Articles

Human Alanyl-tRNA Synthetase: Conservation in Evolution of Catalytic Core and Microhelix Recognition[†,‡]

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ABSTRACT: The class II *Escherichia coli* and human alanyl-tRNA synthetases cross-acylate their respective tRNAs and require, for aminoacylation, an acceptor helix G3:U70 base pair that is conserved in evolution. We report here the primary structure and expression in the yeast *Pichia* of an active human alanyl-tRNA synthetase. The N-terminal 498 amino acids of the 968-residue polypeptide have substantial (41%) identity with the *E. coli* protein. A closely related region encompasses the class-defining domain of the *E. coli* enzyme and includes the part needed for recognition of the acceptor helix. As a result, previously reported mutagenesis, modeling, domain organization, and biochemical characterization on the *E. coli* protein appear valid as a template for the human protein. In particular, we show that both the *E. coli* enzyme and the human enzyme purified from *Pichia* aminoacylate 9-base pair RNA duplexes whose sequences are based on the acceptor stems of either *E. coli* or human alanine tRNAs. In contrast, the sequences of the two enzymes completely diverge in an internal portion of the C-terminal half that is essential for tetramer formation by the *E. coli* enzyme, but that is dispensable for microhelix aminoacylation. This divergence correlates with the expressed human enzyme behaving as a monomer. Thus, the region of close sequence similarity may be a consequence of strong selective pressure to conserve the acceptor helix G3:U70 base pair as an RNA signal for alanine.

In this work, we report the results of investigation of a human class II tRNA synthetase whose *Escherichia coli* counterparts is one of the best examples of a tRNA synthetase that is active on RNA oligonucleotide substrates (Hou et al., 1989). Three different analyses suggest that the acceptor helix-containing domain of tRNAs originated independently of the domain harboring the anticodon (Buechter & Schimmel, 1993b; Maizels & Weiner, 1993; Noller, 1993). In addition, RNA oligonucleotides that recapitulate the acceptor stems of tRNAs are substrates for aminoacylation for at least eight different tRNA synthetases (Francklyn et al., 1992; Martinis & Schimmel, 1992; Nureki et al., 1993; Sampson & Saks, 1993; Frugier et al., 1994). The RNA sequences/structures proximal to the amino acid attachment site constitute an operational RNA code for amino acids that may have predated and then later became part of the genetic code when the two domains of the L-shaped tRNA molecule were joined together (Buechter & Schimmel, 1993b; Schimmel et al., 1993).

The aminoacyl-tRNA synthetases are generally thought to have arisen early in evolution as the agents that interpreted the operational RNA code and ultimately established the genetic code by attaching specific amino acids to tRNAs that contained the cognate trinucleotide anticodons. This historical development is reflected in the structures of contemporary tRNA synthetases, which, to a first approximation, are composed of two domains (Moras, 1992; Buechter & Schimmel, 1993b; Schimmel et al., 1993). The class-defining catalytic domain interacts with the acceptor-TψC stem, while a second domain, idiosyncratic to the synthetases, provides for contacts with the rest of the tRNA structure, including the anticodon (Rould et al., 1989; Ruff et al., 1991; Biou et al., 1994).

The tRNA synthetases are divided into two classes of ten enzymes each (Eriani et al., 1990). Alanyl-tRNA synthetase is one of the class II enzymes, which characteristically have three highly degenerate sequence motifs (Eriani et al., 1990; Ribas de Pouplana et al., 1993; Shi et al., 1994; Davis et al., 1994) associated with specific parts of an eight-stranded antiparallel β-structure (Cusack et al., 1990; Ruff et al., 1991). The *E. coli* enzyme recognizes a G3:U70 base pair as the major determinant for aminoacylation with alanine (Hou & Schimmel, 1988). Transfer of this base pair into other tRNAs confers alanine acceptance (Hou & Schimmel,

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[‡] The nucleotide sequence data reported in this paper will appear in the GSDB, DDBJ, EMBL, and CBI nucleotide sequence databases with the accession number D32050.

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1988, 1989; McClain & Foss, 1988). This base pair is unique to alanine tRNAs in *E. coli* and is also present in eukaryote cytoplasmic alanine tRNAs. Both the purified enzyme from the insect *Bombyx mori* and HeLa cell extracts aminoacylate tRNA substrates in a G3:U70-dependent manner, confirming that the major determinant for alanine acceptance is conserved in evolution (Hou & Schimmel, 1989).

The *E. coli* enzyme also aminoacylates RNA oligonucleotides that recapitulate as few as the first four base pairs of the acceptor stem of $tRNA^{Ala}$, including the G3:U70 base pair (Francklyn & Schimmel, 1989; Musier-Forsyth et al., 1991a; Shi et al., 1992). Although the enzyme makes contact with parts of the tRNA outside the acceptor stem, the anticodon is not protected from nuclease cleavage when $tRNA^{Ala}$ is bound (Park & Schimmel, 1988). In addition, nucleotide sequence changes within the anticodon trinucleotide have no effect on the kinetic parameters for aminoacylation (Park et al., 1989). These data demonstrate a lack of physical interaction between *E. coli* alanyl-tRNA synthetase and the anticodon of $tRNA^{Ala}$ and provide strong evidence that the relationship between the trinucleotide of the genetic code and alanine is indirect and was possibly established when the acceptor helix domain with the sequence/structure representing the operational RNA code for alanine was fused to the template "reading head" of the anticodon-containing domain (Buechter & Schimmel, 1993b; Schimmel et al., 1993).

The historical development of tRNA synthetases thus is closely connected to the evolution of tRNAs and the genetic code. Two classes of synthetases were established quite early and remain fixed, as no synthetase has been reported to switch from one class to the other during evolution. In spite of these constraints, individual enzymes have followed different evolutionary paths while remaining in the same class. These differences are especially apparent when a synthetase from a prokaryote such as *E. coli* is compared with its counterpart in human cells. For example, the class-defining catalytic domain of the class I *E. coli* and a human cytoplasmic isoleucyl-tRNA synthetases clearly are derived from a common ancestor, even though the second domain, which interacts with the anticodon of $tRNA^{Ile}$, is highly diverged between the two proteins (Shiba et al., 1994a). In contrast, the human (and *B. mori* and *Saccharomyces cerevisiae*) class II glycyl-tRNA synthetase is widely diverged throughout its entire primary structure from that of the *E. coli* enzyme, so that the sequences themselves provide no evidence for a common ancestor to the two enzymes (Shiba et al., 1994b). This difference is correlated with the failure of the human and *E. coli* enzymes to cross-acylate their respective tRNAs (Shiba et al., 1994b). This failure to cross-acylate may be due, at least in part, to a U73/A difference in the N73 nucleotide that is needed for aminoacylation of $tRNA^{Gly}$ and microhelix substrates by the *E. coli* enzyme (Francklyn et al., 1992). In addition, *E. coli* glycyl-tRNA synthetase has an $\alpha_2\beta_2$ quaternary structure (Ostrem & Berg, 1974), which is not shared by the other eukaryotic glycine enzymes (Dignam & Dignam, 1984; Shiba et al., 1994b). Similarly, the class II *E. coli* and human histidyl-tRNA synthetases are diverged significantly, although a distant relationship between the sequences of the two enzymes can be discerned (Tsui & Siminovitch, 1987; Raben et al., 1992).

