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Interaction between human tRNA synthetases involves repeated sequence elements

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ABSTRACT **Aminoacyl-tRNA synthetases (tRNA synthetases) of higher eukaryotes form a multiprotein complex. Sequence elements that are responsible for the protein assembly were searched by using a yeast two-hybrid system. Human cytoplasmic isoleucyl-tRNA synthetase is a component of the multi-tRNA synthetase complex and it contains a unique C-terminal appendix. This part of the protein was used as bait to identify an interacting protein from a HeLa cDNA library. The selected sequence represented the internal 317 amino acids of human bifunctional (glutamyl- and prolyl-) tRNA synthetase, which is also known to be a component of the complex. Both the C-terminal appendix of the isoleucyl-tRNA synthetase and the internal region of bifunctional tRNA synthetase comprise repeating sequence units, two repeats of about 90 amino acids, and three repeats of 57 amino acids, respectively. Each repeated motif of the two proteins was responsible for the interaction, but the stronger interaction was shown by the native structures containing multiple motifs. Interestingly, the N-terminal extension of human glycyl-tRNA synthetase containing a single motif homologous to those in the bifunctional tRNA synthetase also interacted with the C-terminal motif of the isoleucyl-tRNA synthetase although the enzyme is not a component of the complex. The data indicate that the multiplicity of the binding motif in the tRNA synthetases is necessary for enhancing the interaction strength and may be one of the determining factors for the tRNA synthetases to be involved in the formation of the multi-tRNA synthetase complex.**

Aminoacyl-tRNA synthetases play an essential role in cellular protein synthesis by catalyzing attachment of their cognate amino acids to tRNAs. Many eukaryotic aminoacyl-tRNA synthetases are distinguished from their prokaryotic counterpart in their abilities to form supracomplexes through self assembly or association with protein synthesis machinery (1–5) and cellular structures (6, 7). Most intriguing among them is a multi-tRNA synthetase complex generated by the assembly of many aminoacyl-tRNA synthetases and a few other protein factors of unknown function (8–10). The exact structure of the complex is still controversial because different forms of the complex have been isolated depending on the purification methods and organisms. The eukaryotic tRNA synthetases have been grouped depending on their abilities to form the multi-tRNA synthetase complex (8). The class I enzymes includes isoleucyl-, leucyl-, methionyl-, aspartyl-, bifunctional (glutamyl- and prolyl-), glutaminyl-, lysyl-, and arginyl-tRNA synthetases that have been consistently identified as the components of the multi-tRNA synthetase complex (11, 12). It is not clear whether other tRNA synthetases are loosely associated with or completely independent of such a complex.

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Understanding the structure of the multi-tRNA synthetase complex is important to find the functional linkage between the catalytic activities of this complex and other cellular processes. Many different approaches have been made to analyze the structure of the complex. Chaotropic salts and detergents (13, 14), centrifugation (15), and chromatography (16) were used to dissociate the components from the complex. The ultrastructure of the complex has been observed by electron microscopy (17), and neighboring proteins within the complex have been studied by chemical cross-linking (18). It has been suggested that a hydrophobic interaction between the protein components is responsible for the assembly of the proteins (19–21). Despite all these efforts, the structural organization of the complex and molecular mechanism for the protein assembly remain obscure and they need to be answered in order to elucidate the functional significance of the complex.

Owing to the rapid accumulation of the sequence information of the aminoacyl-tRNA synthetases, several novel motifs have been found in the proteins that belong to the multi-tRNA synthetase complex. Examples are the N-terminal motifs of cytoplasmic aspartyl-tRNA synthetase (22, 23), arginyl-tRNA synthetase (24, 25), the internal tandem repeats found in the bifunctional tRNA synthetase (26, 27), and the double repeats in the C-terminal region of human isoleucyl-tRNA synthetase (28, 29). Although these motifs have been thought to be responsible for the assembly of the complex, none has been experimentally proven to be the case. In this work, we applied a genetic approach to investigate a protein–protein interaction involving human isoleucyl-tRNA synthetase.

