The EMBO Journal vol.15 no.5 pp.1142–1148, 1996

Single sequence of a helix–loop peptide confers functional anticodon recognition on two tRNA synthetases

Douglas S.Auld and Paul Schimmel[1]

Department of Biology, Massachusetts Institute of Technology, Cambridge, MA 02139, USA

[1]Corresponding author

The specific aminoacylation of RNA oligonucleotides whose sequences are based on the acceptor stems of tRNAs can be viewed as an operational RNA code for amino acids that may be related to the development of the genetic code. Many synthetases also have direct interactions with tRNA anticodon triplets and, in some cases, these interactions are thought to be essential for aminoacylation specificity. In these instances, an unresolved question is whether interactions with parts of the tRNA outside of the anticodon are sufficient for decoding genetic information. *Escherichia coli* isoleucyl- and methionyl-tRNA synthetases are closely related enzymes that interact with their respective anticodons. We used binary combinatorial mutagenesis of a 10 amino acid anticodon binding peptide in these two enzymes to identify composite sequences that would confer function to both enzymes despite their recognizing different anticodons. A single peptide was found that confers function to both enzymes *in vivo* and *in vitro*. Thus, even in enzymes where anticodon interactions are normally important for distinguishing one tRNA from another, these interactions can be 'neutralized' without losing specificity of aminoacylation. We suggest that acceptor helix interactions may play a role in providing the needed specificity.

Keywords: chimeric enzymes/genetic code/tRNA recognition

Introduction

In this work, we investigated the significance of the 'operational RNA code' for amino acids imbedded in the acceptor stems of tRNAs in two systems where tRNA anticodon trinucleotides play a prominent role in determining aminoacylation specificity. The genetic code is determined by the specific aminoacylations of aminoacyl-tRNA synthetases. These enzymes catalyze the attachments of amino acids to the 2′- or 3′-hydroxyls of their cognate tRNAs which harbor the anticodon trinucleotides of the genetic code. In the cases of alanyl-, leucyl- and seryl-tRNA synthetases, the anticodon trinucleotides do not play a role in determining aminoacylation specificity, showing that the relationship between the genetic code trinucleotides and alanine, leucine and serine is indirect (Normanly *et al.*, 1986; Park and Schimmel, 1988; Sampson and Saks, 1993; Biou *et al.*, 1994). In particular, acceptor stem nucleotides near the amino acid attachment site provide signals for aminoacylation (Hou and Schimmel, 1988; McClain and Foss, 1988).

In many other instances, including isoleucyl- and methionyl-tRNA synthetases, the anticodon has a major role in determining aminoacylation specificity and efficiency (Muramatsu *et al.*, 1988a; Schulman and Pelka, 1988). Even in these instances, however, the acceptor stems also contain important signals for aminoacylation, and these signals are sufficient to confer specific aminoacylation on RNA microhelix substrates (Martinis and Schimmel, 1992, 1993; Nureki *et al.*, 1993). Collectively, 10 examples of sequence-specific aminoacylation of RNA microhelices have been reported. Of these 10 examples, eight include systems where the anticodon plays some role in determining aminoacylation specificity and efficiency in the full tRNA substrate (Frugier *et al.*, 1994; Hamann and Hou, 1995; Martinis and Schimmel, 1995).

The specific aminoacylation of microhelix substrates based on tRNA acceptor stems constitutes an operational RNA code for amino acids whereby RNA sequences/structures correspond to specific amino acids (Schimmel *et al.*, 1993). The two domains of the L-shaped tRNA molecule segregate the acceptor helix and anticodon-containing domains. These domains are proposed to have arisen independently, with the acceptor helix being the more ancient structure (Weiner and Maizels, 1987, 1994; Buechter and Schimmel, 1993; Noller, 1993). Although tRNA synthetases are divided into two classes of 10 enzymes each, enzymes in both classes have two major domains which mirror the two domains of tRNA molecules (Moras, 1992). One domain contains the class-defining catalytic core which is believed to be the ancestral tRNA synthetase. This catalytic unit and the insertions within provide for interactions with the tRNA acceptor stem and the interpretation of the operational RNA code. The second domain of tRNA synthetases is idiosyncratic and not conserved amongst all members of the same class. This domain provides for interactions with parts of the tRNA distal to the acceptor stem, including the anticodon, and may have arisen later in evolution (Schimmel and Ribas de Pouplana, 1995).

For both isoleucyl- and methionyl-tRNA synthetase, the anticodon binding domain contains a helix–loop peptide (HLP) which protrudes from the surface and makes contact with the anticodon trinucleotide (Perona *et al.*, 1991; Shiba and Schimmel, 1992). The two anticodon triplets differ by a single nucleotide (G/L)AU (isoleucine; where L is lysidine, a cytosine with the ε-amino group of lysine covalently linked to C-2 of the pyrimidine ring; Muramatsu *et al.*, 1988b) and CAU (methionine). Anticodon recognition is so prominent in these systems that an interchange of the differing nucleotide is sufficient to change aminoacylation specificity of a $tRNA^{Ile}$ isoacceptor from isoleucine to methionine (Muramatsu *et al.*, 1988a). Conversely, by replacing a 10 amino acid segment of the HLP peptide with a novel composite sequence based on

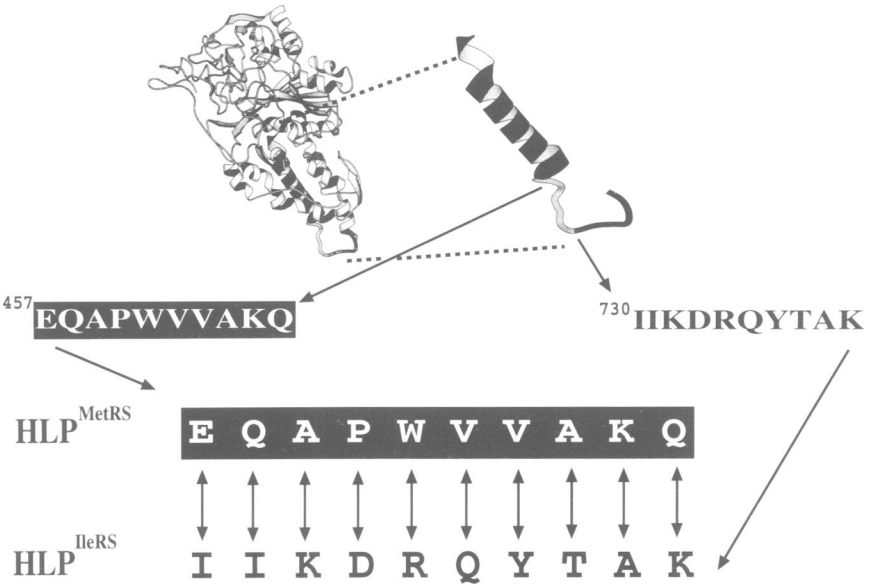

Fig. 1. Ribbon diagram representative of the structure of the monomeric 547 amino acid fragment of *Escherichia coli* methionyl-tRNA synthetase (Brunie *et al.*, 1990). The helix–loop element present in the C-terminal domain is shown enlarged (drawn using the program Molscript; Kraulis, 1991). Also shown is the HLP sequence the amino acids of which were combined. HLP^{MetRS} and HLP^{IleRS} are sequences for methionyl- and isoleucyl-tRNA synthetase, respectively.