With these considerations in mind, we sought to clone human alanyl-tRNA synthetase and, given that it cross-acylates *E. coli* $tRNA^{Ala}$, determine whether its primary structure is better conserved with the *E. coli* enzyme than are the class II human glycyl- and histidyl-tRNA synthetases with their respective *E. coli* counterparts. A better conservation would allow us to use the *E. coli* protein as a template to understand the human enzyme, and that relationship could then be further tested by direct experiments with human alanyl-tRNA synthetase. The *E. coli* enzyme is a tetramer of identical 875-amino acid polypeptides (Putney et al., 1981a,b). The class-defining motifs with the catalytic core can be isolated as a monomeric polypeptide composed of the N-terminal 461 amino acids (Jasin et al., 1983). While the activity (k_{cat}/K_m) of the truncated monomer on *E. coli* $tRNA^{Ala}$ is reduced compared to the native tetramer (Ho et al., 1985), aminoacylation of RNA microhelix substrates is unaffected by the large deletion and monomerization of the enzyme (Buechter & Schimmel, 1993a). In addition, the activity of the truncated monomer on the full $tRNA^{Ala}$ is indistinguishable from that on microhelix substrates (Buechter & Schimmel, 1993a). Thus, the C-terminal domain, including the portion responsible for oligomerization, is dispensable for "reading" the operational RNA code for alanine.

On the other hand, Dignam et al. reported that the rat liver enzyme was a monomer, but with a polypeptide size somewhat larger than that of the *E. coli* protein (Dignam et al., 1991). This raised the question of whether the mammalian enzyme might be slightly different from the *E. coli* protein, even though it required the G3:U70 base pair for aminoacylation. Thus, we also wanted to determine whether the human enzyme could aminoacylate RNA oligonucleotide substrates, as an example of the conservation of the operational RNA code for alanine in evolution, and, if so, whether a close sequence−structure relationship between the enzymes could be discerned in the part of the sequence of the *E. coli* protein (N-terminal 461 amino acids) required for microhelix aminoacylation.

EXPERIMENTAL PROCEDURES

Cross-Species Polymerase Chain Reaction. By using mRNA isolated from the human fetal fibroblast cell strain TIG-2 (Ohashi et al., 1980), a cross-species polymerase chain reaction (PCR)[1] was performed as described previously (Shiba et al., 1994a). Reactions were run for 35 cycles with the Gene Machine II thermal controller (U.S. Scientific Plastics, Ocala, FL), with 2 min at 94 °C, 2 min at 55 °C, and 3 min at 72 °C for each cycle. Amplified DNA fragments were cloned into the *Sma*I site of phagemid pTZ19R (Mead et al., 1986), and their sequences were then determined.

Screening of Human cDNA Library. A cDNA plasmid library derived from the human T-cell line KUT-2 (Shiba et al., 1994a) was screened with an oligonucleotide (TCTCACCCCATGGCAAAGCTG) that is specific for the type A cloned PCR fragment (see Results). For this purpose, approximately 5×10^5 colonies were immobilized onto 25 nylon filters (Duraron-UV, 82 mm, Stratagene, La Jolla, CA) as described (Shiba et al., 1994b). These filters were first

[1] Abbreviations: PCR, polymerase chain reaction; kbp, kilobase pair(s); bp, base pair(s).

preincubated in 50 mL of 6× SSC (Sambrook et al., 1989), 5× Denhardt's solution (Sambrook et al., 1989), 20 mM NaH_2PO_4, and 500 μg/mL denatured salmon sperm DNA (Sigma Chemical Co., St. Louis, MO) at 42 °C for 2 h and then incubated with the radiolabeled (5×10^8 cpm) oligonucleotide probe [labeled with ^{32}P using [γ-^{32}P]ATP (7000 Ci/mmol, ICN Biomedicals, Costa Mesa) and the Megalabel Kit of Takara (Ohtsu)] in 50 mL of 6× SSC, 1% SDS, 20 mM NaH_2PO_4, and 500 μg/mL salmon sperm DNA at 42 °C for 16 h. Filters were washed twice in 6× SSC and 0.1% SDS at 56 °C for 15 min and autoradiographed by exposure to Kodak AR film with the Lightning Plus intensifier screen (DuPont, Wilmington, DE) at −80 °C. Three positive clones were obtained. The plasmid designated pAA1 had the longest insert and was characterized further.

DNA Sequencing. Plasmid pAA1 was digested at the single *Mlu*I site within the vector, made flush-ended by treatment with the Klenow fragment and the four dNTPs (Sambrook et al., 1989), and then digested at the lone *Eco*RI site in the vector. The 3.4 kbp insert released by these digestions was recovered from an agarose gel and then cloned into the *Eco*RI and *Sma*I sites of phagemid pBluescript KS-(+) (Stratagene) to construct plasmid pAA1-11. The *Eco*RI−*Not*I fragment (which includes the entire insert) of pAA1-11 was then recloned into the *Eco*RI−*Not*I sites of pBluescript KS(−) to give plasmid pAA1-12. The *Eco*RI−*Bam*HI fragment from pAA1-12 was recloned into the *Eco*RI−*Bam*HI sites of pTZ19R to give plasmid pAA1-13. Unidirectional deletions were made from pAA1-12 and pAA1-13 by using the Kilo-Sequence deletion kit (Takara Shuzo, Ohtsu). Single-stranded DNA was obtained by superinfection with helper phage M3 K07 (Vieira & Messing, 1989), and sequences were determined with the ALF automated DNA sequencer (Pharmacia, Piscataway, NJ). The sequence of each strand was determined at least once.

Analysis of Sequences. Multiple sequence alignments were performed with the PILEUP program of Genetics Computer Group (Madison, WI), which makes alignments on the basis of the method of Needleman and Wunsch (1970). With an alignmnet of four sequences of alanyl-tRNA synthetase from *E. coli* (Putney et al., 1981b), *Arabidopsis thaliana* (Mireau et al., 1993), *B. mori* (Chang & Dignam, 1990), and *Homo sapiens*, the average similarity at each position was calculated by using the PLOTSIMILARITY program of the Genetics Computer Group. The identity scores were computed by dividing the number of identical residues among all four sequences by the total length of the shortest sequence of the four in a given region, and the results were expressed as a percentage (Shiba et al., 1994b).

