The Expression and Characterisation of C-Terminal Deletion Mutants and Chimeras of Yeast Isoleucyl-tRNA Synthetase

by

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THE EXPRESSION AND CHARACTERISATION OF C-TERMINAL DELETION MUTANTS AND CHIMERAS OF YEAST ISOLEUCYL-tRNA SYNTHETASE

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ABSTRACT:

The isoleucyl-tRNA synthetase (IleRS) is a Class I synthetase which is responsible for coupling isoleucine to its cognate tRNA. All prokaryotic and eukaryotic IleRSs catalyse essentially the identical two-step reaction:

IleRS + isoleucine + ATP --------> PP_i + [IleRS-isoleucine-AMP] (aminoadenylation)

[IleRS-isoleucine-AMP]+$tRNA^{ile}$-------> isoleucine-$tRNA^{ile}$+AMP+IleRS (aminoacylation)

Despite the similarities in the enzymes' structure and function, prokaryotic and eukaryotic forms of IleRS differ in their specificities towards tRNA and their behaviour to certain synthetase inhibitors. Presently, no 3-dimensional structure for IleRS is known. Thus, the purpose of this study was to establish the structural features of IleRS that are responsible for tRNA recognition, aminoadenylate formation, inhibitor specificity and if possible, editing of substrates. To accomplish this study, C-terminal deletion mutants of *Saccharomyces cerevisiae* isoleucyl-tRNA synthetase (1) and genetically engineered chimeric enzymes containing portions of yeast IleRS (yIRS) and the *E. coli* IleRS (bIRS) were created and expressed in an *E. coli* host strain.

The first part of this thesis involves the expression and general characterisation of the yIRS C-terminal deletion proteins and the chimeric IleRS enzymes. *In vivo* and *in vitro* aminoacylation assays on the C-terminal deletion proteins and the chimeric IleRS enzymes were performed. From the results obtained, it appeared that none of the mutant IleRS constructs had aminoacylation activity. However, these assays did not preclude the possibility that the mutant IleRS constructs could perform aminoadenylation, the first step of the reaction mechanism. In order to address the possibility that these mutant IleRS constructs may have aminoadenylation activity, two similar chimeric IleRS enzymes were purified and assayed for aminoadenylate formation in the absence and presence of Pseudomonic Acid A (PA), which is a competitive inhibitor for the isoleucine binding site of bIRS. Of the two chimeras, the Nsi I Chimera showed aminoadenylation activity in the absence and presence of PA whereas the Afl III/Sac II chimera showed no aminoadenylation activity. This suggests that the junction between the

yIRS and bIRS portions of the Afl II/Sac II Chimera disrupted the nucleotide-binding fold whereas the junction made for the Nsi I Chimera did not. The results also confirmed that the amino acid and ATP binding sites and the determinant for PA resistance are located in the N-terminal domain of IleRS.

The second part of the thesis is the characterisation of the monoclonal antibodies which were developed against the wild-type yIRS and bIRS enzymes. The antigenic sites for three monoclonal antibodies to yIRS and one monoclonal antibody to bIRS have been localized and their effect on the synthetase's activity have been studied. Of the three monoclonal antibodies developed against yIRS, one was capable of "inhibiting" both aminoacylation and aminoadenylation activities in yIRS. This monoclonal antibody has been shown to bind to the N-terminal domain of yIRS which is known to contain the catalytic site in Class I synthetases.

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DEDICATION:

To:

Eric

(Who had to put up with a very stressed and often grumpy Grace during the writing of this thesis)

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LIST OF ABBREVIATIONS

aaRS	Aminoacyl-tRNA synthetase
amp	Ampicillan
AMP	Adenosine monophosphate
A.S.T.	Active site titration
ATP	Adenosine triphosphate
β-mer	β-mercaptoethanol
bIRS	*E. coli* isoleucyl-tRNA synthetase
bp	base pair
BSA	Bovine serum albumin
CB	Coupling buffer
CIP	Calf intestinal phosphatase
ddH_2O	Deionised, distilled water
dH_2O	Distilled water
dNTPs	Deoxyribonucleic acids
DTT	DL-dithiothreitol
EB	ELISA blocking
ED	ELISA dilution
EDTA	Ethylenediaminine tetraacetic acid
EGF	Epiderminal growth factor
EGTA	Ethylene glycol-bis (β-aminoethyl ether)-N,N,N',N'-tetraacetic acid
ELISA	Enzyme-linked immunoabsorbent assay
EtBr	Ethidium bromide
FPLC	Fast protein liquid chromatography
IgA	Immunoglobin A