both enzymes, we were able to switch between recognition of $tRNA^{Ile}$ and $tRNA^{Met}$ through a single amino acid swap in the transplanted peptide (Auld and Schimmel, 1995).

Given that the anticodon plays a critical role in determining recognition by isoleucyl- and methionyl-tRNA synthetase, we sought to determine whether interactions outside of the anticodons of $tRNA^{Ile}$ or $tRNA^{Met}$, such as those in the acceptor helix, would confer aminoacylation specificity sufficient to sustain growth of special strains of *E.coli* which encode null alleles for either of these synthetases. A way to approach this question would be to use an anticodon binding peptide motif which preserved much of the physical interaction energy with the anticodon but lost at least some of its specificity. A peptide of this sort would presumably have contacts with the ribose-phosphate backbone and other atoms which were common to $tRNA^{Ile}$ and $tRNA^{Met}$, but would not be able to distinguish between 'G' and 'C' at the first position of the anticodon triplet. For this purpose, we used the previously described combinatorial mutagenesis and cell selections (Auld and Schimmel, 1995) to identify a peptide that does not discriminate between the anticodons of $tRNA^{Ile}$ and $tRNA^{Met}$ as well as does the respective 'wild-type' peptide sequences but confers function to both enzymes due to specific interactions that are outside of the anticodon triplet, such as the acceptor stem.

Results

Basic strategy and selection of active variants of isoleucyl-tRNA synthetase

We used a previously described combinatorial library to search for a peptide sequence which would confer function on both isoleucyl- and methionyl-tRNA synthetases. The library consists of $2^{10} = 1024$ combinatorial variants of the sequences of the two enzymes in the 10 amino acid anticodon binding HLP (Figure 1). The peptide element was encoded by a 61 nucleotide oligomer which could be introduced as a cassette into the appropriate location of the coding sequence of each enzyme. The recombined genes were then introduced into either strain IQ844/pRMS711 or strain MN9261/pRMS615. These strains encode null alleles for *ileS* (strain IQ844) and *metG* (strain MN9261) which are sustained by a gene on a plasmid encoding isoleucyl- (pRMS711) or methionyl-tRNA synthetase (pRMS615). The plasmids have temperature-sensitive replicons, so that strains IQ844/pRMS711 and MN9261/pRMS615 do not grow at 42°C unless rescued by introduction of a second, compatible plasmid encoding the missing activity. With these two tester strains, we were able to determine which, if any, of the peptide elements could confer activity to either or both of the enzymes.

Initially, the recombined genes for isoleucyl-tRNA synthetase were introduced into strain IQ844/pRMS711 on plasmid pDUG03. Neither recombined enzyme containing the HLP of the other enzyme complemented their respective null strains (data not shown). However, ~2.5% of the 1024 members of the HLP combinatorial library introduced into isoleucyl-tRNA synthetase conferred complementation of strain IQ844/pRMS711 (Table I). Western blot analysis of 30 inactive variants showed that, in each case, the recombined enzyme with a combinatorial sequence accumulated *in vivo*, suggesting that the HLP variants composed of many diverse sequences were incorporated into the folded protein structure (data not shown).

The different HLP elements in the library were each assigned a number for accounting purposes. Among isoleucyl-tRNA synthetase variants we found that active peptides (HLPs 1–29; Table Ia) had a consensus sequence 731NNKNRNYNNN, where N is a residue in either isoleucyl- or methionyl-tRNA synthetase. Also, inactive peptides invariably replaced one of the consensus residues (for example, compare HLP^{19} with HLP^{59} in Table I). Of the three conserved residues in the consensus sequence, 732K and 736Y were absolutely conserved and only two

Table I. Helix–loop peptide variants (isoleucyl-tRNA synthetase)

a

Variant	Sequence
HLP^{1}	I Q K D R V Y A A Q
HLP^{2}	I I K D R V Y A K Q
HLP^{3}	E I K D R V Y T K Q
HLP^{4}	I I K P R V Y T K K
HLP^{5}	E I K P R V Y T A K
HLP^{6}	I Q K P R V Y T A K
HLP^{7}	E Q K D R V Y T A K
HLP^{8}	I I K D R V Y T K Q
HLP^{9}	I Q K D R V Y T A Q
HLP^{10}	E I K D R V Y A K
HLP^{11}	E I K D R V Y A A K
HLP^{12}	I I K P W V Y T A K
HLP^{13}	I I K P R V Y A A K
HLP^{14}	E Q K D R Q Y A A K
HLP^{15}	I I K P R V Y T A K
HLP^{16}	I Q K D R V Y T A K
HLP^{17}	E I K D R Q Y T A Q
HLP^{18}	E I K D R V Y T - -
HLP^{19}	I Q K P R Q Y T A K
HLP^{20}	I I K P R Q Y T A Q
HLP^{21}	E Q K D R Q Y T A K
HLP^{22}	I I K D R V Y T K K
HLP^{23}	I I K D R V Y A A K
HLP^{24}	I I K D W Q Y T A K
HLP^{25}	I I K P R Q Y T A K
HLP^{26}	I I K D R Q Y T K K
HLP^{27}	I I K D R Q Y A A K
HLP^{28}	I Q K D R Q Y T A K
HLP^{29}	I I K D R Q Y T A K