Construction of a Pichia pastoris Strain for the Expression of Human Alanyl-tRNA Synthetase. An *Nde*I site was placed at the first ATG codon (nucleotides 111−113) of the coding sequence of the human cDNA using the Oligonucleotide-Directed *In Vitro* Mutagenesis System Kit (Amersham, Buckinghamshire, UK). The *Nde*I−*Not*I fragment was made flush by treatment with the Klenow fragment and the four dNTPs and then ligated with an *Eco*RI linker oligonucleotide. The fragment was then cloned into the *Eco*RI site of the *P. pastoris* expression vector pHIL-D2, which has a *HIS4* marker (Invitrogen), to give a plasmid pKS491. This plasmid has the cDNA clone for human alanyl-tRNA synthetase behind the promoter of the gene for *P. pastoris* alcohol oxidase (*AOX1*). Five micrograms of pKS491 was linearized by digestion with *Not*I (which cleaves at sites within the vector) and was introduced into spheroplasts of strain GS115 [a *HIS4* mutant of *P. pastoris* (Invitrogen)]. HIS^+ transformants were selected by growth for 4 days at 30 °C on plates containing 18.6% D-glucitol, 1.34% yeast nitrogen base without amino acids (Difco, Detroit), 0.4 μg/mL D-biotin, 2% D-glucose, and 50 μg/mL each of L-glutamic acid, L-methionine, L-lysine, L-leucine, and L-isoleucine. Colonies that grew were checked for their utilization of methanol by scoring for growth on MM plates containing 1.34% yeast nitrogen base without amino acids, 0.4 μg/mL D-biotin (Sigma), and 0.5% methanol and, separately, on MD plates containing 1.34% yeast nitrogen base without amino acids, 0.4 μg/mL D-biotin, and 2% D-glucose. If the DNA fragment introduced was integrated into the *AOX1* locus of the host chromosome by homologous recombination between the 5′ and 3′ *AOX1* sequences in the pHIL-D2 vector portion of pKS491 and those in the genome, then the resultant cells are disrupted at the *AOX1* gene and cannot metabolize methanol as the sole carbon source (Mut^- phenotype). One of the His^+/Mut^- transformants (that grow on MD plates but not on MM plates) was purified as the strain designated NOR-Aa6. The NOR-0 control strain was taken as the His^+/Mut^- transformant obtained by introduction of the vector pHIL-D2 without the cDNA insert encoding human alanyl-tRNA synthetase.

Expression of Human Alanyl-tRNA Synthetase in P. pastoris and Preparation of Extracts for Aminoacylation of Bovine and E. coli tRNA. Strains NOR-Aa6 and NOR-0 were grown at 30 °C for 24 h in 5 mL of 1.34% yeast nitrogen base without amino acids, 0.4 μg/mL D-biotin, and 1% glycerol. Cells were harvested and resuspended in 10 mL of MM medium and incubated for an additional 66 h at 30 °C. The cells were then resuspended in 0.2 mL of 50 mM sodium phosphate (pH 7.4), 1 mM phenylmethanesulfonyl fluoride (Sigma), 1 mM ethylenediaminetetraacetic acid, and 5% glycerol and broken with acid-washed glass beads (425−600 μm, Sigma). Aminoacylation activity in cell extracts was assayed as described (Shiba & Schimmel, 1992) by using 30 ng of crude extract (determined by the Protein Assay Kit, Bio-Rad, Richmond) and 120 μCi of L-[3-^{3}H]alanine (76.9 mCi/mmol, New England Nuclear, Boston) in a total reaction mixture of 100 μL containing 0.4 mg/mL of either of *E. coli* MRE 600 or calf liver tRNA (Boehringer Mannheim), 20 μM alanine, 0.1 mg/mL bovine serum albumin, 20 mM KCl, 10 mM $MgCl_2$, 20 mM β-mercaptoethanol, 4 mM ATP, and 50 mM sodium phosphate (pH 7.5).

Purification of Human Alanyl-tRNA Synthetase from P. pastoris. One liter of strains NOR-Aa6 and NOR-0 was grown at 30 °C as described earlier. SDS−PAGE analysis of lysed cell extract before and after methanol induction indicated that human alanyl-tRNA synthetase was present as roughly 3% of total protein. Cells were harvested, resuspended in 25 mL of 40 mM [bis(2-hydroxyethyl)imino]-tris(hydroxymethyl)methane (pH 7.5), 2 mM β-mercaptoethanol, 10% glycerol, 1 mM benzamidine (Sigma), 20 μg/mL leupeptin (Sigma), 25 μg/mL aprotinin (Sigma), 3.5 μg/mL pepstatin A (Sigma), and 1 mM phenylmethanesulfonyl fluoride (Sigma) at 4 °C, and lysed in a French press (SLM Amnica) at 10 000 psi. The remainder of the purification was carried out at 4 °C. Cell debris was removed by centrifugation at 35000*g* for 60 min, and the suspension was applied to a 10 × 2.5 cm DEAE-Superose column. The

column was step-eluted with 60 mL of 40 mM [bis(2-hydroxyethyl)imino]tris(hydroxymethyl)methane (pH 7.5), 2 mM β-mercaptoethanol, 300 mM NaCl, and 1 mM phenylmethanesulfonyl fluoride. The eluate was fractionated further on a Mono Q 10/10 (Pharmacia) column and eluted with a 250 mL gradient of NaCl that extended from 0 to 400 mM in 40 mM [bis(2-hydroxyethyl)imino]tris(hydroxymethyl)-methane (pH 7.5) and 2 mM β-mercaptoethanol. Enzyme activity from the NOR-Aa6 strain (but not from the "vector alone" control strain NOR-0) eluted at 220 mM NaCl. Peak fractions were pooled, concentrated using ultrafiltration, applied to a Superose 6 HR 10/30 (Pharmacia) column, and eluted with 10 mL of 40 mM [bis(2-hydroxyethyl)imino]-tris(hydroxymethyl)methane (pH 7.5) and 2 mM β-mercaptoethanol. The enzyme was estimated to be 90% of total protein by electrophoresis on an 8% polyacrylamide gel in the presence of 0.1% SDS.