IgE	Immunoglobin E
IgG	Immunoglobin G
IleRS	Isoleucyl-tRNA synthetase
IPTG	Isopropylthio-β-galactosidase
IRS	Isoleucyl-tRNA synthetase
kb	Kilobase pair
MM	Minimal media
M_r	Molecular weight
mRNA	Messenger RNA
M.W.	Molecular weight
PA	Pseudomonic acid A
PAGE	Polyacrylamide gel elelctrophoresis
PBS	Phosphate buffered saline
PMSF	Phenylmethyl sulfonyl fluoride
SAS	Saturated ammonium sulphate
SDS	Sodium dodecyl sulphate
RNase A	Ribonuclease A
RNase T_1	Ribonuclease T_1
RS	Transfer RNA-synthetase (recombinant)
Tris	Tris (hydroxymethyl) methylamine
tRNA	Transfer RNA
$tRNA^{ile}$	Cognate transfer RNA for isoleucine
TY	Tryptone yeast
X-gal	5-bromo-4-chloro-3-idoyl-β-D-galactoside
yIRS	Yeast isoleucyl-tRNA synthetase

1. INTRODUCTION:

1.1 AMINOACYL-tRNA SYNTHETASES AND THEIR ROLE IN PROTEIN BIOSYNTHESIS

The biosynthesis of protein, from the transcription of DNA to mRNA, to the translation of mRNA to protein, is mediated by many different enzymes. Some of these enzymes have developed specialised editing functions to maintain the fidelity of information transfer from DNA to protein.

Previous studies have shown that in the process of protein translation the accuracy of amino acid incorporation into protein was dependent on the correct ligation of amino acids to its cognate tRNA. A previous study involving the chemical reduction of cysteinyl-$tRNA^{cys}$ to alanyl-$tRNA^{cys}$ with Raney Nickel, showed the incorporation of alanine into protein at positions normally reserved for cysteine (50). This study showed that there was no further "checking" of the aminoacylated tRNA after the amino acid was ligated to the tRNA.

Aminoacyl-tRNA synthetases are a family of enzymes believed to have arisen early in evolution (73). Their function is to specifically couple amino acids to their cognate tRNAs, thereby maintaining the accuracy of protein translation. There are 20 such aminoacyl-tRNA synthetases, one for each of the 20 amino acids. One aminoacyl-tRNA synthetase (aaRS) can have as many as six different cognate tRNAs and thus, must be able to discriminate its cognate tRNAs from all other tRNAs in addition to recognising its own amino acid substrate.

All aminoacyl-tRNA synthetases catalyse the identical two-step ATP dependent reaction:

(1) aaRS + amino acid + ATP ----> [aaRS-amino acid-AMP]+ $2PO_4^{3-}$

(2) [aaRS-amino acid-AMP] + tRNA -------> aminoacyl-tRNA + aaRS + AMP

Step 1 of the reaction involves the formation of the activated aminoadenylate and step 2 involves the formation of the aminoacylated tRNA. This ligation of the amino acid to the tRNA serves two purposes; 1) to activate the amino acid for protein synthesis and 2) to bring the correct amino acid to the ribosome A site as dictated by mRNA. The ability for the reaction to proceed in the forward direction is dependent on the hydrolysis of pyrophosphate to phosphates by pyrophosphatase. Detailed studies on the reaction mechanisms of aaRSs have shown that the order in which the three substrates bind varies from one aaRS to another (32). Some aaRSs require the binding of the cognate tRNA to facilitate the binding of ATP in the first step of the reaction whereas the majority of aaRSs can form the aminoadenylate in the absence of its cognate tRNA (1, 2, 53). Therefore, the aminoacylation of tRNA can be kinetically much more complex than depicted in equations 1 and 2.

1.2 STRUCTURAL AND FUNCTIONAL RELATIONSHIPS BETWEEN AMINOACYL-tRNA SYNTHETASES

It was first suggested that the large structural diversity observed among the 20 aminoacyl-tRNA synthetases reflected a very weak evolutionary relationship between the enzymes (88). AaRSs range in size from the 334 residues observed in tryptophanyl-tRNA synthetase (TrpRS) to the 1112 residues in phenylalanyl-tRNA synthetase (PheRS) (13) and they exhibit different oligomeric states that include monomers, homodimers, heterodimers and homotetramers (53). Recently, structural correlations among the 20 aaRSs have been established. due mainly to the availability of partially solved three-dimensional crystal structures of five aaRSs; TyrRS, SerRS, MetRS, AspRS and GlnRS, (10, 12, 21, 68, 69). Protein sequence data has also been used to make correlations between aaRSs. From this information, two distinct classes of 10 aaRSs each have been defined according to the differences in their active site sequences (14, 21, 24, 53). Class I aaRSs are distinguished by the N-terminal nucleotide-binding domain (with its characteristic Rossmann Fold) while the Class II aaRSs are characterised by a eight-stranded anti-parallel β-sheet domain as illustrated in figure 1(53).