Human cytoplasmic isoleucyl-tRNA synthetases (IRS) is a component of the multi-tRNA synthetase complex (12), and the whole protein sequence has been reported (28, 29). Two domains in the N-terminal region are conserved with the corresponding regions of the other isoleucyl-tRNA synthetases and are responsible for the enzyme activity. The enzyme contains the C-terminal extension that is not found in other isoleucyl-tRNA synthetases. This extension includes about 180 amino acids consisting of two tandem repeats of about 90 amino acids. We focused on this part of the protein to determine whether it is actually involved in the protein–protein interaction and to identify an interacting protein using a yeast two-hybrid system.

MATERIALS AND METHODS

Bait Construction with Human Isoleucyl-tRNA Synthetase. The yeast two-hybrid system consists of three functional components: one hybrid protein with DNA-binding domain and protein X, another hybrid protein with transcription activation domain and protein Y, and the reporter genes which

Abbreviations: IRS, human cytoplasmic isoleucyl-tRNA synthetase; EPRS, human cytoplasmic bifunctional tRNA synthetase; X-Gal, 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside; ONPG, *o*-nitrophenyl β-D-galactopyranoside.

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are induced by the interaction of the two hybrid proteins via the fused proteins, X and Y (30, 31). Thus, the interaction of the two proteins can be genetically monitored by the expression of the reporter genes. We employed the system developed by Brent and coworkers (32) that uses LexA as a DNA-binding protein and B42 as a transcription activator and *lacZ* and *LEU2* as a reporter.

The cDNA fragment encoding the C-terminal 301 amino acids (spanning from E966 to F1266) of IRS was isolated by PCR with the primers IRS-F (CCGACGCGTATGCTTCAACAAGTTCCAG) and IRS-B (GGCGTCGACATGCTAGAAGTCTGC). The amplified PCR product was cleaved with *Mlu*I and *Sal*I and ligated to the yeast plasmid pLexmpnr derived from pLexA202+PL (33), expressing 202 amino acids of the LexA protein. The resulting plasmid was introduced into yeast strain EGY48 [*MATa, his3, trp1, ura3-52, leu2::pLeu2-LexAop6*/pSH18-34 (*LexAop–lacZ* reporter)] by a modified lithium acetate method (34). The transformants were selected on the yeast synthetic media (Ura$^-$ and His$^-$) (35). The synthesis of the hybrid protein was confirmed by immunoblotting with polyclonal rabbit antibody raised against LexA protein (see Fig. 2*C Left*).

Screening Human cDNA Library. The yeast strain EGY48 containing the LexA–IRS hybrid protein was grown in a yeast synthetic medium (Ura$^-$, His$^-$) with 2% glucose and used as a host for transformation with the HeLa cDNA library (provided from W. G. Seol, Harvard University). The cDNA fragments were cloned in pJG4-5 using *Eco*RI and *Xho*I to generate B42 fusion proteins, and the expression of the fusion proteins was designed to be induced by the presence of galactose (32). The cDNA was introduced into the competent yeast cells and the transformants were selected for the tryptophan prototrophy (plasmid marker) on the synthetic medium (Ura$^-$, His$^-$, Trp$^-$) containing 2% glucose. All the transformants were pooled and respread on the synthetic medium (Ura$^-$, His$^-$, Trp$^-$, Leu$^-$) containing 2% galactose to induce the introduced cDNA. Cells growing on the selection media were retested on the synthetic medium (Ura$^-$, His$^-$, Trp$^-$, Leu$^-$) containing 2% galactose (inducing condition) and 2% glucose (noninducing condition) to confirm the dependency of their growth on the presence of galactose. Cells growing only on the galactose media were subjected to further characterization. The selected cells were also streaked on the synthetic medium (Ura$^-$, His$^-$, Trp$^-$) containing 2% galactose or 2% glucose with 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-Gal) to test the β-galactosidase activity. The cells expressing both reporter genes only in the presence of galactose were finally chosen to isolate the plasmids. The isolated plasmids were transformed into *Escherichia coli* K12 strain KC8 (*pyrF::Tn5, hsdR, leuB600, trpC9830, lacD74, strA, gslK, hisB436*), and the transformants containing the recombinant cDNAs were selected by their growth on M9 minimal medium (Thi$^+$, His$^+$, Ura$^+$, Leu$^+$, Trp$^-$) containing ampicillin. The plasmids were then isolated from Trp$^+$ *E. coli* transformants and used to confirm the selection results and to sequence the inserted cDNA. The LexA–thyroid receptor hybrid protein (36) was used as a control bait to determine whether the isolated plasmids showed specificity to the LexA–IRS for the induction of the reporter genes.