Determination of Molecular Weight of Human Alanyl-tRNA Synthetase. The molecular weight of human alanyl-tRNA synthetase was determined by Superose 6 chromatography. A Superose 6 HR 10/30 (Pharmacia) column was equilibrated with 40 mM [bis(2-hydroxyethyl)imino]tris-(hydroxymethyl)methane (pH 7.5), 100 mM NaCl, and 2 mM β-mercaptoethanol. All samples were applied in 0.1 mL. The column void volume was determined from the elution volume of blue dextran [average molecular weight 2 000 000 (Sigma)]. The elution volumes of standard proteins were determined by separate chromatography of ferritin (5 mg/mL), aldolase (5 mg/mL), catalase (5 mg/mL), and *E. coli* alanyl-tRNA synthetase (7 mg/mL). Elution positions of standards and *E. coli* and human alanyl-tRNA synthetases were determined by optical density (280 nm).

Aminoacylation Activity of Human Alanyl-tRNA Synthetase on RNA Duplex Substrates. Chemical synthesis and purification of RNA substrates were carried out as outlined previously (Usman et al., 1987; Scaringe et al., 1990; Musier-Forsyth et al., 1991a). Purified human enzyme was assayed for its ability to aminoacylate duplex substrates using protocols previously described (Schreier & Schimmel, 1972; Hill & Schimmel, 1989; Musier-Forsyth et al., 1991a).

RESULTS

Cloning of a Human Alanyl-tRNA Synthetase by Cross-Species Polymerase Chain Reaction. An alignment-guided cross-species PCR approach was used recently to clone human isoleucyl- and glycyl-tRNA synthetases (Shiba et al., 1994a,b). A similar strategy was used in this work. Primers for PCR were designed by inspection of a multiple alignment of the complete nucleotide coding sequences for *E. coli* (Putney et al., 1981b) and *B. mori* (Chang & Dignam, 1990) alanyl-tRNA synthetases and the partial sequences of the N-terminal coding regions of *Rhizobium leguminosarum* (Selbitschka et al., 1991) and *R. meliloti* (Selbitschka et al., 1991) alanyl-tRNA synthetases. Two well-conserved regions among the four species start at codons 38 and 86, respectively, of the *E. coli* enzyme (the locations for the primers are shown by long open arrows in Figure 1). These regions were chosen for designing the primers KY-23 (TTYR-CIAAYGCIGGIATGAAYCARTTYAAR) and KY-25 (RT-TICCCATCATYTCRAARAAIGTRTGRTG, where Y is pyrimidine, R is purine, and I is inosine). These primers were used for PCR amplification of cDNA prepared from human fetal fibroblast cell strain TIG-2. Amplified fragments of approximately 170 bp were then cloned into the *SmaI* site of phagemid pTZ19R. Sequence analysis of six cloned PCR products showed two distinct sequences (type A in four clones and type B in two clones) that were related to alanyl-tRNA synthetase.

Isolation and Sequence Determination of Type A cDNA. Poly(A)$^+$ RNA was isolated from human T-cell line KUT-2 and used to construct a cDNA library (Shiba et al., 1994). The library was screened with an oligonucleotide specific to the type A PCR product. Of three positive clones obtained, one had a 3.3 kbp insert and the remaining two had 1.5 kbp inserts. We also screened the library with a probe specific to the type B PCR product. In this case, we failed to obtain clones other than false positives and for that reason focused our efforts on the type A sequence.

We determined the entire 3344-nucleotide sequence (exclusive of the poly(A) segment) of the type A cDNA clone. It codes for a 968-amino acid polypeptide. The size of this coding sequence was similar to that of a single 3.5 kb transcript detected by Northern blot analysis with a type A-specific probe of poly(A)$^+$ RNA resolved by agarose gel electrophoresis (data not shown). The open reading frame starts at the first AUG codon, which encompasses nucleotides 111−113 of the sequenced insert. The AUG-containing sequence of AagAUGG retains the "A" and "G" flanking nucleotides of Kozak's optimal translation initiation sequence of ACCAUGG (data not shown; Kozak, 1986). There is no mitochondrial targeting sequence of the sort seen at the N-terminus of nuclear encoded mammalian mitochondrial proteins (von Heijne et al., 1989), suggesting that the type A sequence corresponds to a cytoplasmic protein. The predicted translation product has a molecular weight of 106 734, which compares with the molecular weight (determined by SDS−polyacrylamide gel electrophoresis) of the 110 000 antigen that reacts with the human PL-12 autoantibody that is directed specifically against human alanyl-tRNA synthetase (Bunn et al., 1986).

Primary Structure and Domain Organization of Human Alanyl-tRNA Synthetase. By using multiple alignments of partial and complete sequences of alanyl-tRNA synthetases with the neural net-based secondary structure program of Rost and Sander (1992) and additional information from the known structures of the class II yeast aspartyl- and *E. coli* seryl-tRNA synthetases (Cusack et al., 1990; Ruff et al., 1991), a model for the secondary structure of the class-defining domain of the *E. coli* enzyme (up to amino acid 249) was developed (Ribas de Pouplana et al., 1993; Shi et al., 1994). The model has the eight-stranded antiparallel β-structure and three motifs characteristic of class II enzymes, including motif 1 (helix−loop−strand), motif 2 (strand−loop−strand), and motif 3 (strand−helix). This model has been further tested and refined by partial sequence information on additional alanine enzymes and by the construction and analysis of mutant proteins with substitutions at more than 40 different positions (Shi et al., 1994; Davis et al., 1994). In analyzing the human alanyl-tRNA synthetase primary structure, we took advantage of this model and of additional information about the locations of critical parts of the *E. coli* enzyme protein obtained from an earlier deletion analysis (Jasin et al., 1983; Regan et al., 1987).

The predicted 968-amino acid polypeptide aligns well with the sequences of *E. coli* (Putney et al., 1981b), *B. mori*