b

Variant	Sequence
HLP^{30}	E Q K P W V V A K Q
HLP^{31}	E I A P W V V A K Q
HLP^{32}	E Q K D W V Y A K Q
HLP^{33}	E I A P R Q V A K Q
HLP^{34}	E Q K P R V Y A K Q
HLP^{35}	I Q K P W Q Y A K Q
HLP^{36}	I Q A P W Q Y A K K
HLP^{37}	E I K P W V Y A K K
HLP^{38}	E Q K D R V Y A K Q
HLP^{39}	E Q K P R V Y T K Q
HLP^{40}	E I A D R V Y A A Q
HLP^{41}	E I A D W V Y T K K
HLP^{42}	E I A D R V V T K K
HLP^{43}	E I A D W V V T A K
HLP^{44}	I Q K P R V Y T K K
HLP^{45}	I I K P W V V A A -
HLP^{46}	I I K P W V V A A K
HLP^{47}	E Q K P W V Y T A K
HLP^{48}	E Q K D R V Y A K K
HLP^{49}	E Q K D R V Y T K Q
HLP^{50}	I I A P R Q V T K K
HLP^{51}	I I K P W V Y A A K
HLP^{52}	E Q A P R Q Y T A K
HLP^{53}	I I K D W Q V T K Q
HLP^{54}	I Q K D R V V T A Q
HLP^{55}	I I K D W Q V T K K
HLP^{56}	E I K D W Q V T A K
HLP^{57}	I I K P W Q V T A K
HLP^{58}	I I K P W Q Y T A K
HLP^{59}	I Q A P R Q Y T A K

Active (a) and inactive (b) mutants of isoleucyl-tRNA synthetase. Residues for isoleucyl-tRNA synthetase are shown in black font and residues for methionyl-tRNA synthetase are shown in blocked white font. HLP^{29} is the wild-type isoleucyl-tRNA synthetase sequence (also called HLP^{IleRS}) and was identified here during the genetic screen. Unintended mutations that arose from single base pair changes of the desired codons (presumably during cassette mutagenesis) are shown in gray-shaded font.

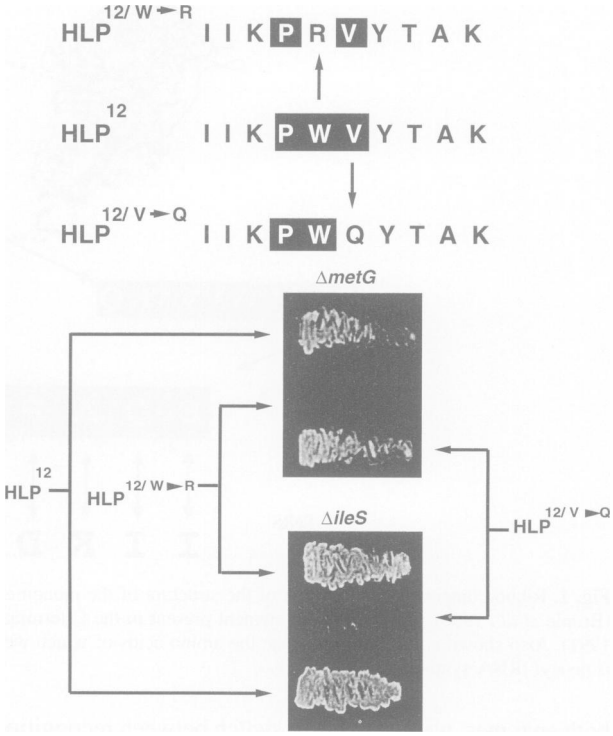

Fig. 2. Top, the sequence of HLP^{12} and the singly-substituted variants $HLP^{12/W\rightarrow R}$ and $HLP^{12/V\rightarrow Q}$. Residues derived from isoleucyl-tRNA synthetase are shown in black font and residues derived from methionyl-tRNA synthetase are shown in blocked white font. Bottom, the resulting phenotype of the *metG* null strain Δ*metG*(MN9261/pRMS615) upon introduction of the different HLPs into methionyl-tRNA synthetase and the resulting phenotype of the *ileS* null strain Δ*ileS*(IQ844/pRMS711) upon introduction of these HLPs into isoleucyl-tRNA synthetase. Conditions for complementation were as described in the text. Cells were streaked in horizontal lines on agar plates containing Luria broth and ampicillin. Photographs of agar plates were processed into bitmap images with an Apple Scanner (model A9M0337) and Applescan software from Apple Computer, Inc. Bitmaps were contrast-equalized using the graphical scanning tool GScan and presented using Showcase version 3.2 (Silicon Graphics IRIS software) on a IRIS 4D Silicon Graphics workstation.

active variants replaced 734R with W from methionyl-tRNA synthetase (HLP^{12} and HLP^{24}, Table Ia). However, inactive mutants such as HLP^{34} and HLP^{38} (Table Ib) show that, although 732K and 736Y are required for an active phenotype, they are not sufficient.

Rationale for and isolation of peptide active in both isoleucyl- and methionyl-tRNA synthetase

The peptide designated as HLP^{12} was the only one among the active variants to have both a P at position 733 and a W at position 734 (Table I). The active variant with HLP^{12} encodes seven amino acids from isoleucyl-tRNA synthetase and three from methionyl-tRNA synthetase. These three include the critical 460P and 461W for methionyl-tRNA synthetase, where 461W is believed essential for binding of the enzyme to the CAU anticodon of $tRNA^{Met}$ and 460P is critical for orientation of 461W (Ghosh *et al.*, 1990). Given that HLP^{12} encodes these essential residues for anticodon binding by methionyl-tRNA synthetase, we were motivated to see if it could confer activity on that enzyme.

HLP^{12} confers function to isoleucyl- and methionyl-tRNA synthetases in vivo

Figure 2 shows that, in addition to conferring complementation of the Δ*ileS* null strain, introduction of HLP^{12} into methionyl-tRNA synthetase confers complementation of the Δ*metG* strain as well. Thus, the single RNA binding peptide HLP^{12} confers function to two tRNA synthetases.

To see if complementation of the Δ*metG* null allele by HLP^{12} was W461-dependent, a single W→R substitution was introduced into HLP^{12} to give $HLP^{12/W\rightarrow R}$ (730IIK-**PRVYTAK**, where residues in bold are from methionyl-tRNA synthetase). This sequence has the critical 732K, 734R and 736Y found in the aforementioned consensus sequence of peptides active in isoleucyl-tRNA synthetase. Introduction of a cassette encoding this peptide into plasmid pKSNB-encoded *metG* yielded a recombined methionine enzyme which could not rescue the growth defect of the Δ*metG* null strain (Figure 2). In contrast, introduction of the $HLP^{12/W\rightarrow R}$ encoding cassette into plasmid pDUG03-encoded *ileS* resulted in an isoleucine enzyme which complemented the growth defect of the Δ*ileS* null strain (see HLP^{15} in Table Ia).