Immunoblotting of Hybrid Protein. Yeast cells grown in 1.5 ml liquid culture were harvested by centrifugation and resuspended in 50 μl of 100 mM Tris·HCl (pH 6.8) containing 0.2% 2-mercaptoethanol, 4% SDS, 0.2% bromophenol blue, 20% glycerol, and 8 M urea and lysed by ultrasonication. The lysed cells were incubated at 75°C for 20 min. Cell debris was removed by centrifugation and proteins in the cell extracts were separated on an 10–12% SDS/polyacrylamide gel. The resolved proteins on the gel were transferred to the nitrocellulose membrane, and immunoblotting was carried out following the method described by the manufacturer using disodium 3-{4-methoxyspiro[1,2-dioxetane-3,2′-(5′-chloro) tricyclo(3.3.1.1^{3,7}) decan]-4-yl} phenyl phosphate (CSPD) as a detecting reagent (Boehringer Mannheim). The monoclonal antibody against hemagglutinin (HA) tag which was inserted between B42 and cDNA was used to detect the hybrid proteins (see Figs. 1*B* and 2*C Right*)

Subcloning of Repeated Sequence Motifs. Deletion mapping was carried out to determine the sequence requirement for the interaction between human isoleucyl-tRNA synthetase and the selected bifunctional tRNA synthetase. The C-terminal region of the IRS contains two repeats of about 90 amino acids, and the DNA fragments encoding these repeats were isolated by PCR using the following primers (IRS-RF1, CCGACGCGTTCCCTTCCTGGTCCT; IRS-RB1, AATGTCGACTCCTGCAGTCACACA; IRS-RF2, CCGACGCGTAGTTCTAGTACTCTT; IRS-B, GGCGTCGACATGCTAGAAGTCTGC). The PCR products spanning one or two of the repeated sequences (A) were cloned into the LexA vector using *Mlu*I and *Sal*I. The constructed plasmids were introduced into yeast EGY48 expressing the selected fragment of the bifunctional tRNA synthetase, and the transformed cells were selected for further experiments.

The three repeated sequence motifs are present in the junction between the glutamyl- and prolyl-tRNA synthetases of the bifunctional enzyme. The DNA fragments encoding different numbers of the repeats were isolated by PCR using the combination of the following primers (EPRS-RF1, CGGAATTCGATTCCTTGGTCCTTTAC; EPRS-RB1, CCGCTCGAGCGGTCAAGGAGGGTTTCCAGGTTT; EPRS-RF2, CGGAATTCGAAAGTAAATCTCTGTAT; EPRS-RB2, CCGCTCGAGCGGTCAGGGGGGCTGACCAGGTAT; EPRS-RF3, CGGAATTCGAAGCGAAAGTACTTTTT; EPRS-RB3, CCGCTCGAGCGGTCAAGTGGCCGACACAGGCTT; EPRS, human cytoplasmic bifunctional tRNA synthase) and then cloned into pJG4-5 digested with *Eco*RI and *Xho*I. The constructed plasmids (three containing each unit and two with two units and one with three units, see Fig. 2*B*) were introduced into yeast EGY48 expressing the LexA–IRS hybrid protein. The transformed yeast cells were then selected by the method described above and used for further experiments. Human glycyl-tRNA synthetase contains a motif homologous to those in the bifunctional tRNA synthetase (see Fig. 4*A*) (37, 38). To test whether this region could interact with the motif in the C-terminal extension of the isoleucyl-tRNA synthetase, the cDNA encoding 291 amino acids of the N-terminal region of human glycyl-tRNA synthetase was isolated by PCR and fused into the pJG4-5 using *Eco*RI site.