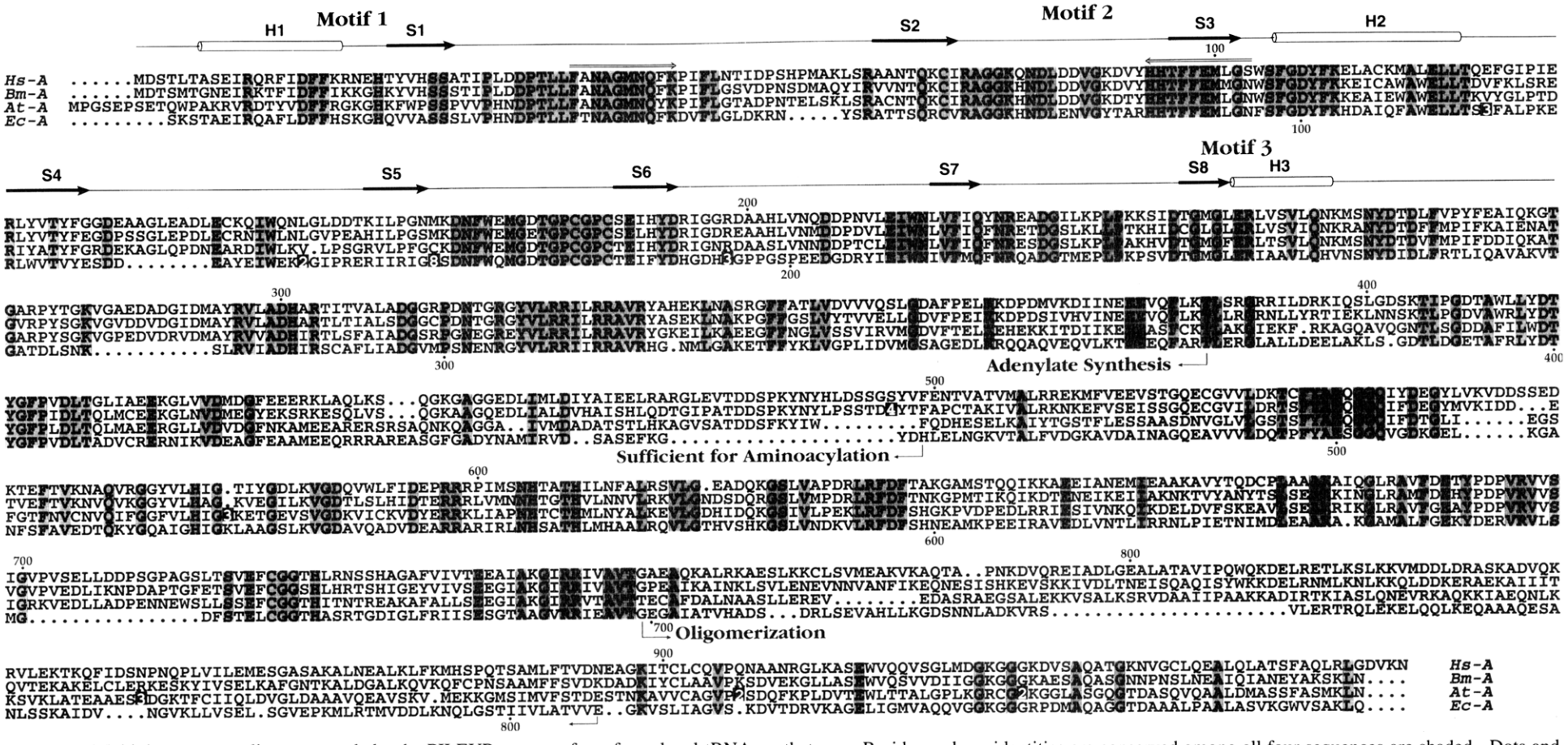

FIGURE 1: Multiple sequence alignment made by the PILEUP program from four alanyl-tRNA synthetases. Residues whose identities are conserved among all four sequences are shaded. Dots and numbers in black boxes show gaps and insertions, respectively. Regions from which primers were designed are shown by white arrows. Numbers above the alignment correspond to amino acid residues of the human alanyl-tRNA synthetase. The predicted secondary structure elements (black arrows and tubes represent β-strands and α-helices, respectively) of *E. coli* alanyl-tRNA synthetase (Ribas de Pouplana et al., 1993; Shi et al., 1994) are shown across the top. Structures needed for adenylate formation, aminoacylation, and oligomerization and the class-defining motifs 1, 2, and 3 are shown. Abbreviations: Hs, *Homo sapiens* (this study); Bm, *Bombyx mori* (Chang & Dignam, 1990); At, *Arabidopsis thaliana*; Ec, *Escherichia coli* (Putney et al., 1981b).

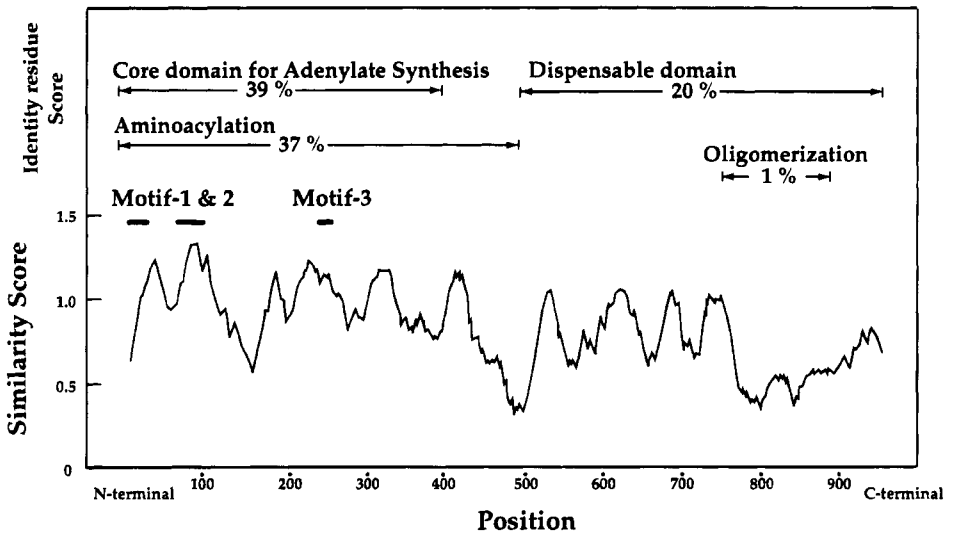

FIGURE 2: Relationship of the functional domains and conserved motifs to sequence conservation among four alanyl-tRNA synthetases as represented by "similarity score" and "identity residue score". The similarity score was obtained with the PLOTSIMILARITY program (Genetic Computer Group, Madison, WI), with a widow range of 30 residues, and its values are shown on the ordinate. The identity residue scores in each functional modular domain were calculated as described in Experimental Procedures and are shown across the top. Numbers on the abscissa represent residues of the human alanyl-tRNA synthetase. The locations of the class-defining motifs 1, 2, and 3 are shown.

(Chang & Dignam, 1990), and *A. thaliana*[2] alanyl-tRNA synthetases (Figure 1). Altogether 214 identifies across all four sequences emphasize the relatedness through evolution of a significant portion of these enzymes. This relatedness is most pronounced for the part that corresponds to the catalytic domain of the *E. coli* enzyme (Figure 2). In particular, this close relatedness includes 16 conserved amino acids in a 22-residue region between predicted strands S1 of motif 1 and S2 of motif 2, 20 of the 34 amino acids of predicted motif 2, 13 of 17 amino acids between predicted strands S5 and S6, and 11 amino acid identities in a 12-residue sequence beyond motif 3 that is centered around L328 of the human enzyme. The strong conservation in these regions of predicted secondary structure provides further support for the secondary structure model.