We found that the peptide HLP^{12} can be rendered inactive in isoleucyl-tRNA synthetase by a single amino acid change at position 735 ($HLP^{12/V\rightarrow Q}$, this variant is the same as HLP^{58} in Table Ib), even though 735Q is

native to wild-type isoleucyl-tRNA synthetase (Table Ia). Conversely, although introduction of a cassette encoding this peptide into plasmid pDUG03-encoded *ileS* resulted in an inactive isoleucine enzyme, the same cassette introduced into plasmid pKSNB-encoded *metG* yielded a recombined methionine enzyme which rescued the growth defect of the Δ*metG* null strain (Figure 2). These results illustrate the sensitivity of specific residue requirements to sequence context. In this connection, we conclude that, while position 735 is the most frequently substituted position among active isoleucyl-tRNA synthetase variants (Table Ia), these substitutions can be highly specific for the particular sequence context.

HLP^{12}-containing enzymes are active in vitro

The results of *in vivo* complementation of two null alleles by the HLP^{12} peptide were extended *in vitro*. For the purpose of comparison, we assayed both enzymes with transplanted HLP^{12} and, in addition, isoleucyl-tRNA synthetase with HLP$^{12/V\rightarrow Q}$ and methionyl-tRNA synthetase with HLP$^{12/W\rightarrow R}$ transplants. Transplantation of HLP^{12} into either enzyme results in the generation of significant aminoacylation activity *in vitro* (Figure 3). In contrast, transplantation of either HLP$^{12/V\rightarrow Q}$ into isoleucyl-tRNA synthetase or of HLP$^{12/W\rightarrow R}$ into methionyl-tRNA synthetase resulted in a large reduction in the rate of aminoacylation. We estimate that the respective activities are decreased at least 20-fold by the respective single amino acid substitution (Table II). For all enzymes, no defect was observed in the rate of amino acid activation (data not shown). This result is consistent with the HLP element being essential for only the tRNA aminoacylation-dependent step and not for amino acid activation.

For each HLP^{12}-transplanted enzyme, the apparent free energy of activation for aminoacylation is raised by ~3.1 to 3.7 ± 0.3 kcal/mol, relative to the respective wild-type enzyme. (This increase corresponds to a reduction of ~150-fold and 400-fold in the activity for isoleucyl- and methionyl-tRNA synthetase, respectively). Thus, HLP^{12} is not an optimal HLP for either enzyme, possibly because it has lost some of the interaction energy associated with discriminating between GAU and CAU anticodons. The residual interaction energy, which may reflect non-specific (e.g. phosphate) contacts with the anticodon sequence, is still sufficient to provide significant activity when combined with interactions with other parts of the tRNA synthetase.

HLP^{12} may be the only peptide in the combinatorial library that confers dual tRNA specificity

We wondered whether HLP^{12} was the only peptide in the combinatorial library to function in both enzymes. This peptide contains 460P and 461W which are important determinants for aminoacylation of tRNA^{Met}. Examination of the HLP^{24} and HLP^{25} variants emphasizes this point. These variants show that neither 461W (HLP^{24}) alone nor 460P (HLP^{25}) alone is sufficient for generating methionyl-tRNA synthetase activity (Tables Ia and II). Conversely, neither 733P alone nor 734W alone is sufficient for inactivation of isoleucyl-tRNA synthetase (Table II). However, 733P;734W (HLP$^{12/V\rightarrow Q}$) together lead to an inactive phenotype when placed in isoleucyl tRNA-synthetase

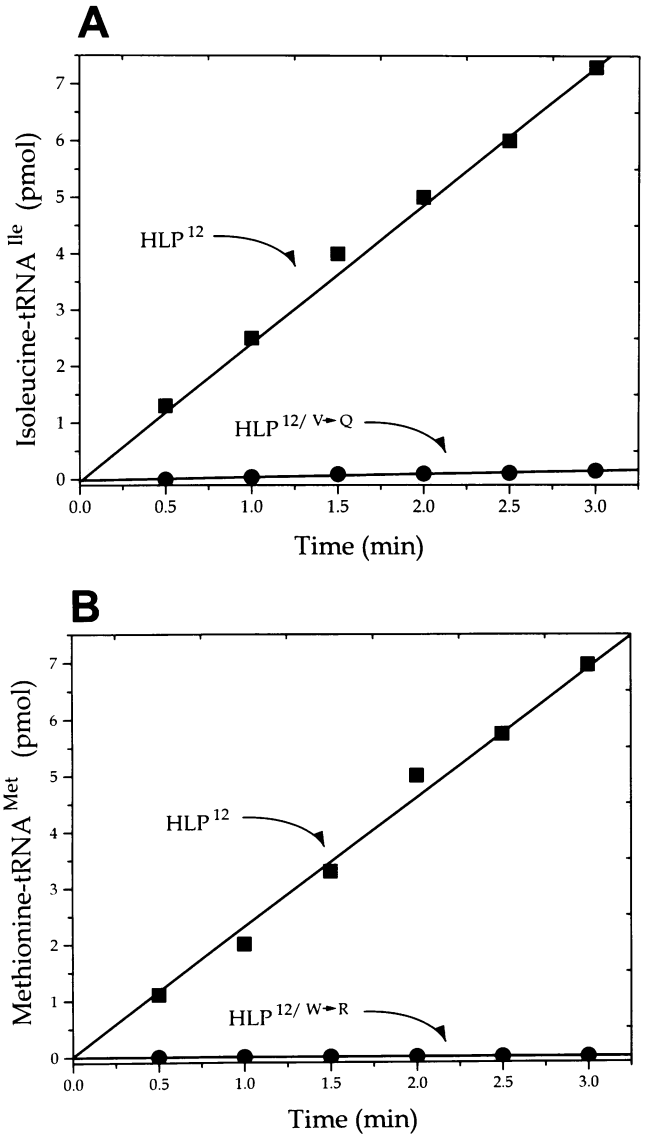

Fig. 3. Activity *in vitro* for the HLP^{12}-containing variants and their single-substituted inactive counterparts. Examples of isoleucine aminoacylation (**A**) and methionine aminoacylation (**B**) for the isoleucyl- and methionyl-tRNA synthetase variants. Conditions for the aminoacylation assay were as described in the text using 50 nM isoleucyl-tRNA synthetase and 5 μM tRNA^{Ile} (A) or 50 nM methionyl-tRNA synthetase and 15 μM tRNA^{fMet} (B) at 37°C (pH 7.5).

