed by subcloning the yIRS gene (coding region and 5′-noncoding sequences) directly into the expression vector pKCR223 (15). A 3.26-kb Hind III restriction enzyme fragment of pZG100 was treated with T4 DNA polymerase to fill in recessed 3′ ends. The fragment was ligated to pKCR223-3 that had been (a) cut with HindIII, (b) blunt ended by treatment with T4 DNA polymerase, and (c) treated with calf intestinal phosphatase to remove terminal 5′-phosphates. The ligation mixture was prepared for ligation with a 155-base pair Hind III/Sau96I fragment of pZG100, encompassing sequences flanking the yIRS start codon, into replicative form phase M13mp19. A 396-base pair Sau96I fragment of pZG100 was blunt ended by treatment with T4 DNA polymerase and ligated with M13mp19 (16) which had been (a) cut with the enzyme XbaI, (b) blunt ended by treatment with T4 DNA polymerase, and (c) treated with calf intestinal phosphatase. The fragment, with the appropriate orientation for mutagenesis, as confirmed by dye-oxynucleotide sequencing (17), was designated M13mp19(395).

The yeast IRS gene was altered by site-directed mutagenesis (18) in order to produce a sequence, 5′ of the coding region, which is a consensus ribosome-binding site of genes that are expressed well in E. coli. Mutagenesis of the yIRS gene was directed by the 25-mer (coding region and 5′-noncoding sequences) directly into the expression vector pKR4 begun with the subcloning of a fragment of pZG100, encompassing sequences flanking the yIRS start codon, into replicative form phase M13mp19. A 396-base pair Sau96I fragment of pZG100 was blunt ended by treatment with T4 DNA polymerase and ligated with M13mp19 (16) which had been (a) cut with the enzyme XbaI, (b) blunt ended by treatment with T4 DNA polymerase, and (c) treated with calf intestinal phosphatase. The fragment, with the appropriate orientation for mutagenesis, as confirmed by dye-oxynucleotide sequencing (17), was designated M13mp19(395).

Expression and Characterization of Yeast IRS—Two liters of 2 × TY media (16 g of Tryptone, 10 g of yeast extract, and 5 g of NaCl/liter of water), containing 100 μg/ml ampicillin, were inoculated with pMT521-infected (21) E. coli strain TG2 and incubated overnight at 37 °C with shaking. bIRS was purified essentially as described above for the E. coli extract, eluted at a salt concentration of 0.5 M KCl to 400 mM KCl at a flow rate of 175 ml/h. The concentration of yeast IRS protein, the major protein component of the E. coli extract, eluted at a salt concentration of 100 mM KCl and 175 mM KCl. Fractions were screened by SDS-PAGE, and yeast IRS was identified by its characteristic mobility (Mw = 123,000).

Fractions containing yeast IRS were pooled and loaded directly onto a hydroxyapatite column (2.5 × 17 cm) that had been pre-equilibrated in Buffer B (0.1 mM potassium phosphate, pH 7.0, 1 mM DTT, 25% glycerol, 0.5 mM PMSF). The column was washed with 100 ml of Buffer B. Yeast IRS was eluted from the hydroxyapatite column in a single peak by a 600-ml linear gradient from 0 to 400 mM potassium phosphate (pH 7.0, 1 mM DTT, 25% glycerol, 0.5 mM PMSF) at a flow rate of 50 ml/h. Fractions containing yeast IRS were pooled, dialyzed against 0.1 mM potassium phosphate, pH 7.0, 1 mM DTT, and the concentration of IRS was quantitated on ice for a further 30 min. Cells debris was removed from the lysate by centrifugation at 39,000 × g for 30 min. All subsequent operations were performed at 4 °C.