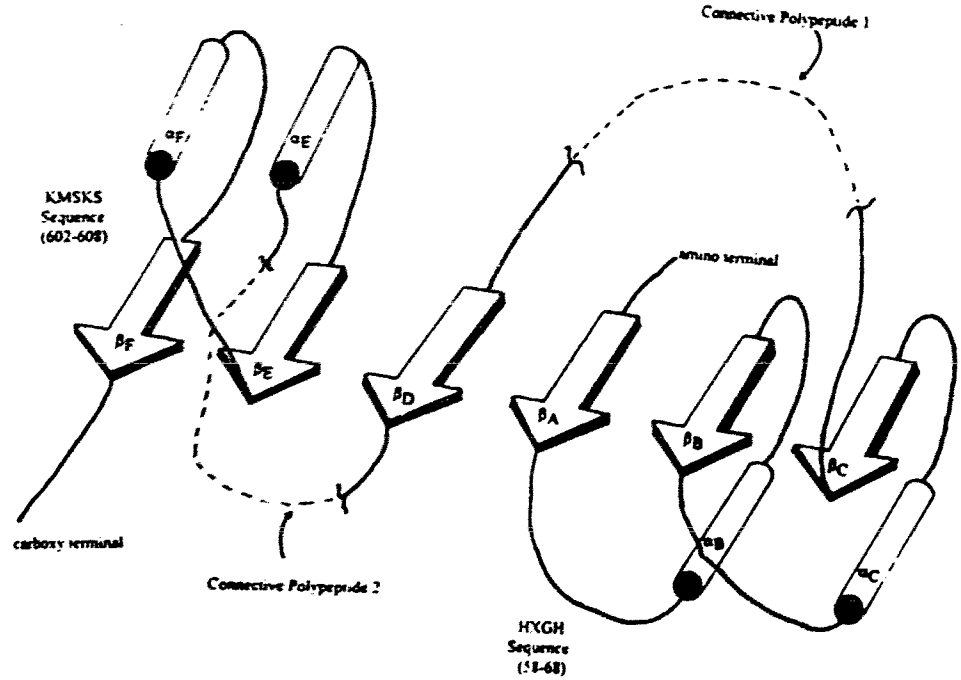
1.2.1 Class I Aminoacyl-tRNA Synthetases

The N-terminal nucleotide-binding domains of all Class I aaRSs contain the catalytic core of the enzyme where the amino acid substrate and ATP are bound (75). In addition, Class I aaRSs have two characteristic motifs located within the Rossmann Fold; an 11 amino acid signature sequence ending with the tetrapeptide His-Xaa-Gly-His (where Xaa is a hydrophobic amino acid) (7, 64, 67) and a pentapeptide sequence Lys-Met-Ser-Lys-Ser (36). In the HXGH sequence, only the first H and G are conserved in the 24 known sequences reviewed (53). The two histidines are believed to be involved in the binding of ATP in *E. coli* MetRS (12). In the KMSKS sequence, only the second lysine is strictly conserved in the 24 known sequences and in *E. coli* MetRS, this lysine is believed to be essential to the amino acid activation (51). In *E. coli* IleRS, it was found that trypsin digest of the peptide between Lysine^{605} and Serine^{606} of the K^{602}MSKS^{606} sequence can be blocked with ATP (90). Another distinguishing characteristic of Class I aaRSs is that they initially attach their amino acid substrates to the 2'OH of the last ribonucleotide in the acceptor stem of the cognate tRNA. Class II aaRSs, on the other hand, initially attach their amino acids to the 3'OH (note: the one exception is PheRS which is a Class II synthetase yet it aminoacylates at the 2'OH) (80). Therefore, the two classes of aaRSs can be partitioned by both structure and function.