(k_{cat}/K_m for aminoacylation of tRNA^{Ile} is reduced ~3000-fold; Table II). Therefore, the Q735V substitution in HLP^{12} is necessary for the methionyl tRNA-synthetase active combination of 733P;734W to also be active in the isoleucyl-tRNA synthetase framework. This observation, together with the observation that further substitutions in HLP^{12} lead to an inactive isoleucyl-tRNA synthetases (see, for example HLP^{51}; Table Ib), suggest that HLP^{12} is the only sequence in the library able to confer *in vivo* activity on both enzymes.

Discussion

Specific interactions with the anticodon trinucleotide have long been considered as essential for the identity of

Table II. Kinetic parameters for a subset of HLP variants

Variant	Sequence	IleRS	MetRS	kcat/Km IleRS	kcat/Km MetRS
HLP ^{MetRS}	E Q A P W V V A K Q	-	+	0.0001	1
HLP ^{IleRS}	I I K D R Q Y T A K	+	-	1	ND
HLP ^{12}	I I K P W V Y T A K	+	+	0.0067	0.0023
HLP $^{12/V-->Q}$	I I K P W Q Y T A K	-	+	0.0003	0.0023
HLP ^{24}	I I K D W Q Y T A K	+	-	0.1	ND
HLP ^{25}	I I K P R Q Y T A K	+	-	0.03	ND
HLP $^{12/W-->R}$	I I K P R V Y T A K	+	-	0.5	<.0001

A subset of active and inactive HLP variants is shown. Phenotype refers to the ability of these mutants to maintain growth of the tester strain Δ*ileS*(IQ844/pRMS711). A '+' refers to complementation of the *ileS* null strain IQ844 and a '–' refers to a lack of complementation. HLP^{IleRS}, HLP^{12} and HLP^{24} in isoleucyl-tRNA synthetase were identified by the genetic screen described in the text whereas the other HLPs shown were individually made and tested separately utilizing cassette mutagenesis. Note that $HLP^{12/W\rightarrow R}$ is the same as HLP^{15} in Table Ia and $HLP^{12/V\rightarrow Q}$ is the a same as HLP^{58} in Table Ib. ND, not determinable due to insufficient yield upon attempts to purify these variants (presumably because of a reduced stability). For the wild-type enzymes, k_{cat}/K_m is ~10^6 M/s. Kinetic parameters were measured at 37°C (pH 7.5).

methionine and isoleucine tRNA (Muramatsu *et al.*, 1988a; Schulman, 1991; Saks *et al.*, 1994). The work presented here is the first example of a single anticodon binding element that is active with two different sets of anticodons. In the approach used here, cell strains were designed so that, in each case, the chimeric synthetase was the only source of enzyme activity. The normal growth phenotype of cells harboring the HLP^{12} peptide, in either isoleucyl- or methionyl-tRNA synthetase, suggests that aminoacylation is highly specific and that neither chimeric enzyme catalyzes a significant level of misacylations *in vivo*. Misacylations are expected to lead to a slow growth or toxic phenotype (Bedouelle *et al.*, 1990; Vidal-Cros and Bedouelle, 1992).

The question posed by this work was whether interactions additional to those which occur at the anticodon were sufficient to achieve specificity of aminoacylation *in vivo*, in a system where anticodon interactions can be manipulated easily to change specificity of charging. That the HLP^{12} sequence element functions in both isoleucyl- and methionyl-tRNA synthetases suggests that interactions other than those at the anticodon are contributing significantly to specificity of aminoacylation. In particular, the acceptor stems of $tRNA^{Met}$ and $tRNA^{Ile}$ provide the basis for at least some anticodon-independent specificity (Nureki *et al.*, 1994; Martinis and Schimmel, 1995). However, because a single CAU→LAU change in an isoleucine tRNA was sufficient to inactivate aminoacylation with isoleucine *in vitro* (Muramatsu *et al.*, 1988a), it seemed unlikely that other determinants (such as in the acceptor stem) could be sufficient to confer aminoacylation.

The HLP^{12} peptide may provide some non-specific contacts at the anticodon that buttress other interactions to the point that a functional enzyme–tRNA complex is made. In a recent analysis, the contributions of the acceptor helix and anticodon stem-loop to the free energy of binding $tRNA^{Met}$ to methionyl-tRNA synthetase were found to be comparable (A.Gale and P.Schimmel, in preparation). For methionyl- and isoleucyl-tRNA synthetase, a polypeptide insertion into the N-terminal domain of these enzymes is believed to provide for interaction with the tRNA acceptor helix (Starzyk *et al.*, 1989; Perona *et al.*, 1991; Landro and Schimmel, 1993). In addition to binding interactions, kinetic studies of mutant $tRNA^{Met}$ and microhelix substrates showed that the transition state of catalysis is particularly sensitive to the sequence of the acceptor stem (Lee *et al.*, 1992; Meinnel *et al.*, 1993). Thus, with enough overall binding energy coming from 'non-specific' anticodon and specific acceptor stem interactions, we imagine that a correct fit of the acceptor stem into the active site might be sufficient to achieve aminoacylation specificity.

While the results are consistent with the above interpretation, we cannot rule out the possibility that 461W still contributes some specificity to the interaction with the CAU anticodon of $tRNA^{Met}$. This possibility is suggested by the dependence of the HLP^{12} peptide on 460P and 461W for activity in methionyl-tRNA synthetase. In addition, the region around 395R of *E.coli* methionyl-tRNA synthetase may provide support for specific anticodon recognition (Despons *et al.*, 1991; Ghosh *et al.*, 1991).

Isoleucyl- and methionyl-tRNA synthetase are two of the most closely related tRNA synthetases and are thought to have arisen from a common ancestor (Webster *et al.*, 1984; Shiba and Schimmel, 1992). The valine at position 735 of isoleucyl-tRNA synthetase in HLP^{12} was derived from the sequence of methionyl-tRNA synthetase, and functions in a peptide that does not discriminate between the anticodons of $tRNA^{Ile}$ or $tRNA^{Met}$. The hybrid sequence of HLP^{12} may be similar to historical peptides that occurred as this subclass of enzymes diverged from their common ancestor.