The domain needed for adenylate synthesis (but that has no aminoacylation activity) in the *E. coli* protein extends from the N-terminus to T368 (Regan et al., 1987), which corresponds to T385 of the human enzyme. Full aminoacylation activity on microhelix substrates for the *E. coli* enzyme is achieved with a fragment that extends to H461 (Buechter & Schimmel, 1993a; aligns with F499 of the human enzyme). The sequence that extends to F499 of human alanyl-tRNA synthetase has 37% identity among four sequences or 41% identity between human and *E. colli* enzymes. This degree of relatedness in the catalytic domain is one of the highest reported between *E. coli* and human tRNA synthetases that have been compared.

Most striking is the complete divergence of sequences in the oligomerization domain mapped by deletion analysis to residues 699−808 of the *E. coli* enzyme (Jasin et al., 1983). G699−E808 in the *E. coli* protein correspond to G753−N894 in the human enzyme (alignment of Figure 1). Although the deletion mapping of the oligomerization domain of the *E. coli* alanine enzyme was inherently approximate, the region of complete divergence from the human sequence almost exactly corresponds to the assignment made by that analysis. After N894 of the human enzyme, identities between the sequences of the four enzymes occur at regularly spaced intervals, and there are many positions where three if not four out of four enzymes have identical amino acids. Similarly, the sequence from F499 to A756 of human alanyl-tRNA synthetase is related closely to the corresponding part of the other three enzymes. For these reasons, we believe that the sequence divergence of the internal piece of the C-terminal domain is precisely bracketed on either side by the previously mapped oligomerization domain of *E. coli* alanyl-tRNA synthetase (Jasin et al., 1983).

The segment in the human enzyme that replaces the oligomerization domain of the *E. coli* enzyme has a distant, if any, relationship, to the corresponding segments of the two eukaryotic alanyl-tRNA synthetases. These, in turn, are also highly diverged from each other (Figure 3).

Although aminoacylation of microhelix substrates by *E. coli* alanyl-tRNA synthetase is unaffected by the removal of the entire C-terminal half-starting from L462 (corresponds to E500 of human protein; Figure 1) (Buechter & Schimmel, 1993b), catalytic and binding activities with the full $tRNA^{Ala}$ substrate are diminished by the deletion (Ho et al., 1985; Regan et al., 1987). The effect on binding activity appears to be specifically due to the deletion of L462−T698 (Regan et al., 1987). This segment is highly homologous among the four enzymes and also encompasses the locations of the G674D and G677D mutations of the *E. coli* enzyme that are responsible for the temperature-sensitive phenotype of the *alaS4* and *alaS5* mutant alleles, respectively (Jasin et al., 1985). These mutant alleles encode enzymes that have a diminished k_{cat} for aminoacylation, with little effect on K_m for $tRNA^{Ala}$ (Jasin et al., 1985). Thus, this segment of the protein may be needed specifically for synthetase−tRNA interactions that stabilize the transition state for aminoacylation, in addition to binding interactions in the ground state, and these interactions may involve parts of the tRNA structure outside of the acceptor helix domain.

[2] EMBL accession number Z22673 (1993, deposited by H. Mireau, D. Lancelin, G. Pelletier, and I. Small).

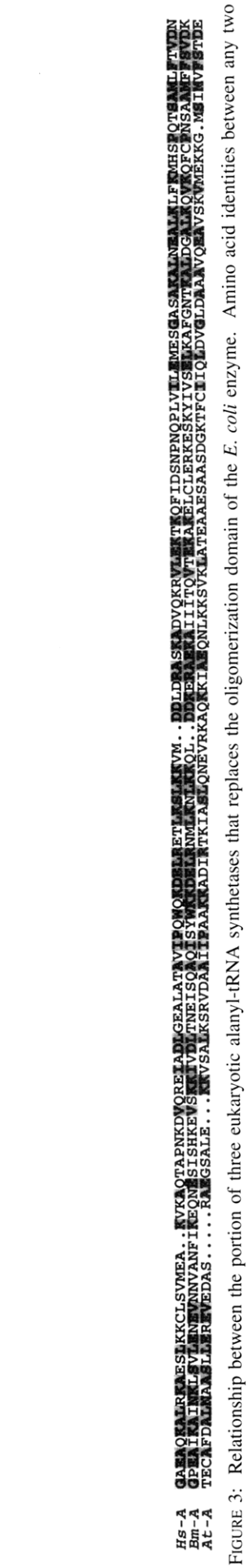

FIGURE 3: Relationship between the portion of three eukaryotic alanyl-tRNA synthetases that replaces the oligomerization domain of the *E. coli* enzyme. Amino acid identities between any two sequences are shaded.

Expression of Human Enzyme in P. pastoris and Detection of Aminoacylation Activity in Extracts. The human enzyme was expressed in *P. pastoris* containing an integrant of the cloned human cDNA (strain NOR-Aa6), and extracts of this strain were compared with an identical strain lacking the integrated human cDNA (strain NOR-0). After induction, extracts prepared from strain NOR-Aa6, but not from strain NOR-0 showed significant aminoacylation activity on bovine tRNA. On the basis of cross-acylation of *E. coli* tRNA with alanine by HeLa cell extracts (Hou & Schimmel, 1989b), we expected that the activity expressed in *P. pastoris* should also cross-acylate *E. coli* tRNA. This expectation was fulfilled (Figure 4). These observations confirmed that the cloned human cDNA encoded an active enzyme.

Human Enzyme Is a Monomer. The function in the eukaryotic enzymes of the segment that replaces the oligomerization domain of *E. coli* alanyl-tRNA synthetase is not known, nor are there sequences of other prokaryotic alanyl-tRNA synthetases to determine whether oligomerization is specific to prokaryotes. Removal of the C-terminal portion (from E700 to Q875) of the *E. coli* enzyme has no effect on tRNA binding, but the aminoacylation activity of the C-terminally truncated protein is diminished (Regan et al., 1987).

Although we successfully expressed the recombinant human enzyme in *E. coli*, no activity for this protein could be detected. Alternatively, we expressed the recombinant human enzyme in *P. pastoris* and obtained active enzyme judged to be 90% homogeneous. By using molecular weight markers that included bovine serum albumin (M_r = 67 000), aldolase (158 000), catalase (232 000), *E. coli* alanyl-tRNA synthetase (380 000), and ferritin (440 000), we used Superose gel filtration chromatography to obtain the molecular weight of the expressed enzyme under native conditions (Figure 5). The data were analyzed in terms of K_{av} values, where $K_{av} = (V_e - V_0)/(V_t - V_0)$ and V_e, V_0, and V_t are the elution, void, and column volumes, respectively. From this analysis, we obtained a molecular weight of 98 000. This compares with the value of 106 734 predicted from the amino acid sequence. The reasonable agreement between these values demonstrates that the human enzyme is a monomer.