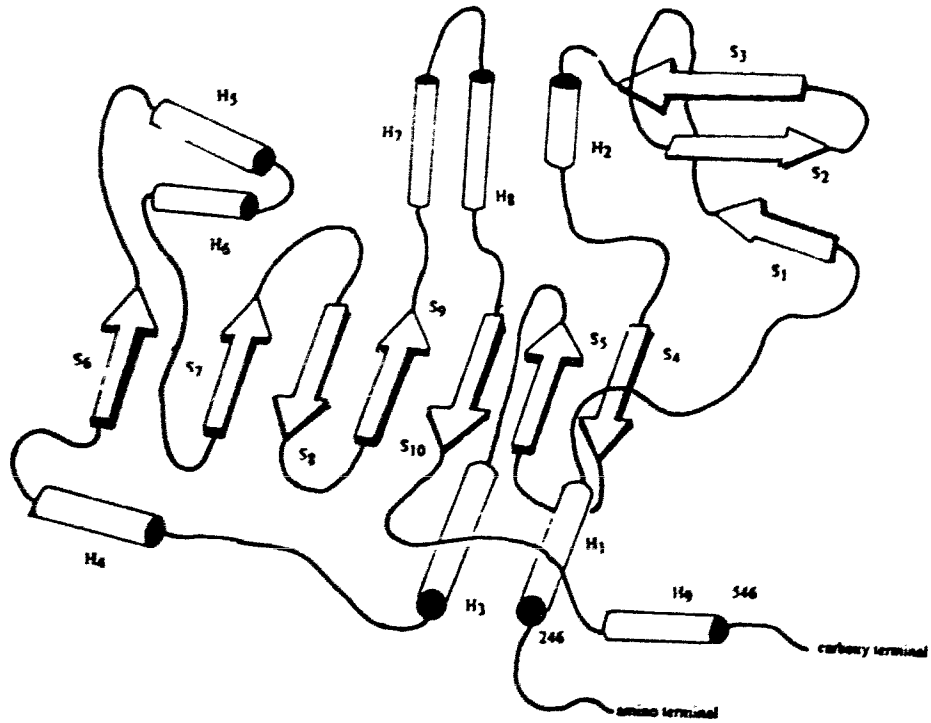
Further refinement of the class distinction was achieved by sequence analysis and comparisons of the partial three-dimensional structures of three known Class I aaRSs. Class I aaRSs can be divided into three subclasses based on the chemical and structural properties of their amino acid substrates (24). The Class and subclass divisions are illustrated in Table 1. Subclass Ia consists of the hydrophobic and sulphur containing residues (Val, Leu, Ile, Met and Cys), Subclass Ib consists of the aromatic tyrosine and tryptophan residues and Subclass Ic consists of the charged residues arginine, glutamic acid and glutamine (53).

Figure 1: Schematic Representation of the Active Site of Aminoacyl-tRNA Synthetases. A) Class I Aminoacyl-tRNA Synthetases and B) Class II Aminoacyl-tRNA Synthetases. Active site structures were derived from the crystal structures of methionyl-tRNA synthetase (91) and aspartyl-tRNA synthetase (69). Cylinders represents a helices and arrows represents b-sheets. Class I synthetases are characterised by a six-stranded parallel b-sheet (Rossman nucleotide-binding fold) and class II synthetases are characterised by an eight-stranded anti-parallel b-sheet. Note that Figure 1A is a modification of Figure 1 from (83) and Figure 1B is a modification of Figure 7 from (69). Note that X denotes a hydrphobic amino acid in Figure 1A.

A


Connective Polypeptide 1
αF
αE
KMSKS
Sequence
(602-608)
amino terminal
βF
βE
βD
βA
βB
βC
αB
αC
carboxy terminal
Connective Polypeptide 2
HXGH
Sequence
(58-68)

B


H5
H7
H8
H2
S3
S2
H6
S1
S9
S6
S7
S5
S4
S8
S10
H1
H4
H3
H9
546
246
carboxy terminal
amino terminal

Subclass	Class I	Class II
a	LeuRS, CysRS, ValRS, IleRS, MetRS	HisRS, ProRS, ThrRS, SerRS
b	TyrRS, TrpRS	AspRS, LysRS, AsnRS
c	ArgRS, GlnRS, GluRS	GlyRS, AlaRS PheRS

Table 1: Classification of Aminoacyl-tRNA Synthetases

Unlike the N-terminal domains of Class I aaRSs, the C-terminal domains of the Class I aaRSs are structurally divergent. Although there is some structural similarity between the C-terminal domains of aaRSs within a subgroup (75), the structural diversity may be important in discriminating cognate tRNAs from all other tRNAs. Studies have shown that the C-terminal domain is important in the recognition of the anticodon in MetRS (32). It has also been observed that the last 15 amino acids at the C-terminal end of TyrRS play an essential role in the binding of the cognate tRNA (6). Other studies involving mutations that affect tRNA binding in the Class I, subgroup Ia aaRSs do not seem to affect aminoadenylate formation, indicating that the C-terminal and N-terminal domains of enzymes in this subgroup may be functionally independent (32, 40, 75).