If such hybrid sequences existed historically, then the ancestral synthetase may have interacted specifically with the acceptor stems of tRNAs, but was indifferent toward the subsequently appended anticodon domain of the tRNA. These anticodon-indifferent peptides may have allowed for active enzymes to exist in a sequence context where single amino acid changes could increase discrimination of one tRNA from another as the trinucleotide codons became established in the genetic code. For isoleucyl- and methionyl-tRNA synthetase, co-evolution with the genetic code may have led to strong binding to the anticodon. However, as shown here, the appropriate combination of these related sequences can lead to peptides that bind non-specifically to the anticodon so that the more ancient interactions between the synthetase and the acceptor stem are again emphasized.

Materials and methods

Design and synthesis of the mutant cassette

The strategy for mutagenesis was based on an alignment of five enzymes involving 21 sequences that define a subclass of class I tRNA synthetases (Schimmel *et al.*, 1992; Shepard *et al.*, 1992; Shiba and Schimmel, 1992). Ten residues in each enzyme that are roughly centered around an important anticodon binding determinant in methionyl-tRNA synthetase (461W) were targeted for combinatorial mutagenesis (Figure 1).

Binary-codon mutagenesis was performed on a Pharmacia Gene Assembler Plus DNA synthesizer model 530 as described (Auld and Schimmel, 1995). The resulting deoxyoligonucleotide results in a 50:50 mix of the methionyl- and isoleucyl-tRNA synthetase-derived codons at each of the 10 positions for a total of $2^{10} = 1024$ sequences.

Cassette mutagenesis

The 61mer oligonucleotide cassette: 5′-GTCTTCTATCTAGAC(ATC/GAA)(ATC/CAG)(AAA/GCT)(GAC/CCG)(CGT/TGG)(CAG/GTG)-(TAC/GTG)(ACC/GCG)(GCC/AAA)(AAA/CAG)GCGGACCGTGTG-CTGA-3′ was synthesized as described above and used for cassette mutagenesis. This cassette encodes both *Xba*I and *Rsr*II restriction endonuclease sites. *In vitro* double-stranded DNA synthesis was performed using the primer 5′-TCAGCACACGGTCCG-3′ by the method of Reidhaar-Olson *et al.* (1991) using DNA polymerase I holoenzyme or the Klenow fragment. Point mutations were made by isolating single-stranded DNA (Vieira and Messing, 1987) and using this as a template for site-directed mutagenesis (Amersham Sculptor™ oligonucleotide mutagenesis system). To introduce the *Xba*I site, two silent point mutations were made [TAC(codon 727)→TAT, CTG(codon 728)→CTA]. However, introduction of the *Rsr*II site required the mutation of S742R (AGT→CGT) and we noticed that arginine is found at the analogous position in methionyl-tRNA synthetase. This substitution is phenotypically silent, with the respective enzyme variant having wild-type behavior *in vitro* and *in vivo*, and therefore is referred to herein as 'wild-type'.

The phagemid pDUG03 was derived from phagemid pKS21 (Shiba and Schimmel, 1992) and contains a cloned copy of *ileS* and *Xba*I and *Rsr*II restriction sites. All mutants of *ileS* discussed herein were made in pDUG03. For cassette mutagenesis of methionyl-tRNA synthetase, oligonucleotides were cloned into *Nar*I and *Bss*HII restriction sites of phagemid pKSNB (Kim *et al.*, 1993). The phagemid pKSNB encodes the 547 amino acid monomeric fragment of methionyl-tRNA synthetase. To test some of the assumptions made about the library, some oligonucleotide cassettes were made separately using a standard DNA synthesis strategy and cassette mutagenesis, and are identified in the text. DNA sequence analysis of all constructs mentioned was performed using dideoxy sequencing (Sanger *et al.*, 1977).

Genetic selection and nomenclature

Selection was performed in the *E.coli* strain IQ844 (*ileS203::kan, recA56*)/pRMS711. This strain contains a null allele (complete ablation) of genomic *ileS* but is viable because it contains the plasmid pRMS711 which harbors a functional *ileS* gene and a temperature-sensitive replicon (Shiba and Schimmel, 1992). Growth at the non-permissive temperature (42°C) is achieved when a second plasmid is introduced that encodes an active isoleucyl-tRNA synthetase. The maintenance plasmid contains the chloramphenicol resistance gene so that loss of the maintenance plasmid can be tested by plating putative functional colonies on Luria broth containing chloramphenicol.

An active phenotype is defined by a mutant that supports growth at 42°C on Luria broth plates containing ampicillin but not on plates containing chloramphenicol. The specific enzymes encoded by these *ileS* alleles are referred to as active variants. An inactive phenotype is defined by a lack of growth after 3 days selection at 42°C, and the specific enzymes encoded by these *ileS* alleles are referred to as inactive variants. Selection for active variants was performed for 3 days at 42°C and, in all cases, no additional colonies appeared after 12–18 h. All inactive variants were initially identified by DNA sequence analysis of pDUG03 plasmids obtained from randomly chosen colonies of *E.coli* MV1184 [*ara, Δ(lac-pro), strA, thi, (φ80ΔlacIZΔM15), Δ(srl-recA) 306::TN10-(tet^r); F′:traD36, proAB, lacI^qZΔm15*; Vieira and Messing, 1987] grown without selection. These plasmids were then transformed separately into the tester strain IQ844/pRMS711 to check the phenotype associated with each specific variant.

The Δ*metG* tester *E.coli* strain MN9261 (*metG::kan, recA*)/pRMS615 (Kim *et al.*, 1993) was used for selecting active variants of methionyl-tRNA synthetase. This strain contains a null allele of *metG* but is viable because it contains the maintenance plasmid pRMS615. Complementation analysis is similar to the IQ844/pRMS711 system described above.

In vivo stability of variants

Small-scale cultures of MV1184 that had been transformed with pDUG03 were grown at 37°C. Expression of isoleucyl-tRNA synthetase was induced by the addition of isopropyl β-D-thiogalactoside to a concentration of 1 mM to cells in mid-logarithmic growth. After an additional 3 h of growth, cells were collected and then lysed by sonication with a Fisher Sonic Dismembrator model 300. Western blots were performed as described (Shepard *et al.*, 1992) using rabbit anti-*E.coli* isoleucyl-tRNA synthetase antiserum (Starzyk *et al.*, 1987) and horseradish peroxidase-linked donkey anti-rabbit antibody (Amersham, Arlington Heights, IL).