Recombinant yeast IRS was purified from TG2 by a modification of a procedure described for the isolation of the native E. coli IRS (20). Cell supernatant was loaded onto an anion-exchange column (Pharmacia LKB Biotechnology Inc., 2.6 × 31 cm) packed with Q-Sepharose and pre-equilibrated in Buffer A (0.05 mM potassium phosphate buffer, pH 7.0, 0.05 M KCl, 0.5 mM DTT, 0.5 mM PMSF). After the addition of 0.6 ml of a 100 mM stock solution of PMSF, the suspension was sonicated with 20-25 bursts at maximum power and recirculated for 5 min at 8 °C. The sample was loaded, the column was washed with 300 ml of Buffer A and eluted with a gradient of 0.5 mM EDTA and 0.5 mM PMSF. Lysozyme was added to the suspension incubated on ice for 30 min. After the addition of 0.6 ml of a 100 mM stock solution of PMSF, the suspension was sonicated with 20-25 bursts at maximum power from a probe sonicator (Fisher Sonic-Dismembrator model 300) fitted with a medium sized probe. Deoxyribonuclease, MgCl2 and EDTA were added to final concentrations of 1 μg/ml, 1 mM, and 25 mM, respectively, and the suspension was incubated on ice for a further 30 min. Cells debris was removed from the lysate by centrifugation at 39,000 × g for 30 min. All subsequent operations were performed at 4 °C.

Expression and Purification of Bacterial IRS—Two liters of 2 × TY media (16 g of Tryptone, 10 g of yeast extract, and 5 g of NaCl/liter of water), containing 100 μg/ml ampicillin, were inoculated with pMT521-infected (21) E. coli strain TG2 and incubated overnight at 37 °C with shaking. bIRS was purified essentially as described above for the E. coli extract, eluted from the Q-Sepharose column at salt concentrations between 130 mM and 160 mM KCl. After column chromatography on hydroxyapatite, fractions were pooled, dialyzed against 20 mM Tris, pH 8.0, and concentrated. The yield of bIRS was approximately 12 mg/liter of
cell culture. The protein was then flash frozen and stored, without added glycerol, in liquid nitrogen until needed.

**Analytical HPLC of yIRS and bIRS**—The homogeneity of the yeast IRS preparation was evaluated by analytical HPLC on a SpectraPhysics model 8810 HPLC system fitted with a Pharmacia 5/5 Mono-Q anion-exchange column (Fig. 3). The column was equilibrated in Buffer C (75 mM potassium phosphate pH 7.0, 1 mM DTT, 50 mM KC1), and proteins were eluted in a salt gradient (Fig. 3) up to 1 M KC1 in Buffer D (75 mM potassium phosphate, pH 7.0, 1 mM DTT, 1 M KC1). Proteins were detected by absorbance at 280 nm with an Applied Biosystems model 757 absorbance detector. Peak fractions were pooled and concentrated by Centricon 10 microconcentrators. The minimal broth samples were tested for IRS activity by the site titration and aminoacylation assay with yeast- and E. coli-specific tRNA (see below).

**Enzyme Assay**—Prior to assay, the concentration of active enzyme in samples was determined by active site titration with [14C]isoleucine and ATP (22).

Charging or amino acylation activities were measured in the standard reaction Buffer E (144 mM Tris, pH 7.78, 10 mM MgCl2, 10 mM KC1, 10 mM 2-mercaptoethanol, and 0.1 mM PMSF) plus 10 mM ATP, 0.002 unit/μl inorganic phosphate, and varying concentrations of [14C]isoleucine, tRNA, and pseudomonic acid A where appropriate. Reaction volumes were typically 5 or 50 μl. In the aminoacylation reactions were initiated by the addition of enzyme, and all incubations were performed at 37 °C in a water bath. Aliquots taken from the reaction mixture at various time intervals were quenched in 3 ml of 5% trichloroacetic acid. The quenched samples were filtered through a Whatman GF/C filter and the filter rinsed with 3 or 3 ml of 5% trichloroacetic acid. The filters were dried then counted in a Beckman LS8301 scintillation counter.

**Inhibitory Constant for Pseudomonic Acid A**—The K, for the inhibitor pseudomonic acid A with yIRS was determined by assay in reaction volumes of 25 μl. The reaction mixture was prepared in Buffer E and contained 10 mM ATP, 0.002 unit/ml inorganic pyrophosphatase, 11.4 mg/ml crude yeast tRNA, concentrations of [14C]isoleucine varying from 6 to 165 mM and concentrations of sodium pseudomonic acid varying from 0 to 200 mM. Reactions were initiated by the addition of yIRS to a final concentration of 3 nM and incubated at 37 °C for 2 min. The reaction was then quenched in 5% (v/v) trichloroacetic acid, filtered, and counted as described above. Each experiment was carried out in triplicate, and the reaction rate was shown to be linear for the concentrations of enzyme and substrates used over the time of incubation.