Human Enzyme Aminoacylates RNA Duplex Substrates on the Basis of Acceptor Stems of Human or E. coli tRNA^{Ala}. Although the human enzyme in HeLa cell extracts was shown previously to aminoacylate human and *E. coli* tRNA^{Ala} (Hou & Schimmel, 1989b), RNA duplex substrates based on part of the acceptor-TψC minihelix domain have not previously been tested. Synthetic RNA duplexes consisting of the first nine base pairs of the *E. coli* and human cytoplasmic tRNA^{Ala} acceptor stems were chemically synthesized and tested for activity (Figure 6A). These duplexes share the critical G3:U70 base pair and differ in sequence at the 5:69, 6:68, and 7:67 positions of the acceptor stem. The recombinant human enzyme aminoacylates the "human" RNA duplex substrate and has no detectable activity on the G3:C70 variant substrate (Figure 6B). (In these initial studies, we did not determine kinetic parameters for the human enzyme, nor did we investigate factors that influences these parameters. Instead, we concentrated on testing for G3:U70-specific aminoacylation.) This enzyme also cross-acylates the *E. coli* RNA duplex substrate (data not shown).

Thus, G3-U70-specific RNA duplex recognition and aminoacylation are conserved from bacteria to man in the

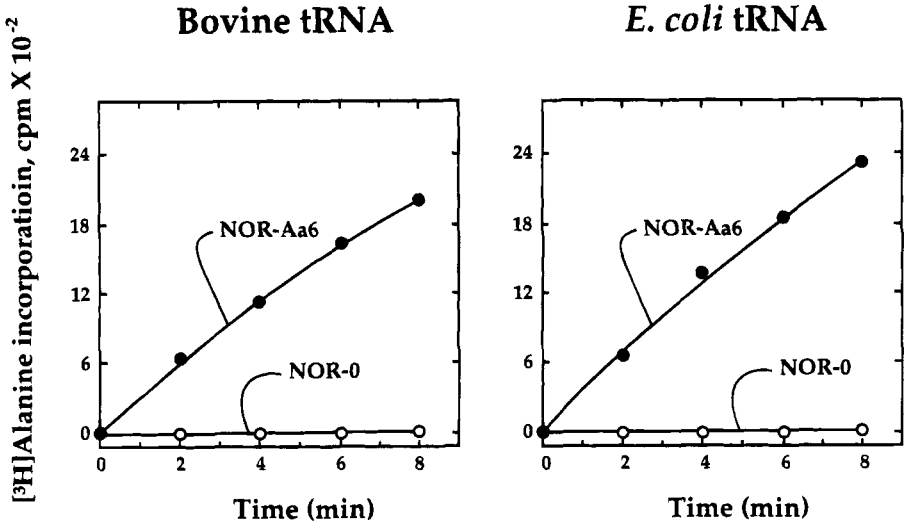

FIGURE 4: Species-specific incorporation of [^{3}H]alanine into bovine and *E. coli* tRNAs as a function of time. The enzyme used was an extract from *P. pastoris* strains NOR-Aa6 (expressing the human enzyme) or NOR-0 (control strain). At this concentration of cell extract (corresponding to 30 ng/100 μL for each graph), the activity from chromosomally encoded *P. pastoris* alanyl-tRNA synthetase was at the background level.

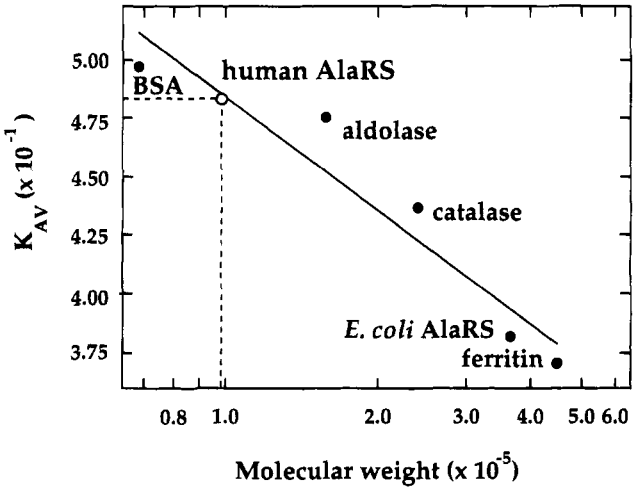

FIGURE 5: Superose 6 chromatography of native human alanyl-tRNA synthetase, *E. coli* alanyl-tRNA synthetase, and standard proteins. Chromatography was performed as described under Experimental Procedures. Molecular weight is plotted versus K_{av} (see text). From these data, the molecular weight of native human alanyl-tRNA synthetase is determined to be 98 000.

alanine system. Because a fragment composed of the first 461 amino acids of the *E. coli* enzyme has full activity for the aminoacylation of these model substrates based on acceptor stem sequences (Buechter & Schimmel, 1993a), we expect that the analogous N-terminal segment (N-terminal segment that extends to F499) of the human enzyme contains all of the determinants needed for RNA duplex aminoacylation.

DISCUSSION

The N-terminal region of the human enzyme, which extends to F499, contains the motif conserved among alanyl-tRNA synthetases for recognition of the acceptor helix G3:U70 base pair (Buechter & Schimmel, 1993a). Although the location of this motif for acceptor helix recognition is not known, an understanding of the active site region and of the residues needed for adenylate synthesis and transfer has been obtained from the studies of the *E. coli* protein. Because of the close similarity between the *E. coli* and human enzyme sequences in this region, we believe that the phenotypes of analogous mutations constructed in the human enzyme would be similar to those found in *E. coli* protein.

For example, the *E. coli* enzyme has been cross-linked to a benzophenone photoaffinity label attached to the 5'-side of U70 (Musier-Forsyth & Schimmel, 1994). The cross-linked peptide spanned the region from G161 at the end of strand S5 to the beginning of strand S8 of motif 3 (Musier-Forsyth & Schimmel, 1994). Alanine-scanning mutagenesis was applied to all residues bearing functional side chains and conserved among sequenced alanyl-tRNA synthetases for this region (Shi et al., 1994). Of the 20 individual alanine substitutions made, 17 resulted in functional proteins and 2 gave proteins that were unstable. The remaining D235→A mutant protein was unable to complement an *alaS* null allele, but accumulated *in vivo* (Shi et al., 1994; D235 corresponds to D239 in the human protein). This protein was active for adenylate synthesis, but was severely reduced in activity for transfer of the activated amino acid from the adenylate to the 3'-end of $tRNA^{Ala}$. By comparison with the structure of the class II yeast aspartyl-tRNA synthetase in complex with $tRNA^{Asp}$, we proposed that D235 is important for interactions needed for adenylate transfer at the 3'-end of $tRNA^{Ala}$ (Shi et al., 1994).