Enzyme purification

Active isoleucyl-tRNA synthetase variants were purified from pDUG03 contained in IQ844. For purification of active methionyl-tRNA synthetase variants, the Δ*metG* null strain MN9261/pRMS615 was used. The procedure for enzyme purification described in Burbaum and Schimmel (1991) for methionyl-tRNA synthetase or in Shepard *et al.* (1992) for isoleucyl-tRNA synthetase was then followed. Purity was estimated to be >95% by SDS–PAGE. Enzyme concentration was determined by Bio-Rad Protein Assay (Bio-Rad Laboratories, Hercules, CA).

For isolation of inactive variants of isoleucyl-tRNA synthetase or methionyl-tRNA synthetase, the *E.coli* strains MI1 (Iaccarino and Berg, 1971) or MJR (Burbaum and Schimmel, 1991) were used, respectively. The *ileS* and *metG* alleles of these strains confer amino acid auxotrophy because they encode enzymes defective in amino acid binding (K_m raised 350- or 2000-fold for the genomic methionyl- or isoleucyl-tRNA synthetase, respectively (Barker *et al.*, 1982; Burbaum and Schimmel, 1991; Schmidt and Schimmel, 1994). Therefore, inactive enzymes can be overexpressed and purified from these strains and the isolated enzyme's activity can be assayed *in vitro* at concentrations of methionine or isoleucine where there is no contribution to activity from any contaminating chromosomal enzyme (Burbaum and Schimmel, 1991; Schmidt and Schimmel, 1993). Additionally, to aid in purification of inactive variants, a glutathione-S transferase (GST) fusion protein purification system was employed (Pharmacia, Piscataway, NJ). In this system, the gene for the desired enzyme is genetically fused to the C-terminal end of the coding sequence for the GST gene and the expressed fusion protein is purified using glutathione–Sepharose® 4B (Pharmacia). Protein is eluted following thrombin cleavage (Sigma, St Louis, MO) of the fusion protein while bound to the column. Wild-type isoleucyl-tRNA synthetase exhibits normal activity following purification by this system (J.A.Landro and P.Schimmel, unpublished data).

Enzyme kinetics

The tRNA-dependent aminoacylation reaction catalyzed by the purified enzymes was assayed as described (Kim and Schimmel, 1992; Shepard *et al.*, 1992). For determination of k_{cat}/K_m values, purified enzyme was added at a concentration of 1–200 nM and tRNA was varied from 0.5 to 80 μM. The k_{cat}/K_m values were derived from the slopes of Lineweaver–Burke plots generated from the data. The increase in the free energy of activation for the variants was calculated from the difference in apparent Gibbs free energy of activation by the designated variant versus the wild-type enzyme. Errors were calculated from two to three separate determinations of k_{cat}/K_m.

The $tRNA^{fMet}$ was purchased from Sigma (St. Louis, MO). To obtain $tRNA^{Ile}$ (major isoacceptor), the following purification scheme was employed. An *E.coli* strain JM109 [*F′ traD36 proA^+ proB^+ lacI^q lacZΔm15\recA1 endA1 gyrA96 (Nal^r) thi hsdR17 supE44 relA1 Δ(lac-proAB) mcrA*; Yanisch-Perron *et al.*, 1985] containing a plasmid-expressed $tRNA^{Ile}$ gene (major) was used. Crude cell preparations were acid-phenol extracted by the method of Varsheny *et al.* (1991). Nucleobond-AX columns (Macherey-Nagel, Düren Germany) were then used to separate total tRNA from plasmid DNA and rRNAs. The nucleic acids contained in the elutant from the Nucleobond-AX column were then precipitated using isopropanol. Following centrifugation, the collected nucleic acid-containing pellet was dissolved in a buffer that contained 1.5 M $(NH_4)_2SO_4$, 0.75% isopropanol and 50 mM sodium MES (pH 6.5). Purification of $tRNA^{Ile/GAU}$ was achieved on a C_4 reverse phase HPLC column (analytical scale VYDAC Peptide and Protein C_4 column) using a linear gradient of 1.5–0 M $(NH_4)_2SO_4$ over 80 min at a flow rate of 1 ml/min in a buffer containing 50 mM MES (pH 6.5) and 0.75%

isopropanol. The isoleucine acceptance activity of the purified $tRNA^{Ile/GAU}$ was estimated to be from 1400 to 1800 pmol/A_{260} unit (1 unit gives an A_{260} of 1 in 1 ml of water, 1 cm pathlength).

The rate of adenylate formation was measured by pyrophosphate exchange at 25°C (Calendar and Berg, 1966). The assay conditions were as described (Kim and Schimmel, 1992; Shepard *et al.*, 1992) using 1 mM isoleucine or methionine. The reaction was initiated by the addition of purified enzyme to 10 nM. Aliquots were removed at 2, 5, 7, 9, 12, 15 and 20 min following the procedures of Kim and Schimmel (1992) or Shepard *et al.* (1992).

Acknowledgements

We thank Eric Schmidt and Dr Stephen Hale for providing thoughtful comments on this manuscript. This work was supported by grant number GM 23562 from the National Institute of Health. Dr Douglas Auld is a NIH post-doctoral fellow (1993–1996).