**Relative Plating Efficiencies**—Two-ml cultures of pKK223-3-infected E. coli strain TG2 were grown in minimal medium broth containing 100 μg/ml ampicillin at 37 °C with shaking until an OD600 = 0.7. At this point, IPTG was added to each culture to a final concentration of 0.4 mM. The cultures were grown for an additional hour, after which the OD600 of each culture was approximately equal to 1.0. The cultures were then diluted with minimal broth to a concentration of approximately 6 x 10^8 colony forming units/ml (estimated by 1 OD600 = 8 x 10^9 colony forming units) and 50-μl aliquots of the diluted cells were plated on minimal media plates containing 200 μg/ml ampicillin and concentrations of pseudomonic acid A varying from 0 to 100 μg/ml. Plates were incubated at 37 °C for 96 h at which time the number of surviving colonies was determined.

**RESULTS AND DISCUSSION**

The mechanisms by which the aminoacyl-tRNA synthetases preserve the fidelity of protein biosynthesis have been the subject of intensive investigation (23). Amino acids with short aliphatic or isosteric side chains are not readily discriminated by a single site reaction mechanism and hence, the valyl-, methionyl-, and isoleucyl-tRNA synthetases have evolved editing mechanisms to correct errors in aminoacylation. The valyl-tRNA synthetase, for example, is known to accept the amino acid threonine at its active site and incorrectly charge valyl-tRNA (tRNAval) with threonine. However, the mischarged tRNAval does not participate in protein synthesis because it is hydrolyzed at a second catalytic site on the enzyme. Similarly, the isoleucyl-tRNA synthetase is known to activate valine (11, 12). The location and structure of editing sites and the mechanisms of editing have not been characterized.

**Expression Vectors**—As a first step toward a description of catalysis and editing in the yeast isoleucyl-tRNA synthetase, by *in vitro* mutagenesis methods, we have engineered the gene that encodes yIRS for expression in *E. coli*. The cloning and sequencing of the *S. cerevisiae* isoleucyl-tRNA gene has been described previously (24). Here, we describe the construction of a multicopy plasmid vector pZG100, bearing the yIRS gene used in our study, was described by Martindale and co-workers (14). Expression in *E. coli* of a protein (M, = 250,000) which is the fusion of yIRS and β-galactosidase has also been reported (25) although authors of this report do not indicate if the fusion protein is active in vivo.

In an initial attempt at the expression of yIRS, we cloned the gene coding region, including some 5′-noncoding sequences, into the expression vector pKK223-3 (Fig. 1). The expression plasmid construct, pKR2, is illustrated in Fig. 2. In this vector system transcription of the yIRS gene is driven by the IPTG-inducible "tac" promoter (26).

Yeast IRS activity was detected in pKR2-infected *E. coli* strain TG2 as judged by the ability of crude cell extracts to aminoacylate yeast tRNA (see tRNA Charging Specificities below). However, the level of expression was consistently lower than anticipated for this vector system. As well, there was a wide batch to batch variation in the levels of enzyme activity. The protein yields obtained with the pKR2 construct were not satisfactory for routine purification and characterization of the enzyme and were, therefore, a potential drawback to the proposed mutagenesis studies. One explanation for the relatively poor expression of the yIRS gene from pKR2-infected cells was the absence of an efficient *E. coli* ribosome-binding site sequence.

The rules that determine efficient or ideal ribosome-binding site sequences have been elucidated by an extensive survey of sequences from a large number of genes expressed in *E. coli* (27-29). These rules pertain to the sequence and to the position of certain nucleotides in relation to the initiation codon of a gene. For example, there should be less than 2 guanine residues in positions -1 to -7 (start codon is +1), and the sequence AGGA (Shine-Delgarno sequence) should be placed within 6-9 nucleotides upstream of the initiation codon. Fortuitously, the yeast gene contains the sequence AGGA immediately 5′ of the initiation codon. However, there are only three nucleotides between this sequence and the start codon, and the putative Shine-Delgarno sequence of the yIRS. The yeast IRS gene, containing the synthetic ribosome-binding site sequence, was subcloned into pKK223-3 (Fig. 1) thereby creating the plasmid pKR4 (Fig. 2).

Insertion mutagenesis simultaneously generated a unique BclI restriction site, overlapping the start codon. The placement of the BclI site enabled us to subclone the yIRS coding region (as a "cassette") into a variety of vector systems of *E. coli*. As we anticipate that certain mutations of yIRS will be toxic to the host cell, we are examining secretion vector systems for the expression of these yIRS mutants.