Quantitation of Interaction. The strength of the interaction between the motifs of human isoleucyl- and bifunctional tRNA synthetase was compared by measuring the expression level of the two reporter genes. The β-galactosidase activity was determined according to the method described by Miller (39) with slight modifications.

Yeast cells containing each of the constructions were cultured in the yeast synthetic media containing 2% glucose until they reached a mid-log phase. The cell growth was monitored by the absorbance at 600 nm. The culture broth (0.2 ml) was taken and mixed with Z buffer (0.7 ml) containing 2-mercaptoethanol. Chloroform (50 μl) and 0.1% SDS (50 μl) were added to the mixture, and the cells were vortex mixed for 30 sec. The reaction substrate *o*-nitrophenyl β-D-galactopyranoside (ONPG) (0.16 ml) was added and the reaction was carried out at 30°C until yellow color appeared. The reaction was quenched by adding 0.4 ml of 1 M Na_2CO_3. Cell debris was removed by centrifuge, and the absorbance of the supernatant was measured at 420 nm. Enzyme activity was also measured by a filter method (40). Briefly, the cells grown on the yeast plate were patched onto a filter paper (Whatman 3MM) and the filters were submerged into liquid nitrogen for 10 sec to freeze the cells. Then the cells were thawed at room temper-

ature for 20 min to permeabilize the cell wall and the filter was incubated in Z buffer containing X-Gal until blue color developed. The expression level of the reporter gene *LEU2* was determined by the growth rate of the yeast cells in the synthetic media lacking leucine.

RESULTS

Construction of LexA–IRS Hybrid Protein. IRS consists of 1266 amino acids, and its C-terminal extension was proposed to be involved in the interaction with other proteins (28, 29). The cDNA fragment encoding the C-terminal 301 amino acids from E966 to F1266 was fused to the carboxyl side of LexA protein (Fig. 1*A*), and the resulting hybrid protein was expressed in yeast strain EGY48. The hybrid protein was stably expressed as determined by immunoblotting with rabbit polyclonal antibody raised against LexA (Fig. 2*C Left*). The hybrid proteins containing larger portions of the isoleucyl-tRNA synthetase did not appear to be stable in the cell (unpublished data).

Screening Interacting Protein. The human HeLa cDNA library fused to the gene for the transcription activator B42 was introduced into yeast cells containing the LexA–IRS hybrid protein. Approximately 3.5×10^5 independent transformants were pooled and respread on the selection media (Ura^-, His^-, Trp^-, Leu^-) containing 2% galactose to induce the expression of cDNAs. If a B42-tagged protein interacts with the C-terminal region of the isoleucyl-tRNA synthetase, it will activate the transcription of *LEU2* gene and allow the host cells to grow on a synthetic medium lacking leucine. Among 33 colonies obtained on the selection media, a total of 17 colonies showed galactose dependency. The plasmids were isolated from the selected yeast cells and introduced into *E. coli* KC8