1.2.2 Class II Aminoacyl-tRNA Synthetases

Class II aaRSs exhibit a greater structural diversity than Class I aaRSs. Seven of the ten members of this class are known to be homodimers while AlaRS is a homotetramer and GlyRS and PheRS are heterodimers. Although all Class II aaRSs are predicted to have the eight-stranded antiparallel β-sheet structure, the position of this structure relative to the N-terminal and the C-terminal ends of the protein varies from one Class II aaRS to another (20). Three homologous sequence motifs were found in Class II aaRSs. Motif 1, a span of 20 amino acids containing one invariant proline residue, is believed to be an amphiphilic α-helix associated with the dimeric interface between the subunits. Putative motif 1 sequences have been identified in all known Class II aaRS sequences. Motifs 2 and 3, however, are even less conserved with one invariant arginine residue in key position in both motifs. Therefore, motifs 2 and 3 are believed to be constituents of the active site in Class II aaRSs (69).

The ten Class II aaRSs can be roughly divided into three subclasses. Subclass IIa is comprised of the homodimers, ThrRS, ProRS, HisRS and SerRS. The synthetases, ThrRS, ProRS and HisRS contain a C-terminal 100 amino acid residue span which show some degree of

homology (22). This subclass is distinguished by cross-subunit tRNA binding (20), where the binding of one tRNA molecule spans both subunits of the dimer. Subclass IIb is characterised by a homologous N-terminal domain which is implicated to be the anticodon binding region. Unlike subclass IIa, the members of subclass IIb, which consist of the homodimers AsnRS, AspRS and LysRS, do not exhibit cross-subunit tRNA binding (20). The cognate tRNA of subclass IIb synthetases binds only to one subunit of the homodimer. And lastly, subclass IIc is comprised of the "odd" aaRSs, the homotetramer AlaRS and the $\alpha_2\beta_2$ heterotetramers, GlyRS and PheRS. PheRS is unusual for a Class II aaRS as it aminoacylates the 2'OH of tRNA as opposed to the 3'OH as seen in the other Class II aaRSs (80).

1.3 ISOLEUCYL-tRNA SYNTHETASE

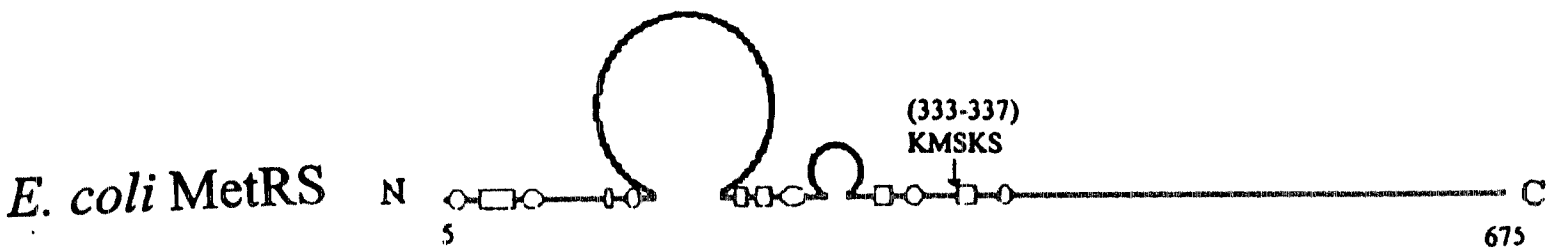
Isoleucyl-tRNA Synthetase (IleRS) is a Class I, subgroup Ia monomer that is predicted to have two large insertion sequences within the N-terminal nucleotide-binding domain (83) (see figure 2). These insertion sequences were first identified in MetRS and GlnRS whose three dimensional crystal structures have been partially solved (12, 58, 68). Because of the lack of conserved amino acids in the insertion sequences, the sequences are probably not required for enzyme activity (83). In MetRS, the prototypical Rossmann fold consists of six strands of parallel β-sheet with four α-helices, two above and two below the plane of the β-sheet (66) (see Figure 1). This Rossmann fold is interrupted twice by insertion sequences, with one large sequence, designated CP1, roughly dividing the fold into two halves of three β-sheet and two α-helices each. The CP1 insertion begins just after the carboxyl terminal end of β_C (83). The second, smaller insertion sequence (CP2) interrupts the second half of the Rossmann fold, beginning at the carboxyl terminal end of β_d. Alignment studies between MetRS and IleRS have identified putative CP1 and CP2 sequences in IleRS (see Figure 2). Some small internal deletions have been made within the predicted CP1 and CP2 sequences which do not affect the activity of the enzyme (82). More extensive deletions may have generated either unstable enzymes or enzymes lacking