A strong selective pressure was exerted when the yIRS gene variant was subcloned from the pUC18 derivative, pKR3, into the expression plasmid pKK223-3. Since the ligation involved the recombination of blunt-ended fragments, the transformation mixture theoretically contained plasmid constructs with the yIRS gene in both correct and incorrect orientations for transcription from the tac promoter. However, of 29 clones selected at random from a transformation of *E. coli* strain...
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FIG. 1. Pedigree of expression plasmids pKR2 and pKR4. Details of cloning procedures are contained under “Experimental Procedures.” The plasmid pZG100 contains the entire coding region of the yeast isoleucyl-tRNA synthetase gene (shaded arrow) as well as 5’- and 3’-noncoding regions (solid boxes). The 5’-noncoding region altered by site-directed mutagenesis in Step 3 is indicated by an open box. Step 1, the expression plasmid pKR2 was constructed by subcloning the coding region and part of the 5’-noncoding region of the IRS gene from pZG100 into the vector pKK223-3. Step 2, the stepwise creation of the expression plasmid pKR4 began with subcloning of a portion of pZG100 (encompassing sequences flanking the IRS start codon) into M13 mp19. Step 3 site-directed mutagenesis introduced an efficient E. coli ribosome-binding site into the IRS fragment in M13 mp19(395). Step 4 + 5, fragments of pZG100 and M13 mp19(395)-mut1 were recombined in pUC18 to reconstruct the yIRS gene containing the consensus ribosome-binding site. Step 6, the reconstructed IRS gene in pKR3 was subcloned into pKK223-3 to create the expression plasmid pKR4. The tac promoter (open arrow) and termination sequences (open boxes) of pKK223-3 are labeled in the expression plasmids pKR2 and pKR4.

DH5α, 27 were in the incorrect orientation for expression. When the same ligation mixture was transformed into the strain TG2, of 10 clones selected at random, seven constructs were isolated with the IRS gene in the correct orientation, three constructs had tandem inserts (with the correct polar-

ity), and there were no constructs in which the IRS gene was in an incorrect orientation. TG2 is a lacIq (27) strain of E. coli, and hence, transcription of the IRS gene is more efficiently repressed (in the absence of IPTG) than it is in the strain DH5α. Since DH5α is not a lacIq strain the IRS gene will be constitutively overexpressed.

yIRS Expression—In contrast to the result observed with pKR2-infected cell cultures, a high level of IRS expression was observed in pKR4-infected and IPTG-induced TG2 cultures. The relative levels of yIRS protein expression were visualized by SDS-PAGE and are presented in Fig. 3.

Our results support the hypothesis that, in some instances, levels of heterologous gene expression can be significantly elevated simply by altering the ribosome-binding site. However, it is well known that the sequence of the ribosome-binding site is not the only factor that will influence recombinant gene expression in E. coli. For example, the position and sequence of gene promoters and terminators will have an effect on the levels of expression of recombinant proteins (30).

Purification—The large scale expression of yeast and bacterial IRS enzymes is described under “Experimental Procedures.” bIRS was expressed from the plasmid pMT521 (21) in E. coli strain DH5α and both the yIRS and bIRS were purified by a combination of anion-exchange and hydroxyapatite chromatography according to a modification of a procedure described elsewhere (19). Ultimately, 10 mg of recombinant yeast IRS protein were purified from each liter of cell culture.

Purified proteins were analyzed by HPLC to ensure their homogeneity (Fig. 4). Yeast IRS was eluted in a single peak from the analytical column whereas E. coli IRS eluted in two closely spaced peaks. There was no overlap in the retention times of the recombinant yeast and bacterial proteins and it
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Fig. 3. SDS-polyacrylamide gel electrophoresis of crude cell extracts of E. coli strain TG2 infected with plasmids pKK223–3, pKR2, and pKR4. Cultures were grown to cell densities of OD₆₀₀ = 0.8 at which point aliquots were withdrawn and induced with IPTG. Both induced and uninduced cultures were grown for a further 3 h at 37 °C and cells were pelleted by centrifugation. The cytosolic fractions of cell lysates were prepared and analyzed as described under “Experimental Procedures.” A band corresponding to yeast IRS was seen in both the induced (IPTG) and uninduced cultures of E. coli strain TG2 infected with pKR2 and pKR4. Purified yIRS (123 kDa) and bIRS (105 kDa) proteins are shown for reference. Qualitatively, the intensities of bands at 123 kDa correlates with yeast specific charging activity in the preparations. Yeast IRS protein was the major component of pKR4-infected and IPTG-induced E. coli strain TG2 preparations.