References

Auld,D.S. and Schimmel,P. (1995) *Science*, **267**, 1994–1996.
Barker,D.G., Ebel,J.-P., Jakes,R. and Bruton,C.J. (1982) *Eur. J. Biochem.*, **127**, 449–457.
Bedouelle,H., Guez,V., Vidal-Cros,A. and Hermann,M. (1990) *J. Bacteriol.*, **172**, 3940–3945.
Biou,V., Yaremchuk,A., Tukalo,M. and Cusack,S. (1994) *Science*, **263**, 1404–1410.
Brunie,S., Zelwer,C. and Risler,J. (1990) *J. Mol. Biol.*, **216**, 411–424.
Buechter,D.D. and Schimmel,P. (1993) *Crit. Rev. Biochem. Mol. Biol.*, **28**, 309–322.
Burbaum,J.J. and Schimmel,P. (1991) *Biochemistry*, **30**, 319–324.
Calendar,R. and Berg,P. (1966) *Biochemistry*, **5**, 1690–1695.
Despons,L., Walter,P., Senger,B., Ebel,J.-P. and Fasiolo,F. (1991) *FEBS Lett.*, **289**, 217–220.
Frugier,M., Florentz,C. and Giegé,R. (1994) *EMBO J.*, **13**, 2218–2226.
Ghosh,G., Pelka,H. and Schulman,L.H. (1990) *Biochemistry*, **29**, 2220–2225.
Ghosh,G., Kim,H.Y., Demaret,J.-P., Brunie,S. and Schulman,L.H. (1991) *Biochemistry*, **30**, 11767–11774.
Hamann,C.S. and Hou,Y.-M. (1995) *Biochemistry*, **34**, 6527–6532.
Hou,Y.-M. and Schimmel,P. (1988) *Nature*, **333**, 140–145.
Iaccarino,M. and Berg,P. (1971) *J. Bacteriol.*, **105**, 527–537.
Kim,S. and Schimmel,P. (1992) *J. Biol. Chem.*, **267**, 15563–15567.
Kim,S., Ribas de Pouplana,L. and Schimmel,P. (1993) *Proc. Natl Acad. Sci. USA*, **90**, 10046–10050.
Kraulis,P.J. (1991) *J. Appl. Crystallogr.*, **24**, 946–950.
Landro,J.A. and Schimmel,P. (1993) *Proc. Natl Acad. Sci. USA*, **90**, 2261–2265.
Lee,C.-P., Dyson,M.R., Mandal,N., Varshney,U., Bahramian,B. and RajBhandary,U.L. (1992) *Proc. Natl Acad. Sci. USA*, **89**, 9262–9266.
Martinis,S.A. and Schimmel,P. (1992) *Proc. Natl Acad. Sci. USA*, **89**, 65–69.
Martinis,S.A. and Schimmel,P. (1993) *J. Biol. Chem.*, **268**, 6069–6072.
Martinis,S.A. and Schimmel,P. (1995) In Söll,D. and RajBhandary,U. (eds), *Small RNA Oligonucleotide Substrates for Specific Aminoacylations*. American Society for Microbiology, Washington, DC, pp. 349–370.
McClain,W.H. and Foss,K. (1988) *Science*, **240**, 793–796.
Meinnel,T., Mechulam,Y., Lazennec,C., Blanquet,S. and Fayat,G. (1993) *J. Mol. Biol.*, **229**, 26–36.
Moras,D. (1992) *Trends Biochem. Sci.*, **17**, 159–164.
Muramatsu,T., Nishikawa,K., Nemoto,F., Kuchino,Y., Nishimura,S., Miyazawa,T. and Yokoyama,S. (1988a) *Nature*, **336**, 179–181.
Muramatsu,T., Yokoyama,S., Horie,N., Matsuda,A., Ueda,T., Yamaizumi,Z., Kuchino,Y., Nishimura,S. and Miyazawa,T. (1988b) *J. Biol. Chem.*, **263**, 9261–9267.
Noller,H.F. (1993) In Gesteland,R.F. and Atkins,J.F. (eds), *On the Origin of the Ribosome: Coevolution of Subdomains of tRNA and rRNA*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 137–156.
Normanly,J., Ogden,R.C., Horvath,S.J. and Abelson,J. (1986) *Nature*, **321**, 213–219.
Nureki,O. *et al.* (1993) In Nierhaus,K.H., Franceschi,F., Subramanian, A.R., Erdmann,V.A. and Wittmann-Liebold,B. (eds), *Conformational Change of tRNA upon Interaction of the Identity-Determinant Set with Aminoacyl-tRNA Synthetase*. Plenum Press, New York, pp. 59–66.
Nureki,O., Niimi,T., Muramatsu,T., Kanno,H., Kohno,T., Florentz,C., Giegé,R. and Yokoyama,S. (1994) *J. Mol. Biol.*, **236**, 710–724.
Park,S.J. and Schimmel,P. (1988) *J. Biol. Chem.*, **263**, 16527–16530.
Perona,J.J., Rould,M.A., Steitz,T.A., Risler,J.-L., Zelwer,C. and Brunie,S. (1991) *Proc. Natl Acad. Sci. USA*, **88**, 2903–2907.
Reidhaar-Olson,J.F. *et al.* (1991) *Methods Enzymol.*, **208**, 564–586.
Saks,M.E., Sampson,J.R. and Abelson,J.N. (1994) *Science*, **263**, 191–197.
Sampson,J.R. and Saks,E. (1993) *Nucleic Acids Res.*, **21**, 4467–4475.
Sanger,F., Niklen,S. and Coulson,A.R. (1977) *Proc. Natl Acad. Sci. USA*, **74**, 5463–5467.
Schimmel,P. and Ribas de Pouplana,L. (1995) *Cell*, **81**, 983–986.
Schimmel,P., Shepard,A. and Shiba,K. (1992) *Protein Sci.*, **1**, 1387–1391.
Schimmel,P., Giegé,R., Moras,D. and Yokoyama,S. (1993) *Proc. Natl Acad. Sci. USA*, **90**, 8763–8768.
Schmidt,E. and Schimmel,P. (1993) *Proc. Natl Acad. Sci. USA*, **90**, 6919–6923.
Schmidt,E. and Schimmel,P. (1994) *Science*, **264**, 265–267.
Schulman,L.H. (1991) *Prog. Nucleic Acid Res. Mol. Biol.*, **41**, 23–87.
Schulman,L.H. and Pelka,H. (1988) *Science*, **242**, 765–768.
Shepard,A., Shiba,K. and Schimmel,P. (1992) *Proc. Natl Acad. Sci. USA*, **89**, 9964–9968.
Shiba,K. and Schimmel,P. (1992) *Proc. Natl Acad. Sci. USA*, **89**, 1880–1884.
Starzyk,R.M., Webster,T.A. and Schimmel,P. (1987) *Science*, **237**, 1614–1618.
Starzyk,R.M., Burbaum,J.J. and Schimmel,P. (1989) *Biochemistry*, **28**, 8479–8484.
Varshney,U., Lee,C.-P. and RajBhandary,U.I. (1991) *J. Biol. Chem.*, **266**, 24712–24718.
Vidal-Cros,A. and Bedouelle,H. (1992) *J. Mol. Biol.*, **223**, 801–810.
Vieira,J. and Messing,J. (1987) *Methods Enzymol.*, **153**, 3–11.
Webster,T.A., Tsai,H., Kula,M., Mackie,G.A. and Schimmel,P. (1984) *Science*, **226**, 1315–1317.
Weiner,A.M. and Maizels,N. (1987) *Proc. Natl Acad. Sci. USA*, **84**, 7383–7387.
Weiner,A.M. and Maizels,N. (1994) *Curr. Biol.*, **4**, 560–563.
Yanisch-Perron,C., Vieira,J. and Messing,J. (1985) *Gene*, **33**, 103–119.

Received on September 25, 1995; revised on October 31, 1995