The intervening loop between the two antiparallel strands (S2 and S3) of motif 2 contains an acidic residue that makes contacts with G73 and C74 nucleotides of $tRNA^{Asp}$ in the crystal structure of the yeast aspartyl-tRNA synthetase–$tRNA^{Asp}$ complex (Cavarelli et al., 1993, 1994) and with N-6 and N-7 of the adenine ring of ATP and seryl adenylate analogs in the crystal structures of seryl-tRNA synthetase complexes (Belrhali et al., 1994). For these reasons, the entire motif 2 region of *E. coli* alanyl-tRNA synthetase was sujected to alanine-scanning mutagenesis at 21 positions bearing conserved functional side chains and phenylalanine residues thought to be possible candidates for making acceptor helix contacts (Davis et al., 1994). Three of the twenty-one mutated positions yielded nonfunctional proteins: R69, D76, and F90 (R77, D84, and F98 in the human enzyme). In all three instances, the apparent K_m values for

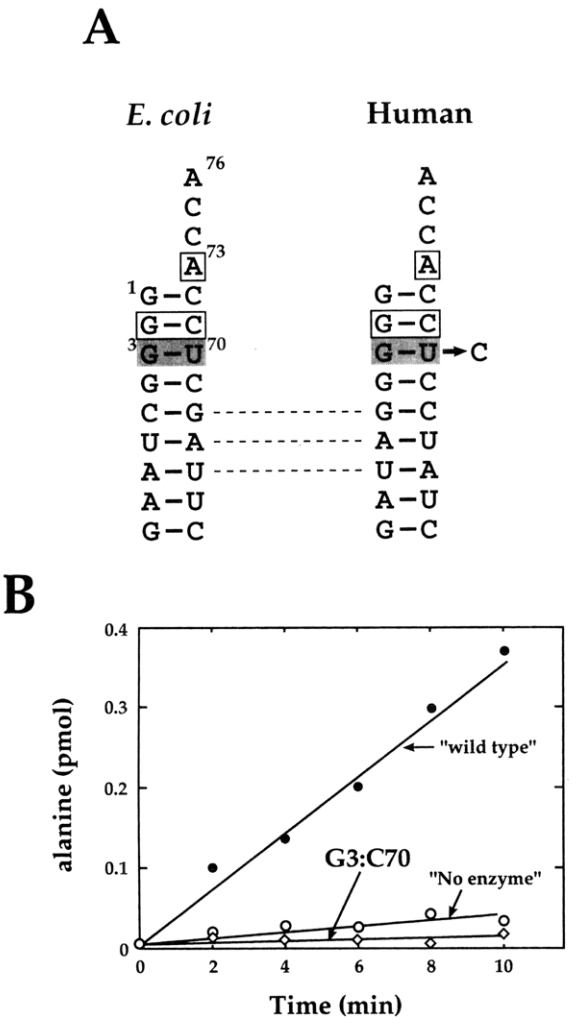

FIGURE 6: (A) RNA duplex substrates based on sequences of an *E. coli* and a human $tRNA^{Ala}$ (Steinberg et al., 1993). These substrates consist of the acceptor stems from *E. coli* $tRNA^{Ala}$ or human $tRNA^{Ala}$ and the first two base pairs of the *E. coli* TψC helix. The critical G3:U70 base pair is shaded, and other nucleotides shown to be important in the *E. coli* system (A73 and G2:C71) (Musier-Forsyth et al., 1991b; Shi et al., 1992; Musier-Forsyth & Schimmel, 1992) are boxed. Positions where the substrates differ are joined by solid lines. A U70→C mutant human duplex was also investigated in this work. (B) Specificity of aminoacylation of RNA duplexes. The assays were carried out at pH 7.5, 25 °C. Enzyme concentrations were 300 nM and RNA concentrations were 50 μM. Aminoacylation of a duplex based on the acceptor helix of human cytoplasmic $tRNA^{Ala}$ is labeled wild type. The aminoacylation rate using identical fractions prepared from non-recombinant *P. pastoris* is indicated as no enzyme.

$tRNA^{Ala}$, ATP, and alanine were little affected by the alanine substitution, but k_{cat} values for amino acid activation and aminoacylation were reduced significantly (Davis et al., 1994). Thus, the loop of motif 2 contains residues for amino acid activation and adenylate transactions.

Mutation analysis has not yet identified the residue(s) that interacts directly with the critical exocylic 2 amino group of G3 of the G3:U70 base pair in the minor groove (Musier-Forsyth et al., 1991). Because both *E. coli* and human proteins have been shown to aminoacylate G3:U70 containing RNA duplexes, further mutation analysis will take advantage of additional conserved residues that are shared by the two proteins, particularly those beyond motif 3 but included within the N-terminal 461 amino acids of the *E. coli* protein (499 of the human enzyme).

Because most class II enzymes are α_2 dimers, the monomeric structure of the human enzyme is unusual, as is the tetrameric form of the *E. coli* protein. [The rat (Dignam et al., 1991) and *B. mori* (Nishio & Kwakami, 1984; Dignam & Dignam, 1984) alanine enzymes are also reported to be monomers.] Crystal structures of the class II aspartyl- and seryl-tRNA synthetases suggested that motif 1 has a role in the formation of the dimer interface (Cusack et al., 1990; Ruff et al., 1991). The conserved motif 1 of the monomeric human enzyme shows that dimerization *per se* is not correlated with the presence of motif 1. Recent mutagenesis of motif 1 in yeast aspartyl-tRNA synthetase also raised questions about the role of motif 1 in dimer formation (Eriani et al., 1993). The *E. coli* protein might be viewed as a dimer of α_2 dimers, and mutagenesis of motif 1 could determine whether it acts in cooperation with the C-terminal oligomerization domain for subunit interactions.

The α_4-structure of the *E. coli* protein has been proposed as being required for making a 2-fold symmetry axis for binding to a pallindromic sequence in the *ala*S promoter (Putney & Schimmel, 1981). This interaction is alanine-dependent and has been demonstrated to autoregulate transcription *in vitro*. The lack of oligomerization of the human protein suggests that it lacks a directly analogous gene regulatory mechanism. However, an alternative function for the human-specific domain is not known and remains a subject for further investigations.

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