Fig. 4. Column profiles of purified yeast and E. coli isoleucyl-tRNA synthetase enzymes analyzed by Fast Protein Liquid Chromatography. Conditions for fast protein liquid chromatography are described under “Experimental Procedures.” A, recombinant yeast IRS (90 μg); B, E. coli IRS (56 μg); C, coinjection of yeast and E. coli IRS. Peaks fractions were pooled, concentrated, and tested for charging activity with both yeast and E. coli-specific tRNA (cf. Fig. 5). The peaks which are cross-hatched incorporated [¹⁴C]isoleucine into both yeast tRNA and E. coli tRNA; shaded peaks incorporated [¹⁴C]isoleucine into E. coli tRNA only. There was no charging activity associated with unshaded peaks.

is, therefore, unlikely that E. coli IRS is a contaminant of the yIRS preparation.

tRNA Charging Specificities—The tRNA specificities of yIRS and bIRS enzymes were tested with commercial preparations of yeast tRNA and E. coli tRNA (Fig. 5). yIRS had the broader specificity of the two enzymes and, under the assay conditions used, appeared to charge the pool of E. coli tRNAs to an even greater extent than the bIRS enzyme. This result may indicate that yIRS recognizes E. coli tRNAs other than tRNA₆₀ or it may be an artifact of the assay conditions and concentrations chosen.

It is worth noting that although the results presented in Fig. 5 were obtained from enzyme assays performed at 37 °C (the optimum growth temperature of E. coli), the relative charging specificities of yIRS and bIRS were identical when the assays were repeated at 30 °C (the optimum growth temperature of S. cerevisiae). Bacterial IRS did not have a significant activity toward yeast tRNA at either 30 or 37 °C.

Although the cross-species charging behavior of prokaryotic and eukaryotic synthetases has been documented (31), this is the first unambiguous examination of heterologous (cross-species) charging by yIRS and bIRS enzymes. In the previous studies, analyses were performed with crude eucaryotic cell extracts and, therefore, the activities of cytoplasmic and mitochondrial synthetases were not distinguished. Since the publication of these early studies it has become known that yeast has a mitochondrial tRNA₆₀. Consequently, the existence of a yeast mitochondrial IRS activity cannot be discounted (32).

Inhibitor Specificity—Pseudomonac acid A is a strong competitive inhibitor of the E. coli isoleucyl-tRNA synthetase (Kᵢ = 2.5 × 10⁻⁹ M) (13), yet the eukaryotic rat liver IRS is reported to be several orders of magnitude less sensitive to the inhibitor (Kᵢ = 2.0 × 10⁻⁵ M). Similarly, we found that the yeast IRS differs from the bacterial IRS in specificity for the antibiotic. Inhibition curves of the yIRS and pseudomonic acid A (as sodium pseudomonate) were determined and the results are shown in Fig. 6. The Kᵢ of pseudomonic acid A with yIRS (1.5 × 10⁻⁵ M) is comparable to that reported for rat liver IRS, four orders of magnitude greater than the value reported for the E. coli enzyme.

We were able to exploit the large difference in yIRS and bIRS sensitivities to the inhibitor to demonstrate expression and function of the recombinant yIRS in E. coli in vivo (Fig. 7). E. coli strain TG2 harboring either pKK223–3 (vector alone) or pKR4 (vector containing yeast IRS gene) were plated on minimal agar plus ampicillin and varying concentrations of pseudomonic acid A. The relative plating efficiencies of the pKK223–3-infected E. coli strain TG2 and pKR4-infected E. coli strain TG2 were determined after growth for
the expression of IRS. Although several strains of vector alone had no colonies. The recombinant yeast IRS gene pseudomonic acid A, cells bearing the yeast IRS gene had tRNA synthetase (PheRS), expressed in acid A of TG2 cells expressing the yIRS gene does not result domonic acid A. Hence, increased resistance to pseudomonic acid A and, hence, yIRS is capable of aminoa-

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At 25 pg/ml pseudomonic acid A. The plating efficiency of the pKR4-infected TG2, expressing yIRS (open circles), was virtually 100% at all concentrations of pseudomonic acid A up to 25 μg/ml. The plating efficiency of the pKK223-3 control (closed circles) was less than 1% at 25 μg/ml pseudomonic acid A. The percent surviving is the number of colonies at a specific pseudomonic acid A concentration as a percentage of the number of colonies at 1 μg/ml pseudomonic acid A.