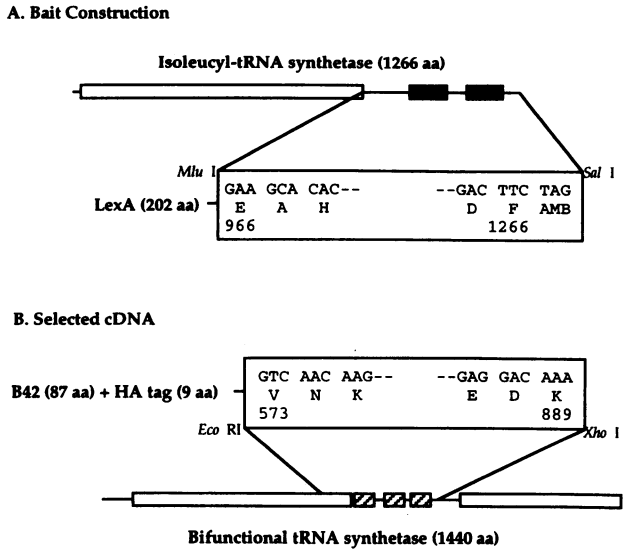

FIG. 1. The C-terminal region of IRS fused to LexA protein (*A*) and the region of human bifunctional tRNA synthetase fused to B42 (*B*). (*A*) Human isoleucyl-tRNA synthetase contains 1266 amino acids, and the amino acids from P6 to N1086 (open box) consist of the two conserved domains responsible for the aminoacylation activity (29). The enzyme contains the unique C-terminal extension with two tandem repeats of about 90 amino acids (solid boxes). The 301-amino acid C-terminal region including these two repeats was fused to LexA and used as a bait to screen an interacting protein. (*B*) The amino acid sequence of the two selected cDNA fragments represented a part of the human bifunctional tRNA synthetase which consists of the glutamy-tRNA synthetase (from G92 to Q687) and the prolyl-tRNA synthetase (from G935 to Y1440) (27). The larger and smaller inserts started at K514 and V573, respectively, and both ended at K889. (Only the smaller insert was shown above.) This region contains three repeats of 57 amino acids (hatched boxes).

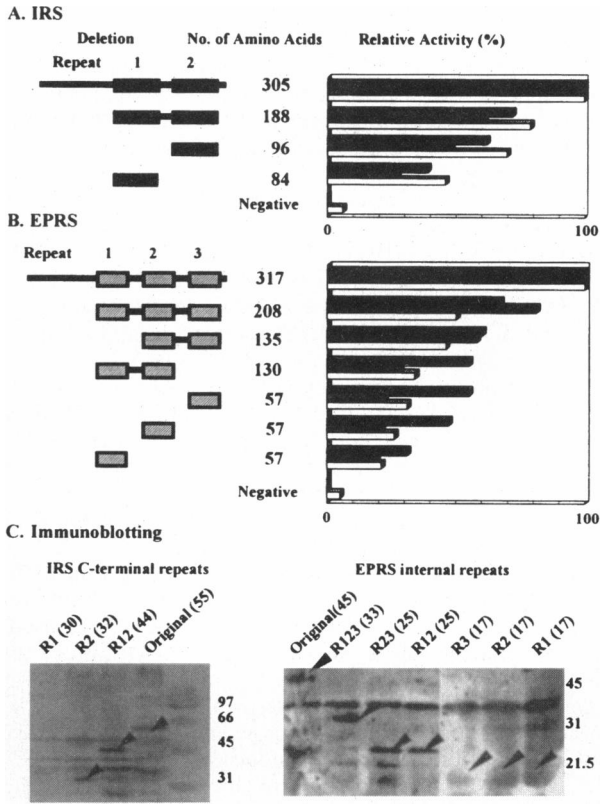

FIG. 2. Interaction mapping between human isoleucyl-tRNA and bifunctional tRNA synthetases. (*A*) Various mutants containing different numbers of repeats (solid boxes) in the isoleucyl-tRNA synthetase (*Left*) were introduced into the yeast cells containing the originally selected fragment of the bifunctional tRNA synthetase, and their interaction activities were compared by the induction level of the reporter genes (*Right*). (*B*) Various mutants containing different numbers of repeats (hatched boxes) in the bifunctional tRNA synthetase (*Left*) were introduced into the yeast cells containing the original bait fragment of the isoleucyl-tRNA synthetase, and their interaction activities were compared (*Right*). The black and gray bars represent the β-galactosidase activity determined by the assay using ONPG and X-Gal, respectively, and the white bar represents the activity of another reporter gene *LEU2* determined by the relative growth rate on the leucine-depleted media. Negative stands for the yeast cells expressing the LexA–TR and B42–EPRS–R123. The β-galactosidase assay using ONPG was carried out as described by Miller (39), and the activity units of the various deletion mutants were divided by that of the original construction to get the relative activity. The β-galactosidase activity was also determined by measuring the time required to show blue color of X-Gal on the filter paper containing the same amount of yeast cells. (See *Materials and Methods* for details.) The growth rate of the various mutants was compared by measuring the time until the isolated colonies appeared on the leucine-depleted media. (*C*) Immunoblotting of the hybrid proteins with various number of the repeats. Polyclonal rabbit antibody against LexA and monoclonal antibody against HA tag were used to detect LexA–IRS and B42–EPRS hybrid proteins.

to isolate the plasmids carrying B42-cDNA inserts. The plasmids were then isolated by the plasmid marker *trp* in the *E. coli* host, and the purified plasmids were reintroduced into the yeast containing the LexA–IRS (the original bait) and the LexA–thyroid receptor (control) to test whether the phenotype was specific to the isoleucyl-tRNA synthetase. Plasmids inducing the reporter genes (*LEU2* and *lacZ*) only in the presence of LexA–IRS were finally selected for sequencing.