activity (82). In all cases, deletions showing no tRNA-charging activity also showed no aminoadenylate formation (82). This indicates that the CP1 and CP2 sequences may be required for maintaining the structure of the active site. Studies have also shown that the CP1 and CP2 sequences are involved in the recognition of the tRNA acceptor stem in GlnRS (68). A recent study has shown that none of the amino acids in the insertion sequences of GlnRS interact directly with the tRNA. This observation supports the idea that the insertion sequences may play a structural role in the stabilisation of active site (58). Insertions have also been identified in TyrRS but because of the small size of this enzyme, the amount of dispensable peptide may be small (83).

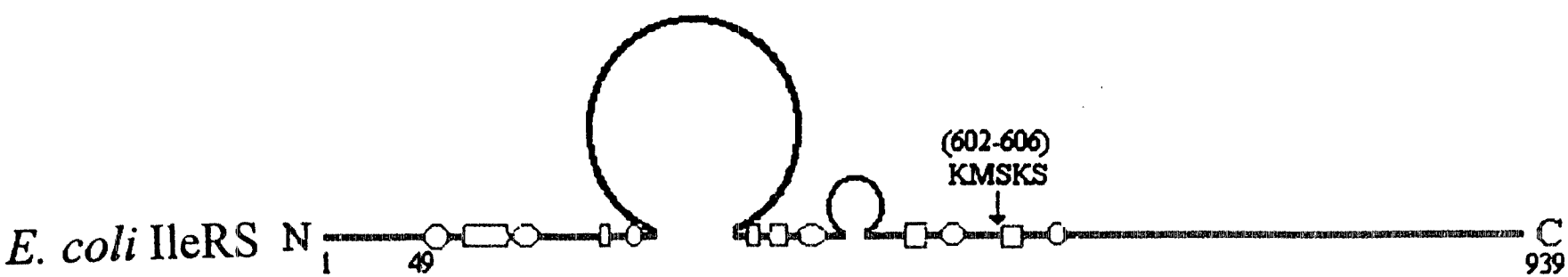
On the basis of sequence homology with MetRS and GlnRS, it was predicted that the large monomeric Class I aaRSs, IleRS, ValRS and LeuRS, all have large insertion domains in the N-terminal domain. These insertion domains vary in size from one Class I synthetase to another and are the reason behind the different sizes of the N-terminal domains in Class I synthetases (75). If the CP1 and CP2 insertion sequences were excluded from comparative sequence analysis, one would find more N-terminal sequence homology between IleRS and the other members of subgroup Ia, (ValRS, LeuRS, MetRS and CysRS). This homology within the members of this subgroup may be due to the similarity of their amino acid substrates and it is this similarity that necessitated the evolution of an "editing" or "proof reading" mechanism in order to maintain the fidelity of protein translation (30).

Comparative sequence analysis between IleRS and other known sequences of Class I aaRSs, especially those sequences with known partially solved three-dimensional structures, can be a powerful tool in the design of mutant IleRS enzymes. Presently, the three dimensional structure of IleRS has not been solved. A study of sequence homologies between MetRS, TyrRS and IleRS have led to the identification of putative binding sites for isoleucine and ATP in IleRS (17, 88). In *E. coli* IleRS, the putative isoleucine binding site has been identified as:

Figure 2: Primary Sequence Alignment Between *E. coli* Methionyl-tRNA Synthetase and *E. coli* Isoleucyl-tRNA Synthetase. Alignments are based on the crystal structure of methionyl-tRNA synthetase (91). Predicted secondary structures are illustrated along the primary sequence as rectangles for β-sheets and ovals for α helices. Note that this figure is a modification of Figure 1 from (75).



M.W. 76 KDa



M.W. 105 KDa

^{93}Pro Gly Trp Asp Cys His Gly Leu Pro Ile Glu Leu^{104}

In *E. coli* TyrRS, the conserved aspartate at position #78 is believed to hydrogen-bond with the amine of the amino acid tyrosine (29). Studies of the three dimensional structures of *E. coli* MetRS and TyrRS predicted that the HXGH and the KMSKS sequences are held in similar positions within the nucleotide-binding fold and are believed to have a similar function in the two synthetases. A recent preliminary study of the KMSKS loop suggests that movement of this loop, during the transition state of the formation of the aminoadenylate, brings lysine residues closer to the phosphates of the bound ATP molecule (74).