The expression of the isoleucyl-tRNA synthetase. There are several advantages to expression of the eukaryotic yeast IRS in E. coli over systems in which recombinant bIRS is expressed in E. coli (8). The principle advantage is that yeast mutants (in particular those with single residue substitutions) expressed in E. coli are readily separated from the host IRS enzyme (Fig. 4) and thereby made available for characterization in vitro. Yeast IRS and bacterial IRS have distinct molecular masses (14, 21) and distinct isoelectric points (Fig. 4). Another advantage of the heterologous expression system, described herein, is the ability to distinguish the activities of recombinant (yIRS) and host (bIRS) IRS enzymes in vivo. This has been made possible 1) by the difference in the affinities of yeast and E. coli IRS for the inhibitor pseudomonic acid A (Fig. 6) and 2) by the ability of the yeast IRS enzyme to aminoacylate E. coli tRNA<sup>56</sup> in vivo as well as in vitro. By comparison, other investigators have found that a yeast phenylalanyl-tRNA synthetase (PheRS), expressed in E. coli, was unable to rescue or complement an E. coli strain with a thermostable PheRS (34).

In a separate experiment we determined that overexpression of the bIRS enzyme in pMT521-infected (21) E. coli strain TG2 was not sufficient to rescue the host from pseudomonic acid A. Hence, increased resistance to pseudomonic acid A of TG2 cells expressing the yIRS gene does not result from an increased level of IRS but is due to the specific action of yIRS in vivo.

Pseudomonic acid A becomes a tool for the selection of functional variants of the yIRS expressed in E. coli, fulfilling the role of an E. coli strain which is temperature sensitive for the expression of IRS. Although several strains of E. coli have been isolated which are temperature sensitive for the expression of aminoaacyltRNA synthetases (35), to date, there are no reports of E. coli strains that are temperature sensitive in

Fig. 6. The kinetics of inhibition of yIRS by sodium pseudomonic acid (PA). The rate of [³H]isoleucyl-tRNA formation was determined over a 2-min period at 37°C. Assays were performed with varying concentration of isoleucine (6-165 μM) and sodium pseudomonic acid (0-200 μM). A, Lineweaver-Burke plot ($K_m = 1.6 \times 10^{-5} \text{M}$). Concentrations of pseudomonic acid are indicated below each line; B, Dixon plot ($K_i = 1.5 \times 10^{-3} \text{μM}$). Concentrations of isoleucine are indicated below each line.

96 h at 37°C (Fig. 7). It is important to note that yIRS is expressed at a low level in pKR4-infected cells even in the absence of the inducer IPTG (Fig. 3) (33). At 25 μg/ml pseudomonic acid A, cells bearing the yeast IRS gene had virtually 100% plating efficiency, whereas at the same concentration of pseudomonic acid A, control plates bearing the vector alone had no colonies. The recombinant yeast IRS gene was able to rescue E. coli strain TG2 from the effects of pseudomonic acid A and, hence, yIRS is capable of aminoacylating E. coli tRNA<sup>56</sup> in vivo as well as in vitro. By comparison, other investigators have found that a yeast phenylalanyl-tRNA synthetase (PheRS), expressed in E. coli, was unable to rescue or complement an E. coli strain with a thermostable PheRS (34).

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Fig. 7. Plating efficiencies of E. coli TG2 infected with either pKR223-3 or pKR4 in the presence of pseudomonic acid A. Colonies were counted after incubation at 37°C for 96 h on plates containing ampicillin and varying concentrations of pseudomonic acid A. The plating efficiency of the pKR4-infected TG2, expressing yIRS (open circles), was virtually 100% at all concentrations of pseudomonic acid A up to 25 μg/ml. The plating efficiency of the pKK223-3 control (closed circles) was less than 1% at 25 μg/ml pseudomonic acid A. The percent surviving is the number of colonies at a specific pseudomonic acid A concentration as a percentage of the number of colonies at 1 μg/ml pseudomonic acid A.

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