Identification of the Interacting Protein. The sequence patterns of the finally selected cDNA fragments were characterized by restriction mapping. The results indicated that the selected inserts could be grouped into two sizes (≈1.0 and 1.2

kb) of the same DNA sequence origin (unpublished data). The sequence determination of the two inserts showed that the longer one had about 200 additional bases toward the 5′ side compared with the shorter one, but both of them had the same 3′ end. The homology search of the cDNA sequence showed that the selected cDNA encoded an internal part of human bifunctional tRNA synthetase. The longer and shorter inserts encoded 376 (from K514 to K889) and 317 amino acids (from V573 to K889) of the protein, respectively (Fig. 1*B*). The human bifunctional tRNA synthetase comprises the glutamyl-tRNA synthetase activity in the N-terminal side (from G92 to Q687) and the prolyl-tRNA synthetase activity in the C-terminal side (from G935 to Y1440) and the junctional region between them. The identified part of the cDNA encodes the protein fragment from the C-terminal region of the glutamyl-tRNA synthetase to the junctional region of the two proteins. Therefore, the result indicated that a part or all of this protein region should be involved in the interaction with the C-terminal region of the isoleucyl-tRNA synthetase.

Refining Interaction Motifs. Human isoleucyl-tRNA synthetase contains two repeats in the C-terminal region and they were included in the bait construction. Human bifunctional tRNA synthetase has three repeats between the glutamyl- and prolyl-tRNA synthetases. The selected cDNA fragment encodes all of these three repeats with the flanking sequences at both ends (mostly to the N side) (Fig. 1*B*). We suspected that the repeats of the two enzymes might be responsible for the interaction. To obtain a refined map for the interaction between these two proteins, the DNA fragments encoding different numbers of the repeated motifs were isolated by PCR and subcloned to their corresponding plasmids and their interaction activities were compared by the expression of the two reporter genes, *lacZ* and *LEU2*.

The DNA fragments, encoding either one or a pair of the repeats located in the C-terminal region of the isoleucyl-tRNA synthetase, were subcloned into the LexA vector (Fig. 2*A*). The constructs were introduced into the yeast cells expressing the originally selected cDNA fragment of the bifunctional tRNA synthetase. The expression level of the reporter genes was highest in the original C-terminal fragment and the fragment of the double repeats showed higher activities than the fragments of the single repeat. Immunoblotting with anti-LexA antibody showed that the hybrid protein containing the repeat 1 was less stable than the protein containing the repeat 2 (Fig. 2*C Left*). This may explain its lower reporter activities than those of the protein with the repeat 2 (Fig. 2 *A* and *C Left*). Nevertheless, the protein with the repeat 1 showed significantly higher activities than the background activities.

The six DNA fragments encoding various combinations of the repeated motifs in the bifunctional tRNA synthetase were also constructed and coexpressed with the original bait fragment of the isoleucyl-tRNA synthetase. As shown in Fig. 2*B*, the reporter activities were decreased according to the number of the repeats. The fragments containing a pair of the two consecutive repeats showed lower activities than that with all three repeats. The activities of the repeated motif 2–3 pair seemed to be slightly higher than those of the 1–2 pair. The fragments with the single repeat showed lower activities than those with the double repeats. The subtle difference in activity was also noticed among the fragments with the single repeat, the highest from the repeat 3 and the lowest from the repeat 1. The hybrid proteins containing different numbers of the repeats did not show a significant difference in the level of protein expression, although the signal of the single repeat was slightly weaker (Fig. 2*C Right*). The fragment with the three repeats lacking the flanking sequences showed lower activities than the original fragment, suggesting that the flanking sequences are required for efficient interaction.