Further studies showed that the C-terminal domain of Class I aaRSs are structurally divergent. Attempts to identify the anticodon binding site of IleRS, based on comparative sequence analysis with the anticodon binding site of MetRS, have been unsuccessful (3, 32, 75). Therefore, the structure of the C-terminal domain may be different for each synthetase.

1.4 INHIBITORS OF ISOLEUCYL-tRNA SYNTHETASE

Competitive inhibitors can be a useful tool in the study of enzyme structure and function. There are several inhibitors of IleRS yet the effect of these competitive inhibitors varies from one species' IleRS to another. Some examples of IleRS inhibitors are furanomycin, L-O-methylthreonine and pseudomonic acid A (mupirocin (92)).

The novel amino acid, furanomycin, has been found to inhibit the formation of isoleucyl-tRNA^{ile} (85). In a recent study, furanomycin, which has a five-member heterocyclic ring with a double bond, was found to be incorporated into protein by *E. coli* IleRS (41). The chemical structure of furanomycin is illustrated in figure 3. A study by Kohno *et al.* has shown that furanomycin can adopt one of three different conformations, gauch$^+$, gauch$^-$ and trans forms (41). The gauche$^-$ form of furanomycin had been found to be similar in conformation to IleRS-

bound L-isoleucine. Further studies showed that furanomycin binds more tightly to IleRS than the natural non substrate amino acids L-valine and L-leucine and almost as tightly as L-isoleucine (41). Kohno *et al.* has also shown that the high efficiency to which furanomycin was aminoacylated to *E. coli* $tRNA^{ile}$ may be attributed to the tight binding of furanomycin at the amino acid binding site and the inability of *E. coli* IleRS's editing function to hydrolyse the adenylated furanomycin (41). This lends support to the double-sieve editing model. This model predicts that the editing site in IleRS has two separate editing functions; the first sieve rejects amino acids larger than isoleucine and the second sieve rejects amino acids smaller than isoleucine. Furanomycin was not rejected by the first sieve and the second sieve because of furanomycin's similarity in size to L-isoleucine. Although furanomycin was recognised and activated by *E. coli* IleRS, the conformation of furanomycin is not exactly the same as L-isoleucine. It was proposed that the bulkiness of the ringed side chain of furanomycin may disrupt the native conformations of cellular proteins when furanomycin is incorporated in protein (41). Thus, the disruption of proteins is probably responsible for the cytoxicity of furanomycin.

L-O-methylthreonine is a known competitive inhibitor of IleRS from Sarcoma-37 ascites cells and is required at high concentrations to inhibit protein synthesis (79). This led to the conclusion that L-O-methylthreonine forms an unstable, non-transferable adenylate with IleRS, thereby affecting the rate at which isoleucine is charged to $tRNA^{ile}$, yet does not seem to influence the extent of isoleucine charged to $tRNA^{ile}$. Similar results were obtained from crude aaRS preparations from rat liver and rabbit reticulocytes but not for *E. coli* where L-O-methylthreonine seems to inhibit both the rate and the extent of isoleucine charged to $tRNA^{ile}$ (79). It has also be noted that L-O-methylthreonine can be incorporated into protein by *E. coli* IleRS (78).

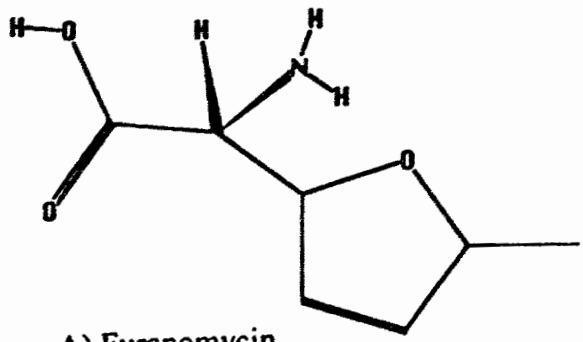
Pseudomonic acid A, also known as mupirocin (92), was discovered to be a competitive inhibitor for the isoleucine binding site in *E. coli* IleRS at low concentrations and is known to cause a marked decrease in protein synthesis in *E. coli* (37). Pseudomonic acid A is different