Single Motif Interaction. Mapping of the interaction was further carried out to determine whether the single repeated motif of the isoleucyl-tRNA synthetase could interact with the single repeat of the bifunctional tRNA synthetase. The repeat 2 of the isoleucyl-tRNA synthetase was chosen as a bait because it showed a slightly higher activity than the repeat 1. It was coexpressed with the B42 hybrid proteins containing different numbers of the internal repeats in the bifunctional tRNA synthetase. As shown in Fig. 3, single copies of the repeats in the two proteins were able to interact to support the growth of the host cells on the leucine-depleted media. The repeats in the bifunctional tRNA synthetase have several homologous motifs in other eukaryotic tRNA synthetases (Fig. 4*A*). The interaction capability of the single repeat in the bifunctional tRNA synthetase suggested the possibility that the other tRNA synthetases containing these motifs could also interact with the C-terminal repeated motifs in the isoleucyl-tRNA synthetase. Human cytoplasmic glycyl-tRNA synthetase is one of the aminoacyl-tRNA synthetases containing a single copy of the homologous motif in its N-terminal region (Fig. 4*A*). This region could also induce the reporter gene *LEU2* by the interaction with the repeat 2 of the isoleucyl-tRNA synthetase (Fig. 3).

DISCUSSION

Experimental evidence suggested that the complex formation of tRNA synthetases may facilitate the amino acid transfer to the protein synthesis machinery (46–48) or may be necessary for efficient performance or regulation of the translation (8, 9, 10, 49). Many biochemical and biophysical approaches appeared to have a limit in the resolution and sensitivity to investigate the structure of this multicomponent complex. This limitation was alleviated by the genetic method used in this work. Only a part of the protein could be used to identify an interaction motif and the genetic selection was sensitive enough to monitor even a weak interaction. Also, this type of approach makes possible to find the interactions of the tRNA synthetases with other noncomplex proteins. Owing to these advantages, the interaction between human cytoplasmic isoleucyl- and bifunctional tRNA synthetases was identified and the binding regions were determined. Both of the interaction regions contain unique repeated sequence elements (Fig. 1) and these repetitions enhanced the interaction strength (Fig. 2 *A* and *B*).

The motifs homologous to those in the junction of human bifunctional tRNA synthetase are present in the equivalent location of the bifunctional tRNA synthetase of *Drosophila melanogaster* as six tandem repeats (26). The similar motifs have been also found in eukaryotic tryptophanyl-, histidyl-,

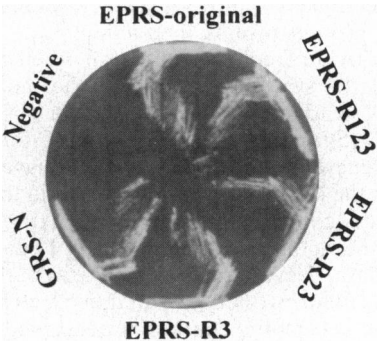

FIG. 3. Cell growth of yeast cells on the *LEU2* selection media induced by the interaction of the second repeat of IRS with various numbers of internal repeats (R) in the bifunctional tRNA synthetase and the N-terminal region of human glycyl-tRNA synthetase. Note that the cDNA encoding 291 amino acids of the N-terminal region of human glycyl-tRNA synthetase was isolated and fused into the pJG4-5 using *Eco*RI site. Negative indicates the yeast cells expressing the LexA–TR and B42–EPRS–R123.

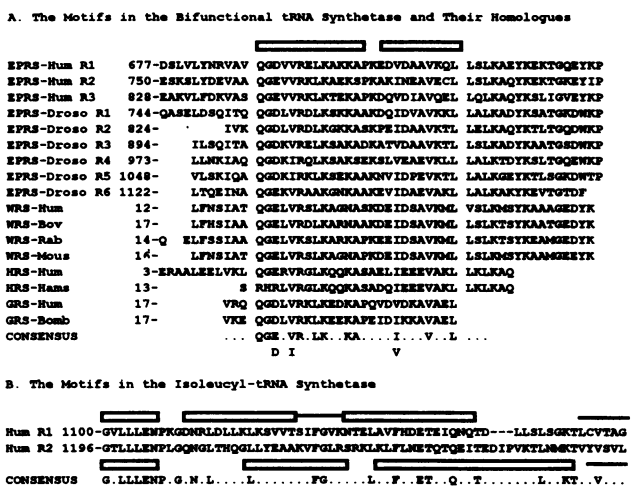

FIG. 4. The motifs homologous to the internal repeats of the bifunctional tRNA synthetase (*A*) and the repeated motifs in the human isoleucyl-tRNA synthetase (*B*). The selected motifs (EPRS-Hum R2 and IRS-Hum R1 and 2) were submitted to Chou–Fasman algorithm to predict the secondary structure. The α-helix region was labeled with an open box and the β-sheet was labeled with a solid line. The structure prediction by Granier–Robson algorithm generally agreed to the patterns shown above except for the first α-helices in the two repeats of the isoleucyl-tRNA synthetase which were predicted to be β-sheet. EPRS-Hum (27) and -Droso (26), WRS-Hum (41), -Bov (42) and -Rab (43) and -Mous (42); HRS-Hum (44), -Hams (45), GRS-Hum (38), -Bomb (37).

and glycyl-tRNA synthetases although they have not been found in the multi-tRNA synthetase complex (Fig. 4*A*). The difference between these enzymes and the bifunctional tRNA synthetase is that they have a single copy of the motif in the N-terminal region while the bifunctional enzyme contains the multiple copy in the internal region.

The N-terminal region of human glycyl-tRNA synthetase containing this motif also showed the capability of interacting with the C-terminal motif of the isoleucyl-tRNA synthetase. This result gives a question about the reason why these proteins have not been found in the complex. Perhaps the interaction with the C-terminal motifs of the isoleucyl-tRNA synthetase is possible, but the proteins with a single motif are prevented from binding by the competition with the bifunctional tRNA synthetase with three copies of the motif. Alternatively, the tRNA synthetases with a single motif may be associated with the complex at lower affinity in the cell and dissociated from the complex during the isolation. It is also possible that the motifs in noncomplex form tRNA synthetases may be used for other type of protein–protein interaction.

These motifs were predicted to form an α-helical coiled–coil (26, 27), and the synthetic peptide of the motif in human histidyl-tRNA synthetase was observed to actually form a typical α-helix (50). The two repeats in the C-terminal region of human isoleucyl-tRNA synthetase do not have homologous motifs among the known proteins (28, 29), and the probability to form a coiled–coil structure was low when the sequence was submitted to the algorithm developed by A. Lupas (ref. 51 and unpublished data). Nevertheless, the majority of the two motifs were predicted to form α-helices with short stretches of β-sheet (Fig. 4*B*). The two motifs contains a conserved hexapeptide LLLENP in the N-terminal side and blocks of basic amino acids which could be involved in the interaction in some way.

The motif of histidyl-tRNA synthetase homologous to the internal repeats of the bifunctional tRNA synthetase has been thought to be a major antigentic site causing an autoimmune disease, myositis (50). The eukaryotic tryptophanyl- and glycyl-tRNA synthetases containing these motifs also serve as an autoantigen (52, 53). Interestingly, some myositis patients also accumulate an autoantibody against the isoleucyl-tRNA synthetase (28). It would be interesting to see whether the antigenicity of these proteins are related to their interactions found in this work.

The results obtained in this work has other experimental support. First, the similar tandem repeats in the internal region of the bifunctional tRNA synthetase isolated from *D. melanogaster* was previously predicted to be involved in the complex formation (26). Second, one of the subcomplexes derived from the rabbit multi-tRNA synthetase complex was composed of the isoleucyl- and glutamyl- (and probably prolyl-) tRNA synthetases, suggesting their association in the complex (13). Conclusively, this work provides evidence showing the identities of the two interacting protein components and their binding motifs in the multi-tRNA synthetase complex. This interaction was enhanced by the multiplicity of the sequence elements in both proteins. We do not know whether these sequence repetitions are simply to increase the interaction strength between the two proteins or to accommodate other protein interactions in addition to the one we found in this work. More investigations are needed to understand the molecular mechanism for the interaction between the two proteins and to see how these proteins are structurally and functionally connected to other proteins within and outside of the complex.

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