from L-O-methylthreonine in that it probably binds more strongly to the isoleucine binding site and it was not found to be incorporated into protein. In a recent study, it was proposed that pseudomonic acid A is a bifunctional inhibitor that interacts with IleRS in such a way that it mimics both isoleucine and ATP. Thus, it was postulated that pseudomonic acid A may be an analogue to the isoleucyladenylate (90). The structure of pseudomonic acid A is shown in figure 3. Pseudomonic acid A has been shown to have a very weak inhibitory effect on crude aaRS preparations from rat liver (37). A comparison of the inhibitory effect of pseudomonic acid A on *E. coli* IleRS and yeast IleRS showed that whereas the addition of pseudomonic acid A inhibits the activity of *E. coli* IleRS, the same concentration of the antibiotic does not inhibit the activity of yeast IleRS (61). The different "affinity" of prokaryotic and eukaryotic IleRS for pseudomonic acid A may be due, in part, to a single amino acid substitution near the KMSKS sequence. A comparative study of the amino acid sequences of wild-type *E. coli* IleRS and the pseudomonic acid A resistant *E. coli* strain PS102 showed a single amino acid substitution of leucine for phenylalanine at position #594 (89). It was observed that yeast IleRS has an isoleucine residue at the equivalent site. Thus, this single amino acid change may contribute to a conformational change which reduces the binding of pseudomonic acid A (89).

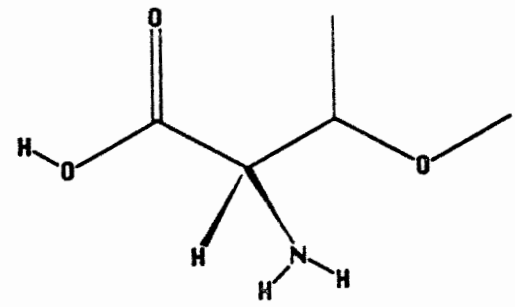
1.5 A STUDY OF ISOLEUCYL-tRNA SYNTHETASE

Isoleucyl-tRNA synthetase enzymes from two different species, Escherichia coli and Saccharomyces cerevisiae (abbreviated bIRS and yIRS respectively) were chosen as the subjects of study because of the relatively high degree of homology in the N-terminal domains of the two enzymes (34). Yet, the overall degree of homology between the two enzymes is only 27%. Although the two enzymes differ in size, bIRS at 939 residues in length and yIRS at 1072 residues in length, the two enzymes catalyse exactly the same reaction. It has been observed that

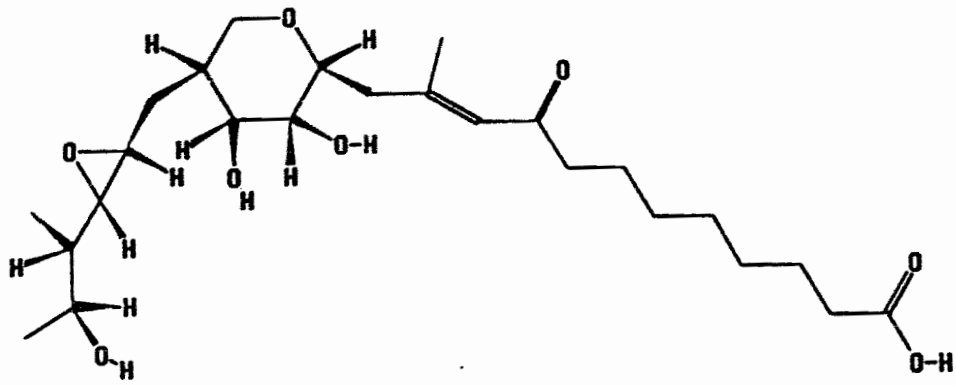
Figure 3: Inhibitors of Isoleucyl-tRNA Synthetase. A) Furanomycin, B) L-O-methylthreonine, C) Pseudomonic Acid A in comparison to the natural substrate D) L-Isoleucine. Furanomycin and L-O-Methylthreonine are amino acid analogues of L-isoleucine and Pseudomonic acid A is an analogue of the isoleucyladenylate.



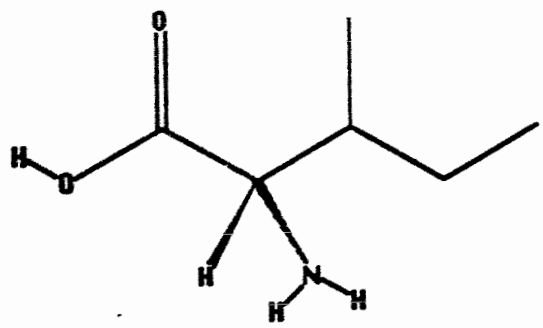
A) Furanomycin



B) L-O-methylthreonine



C) Pseudomonic Acid A



D) L-Isoleucine

the conserved motifs that are characteristic of Class I aaRSs are almost perfectly aligned between yIRS and bIRS. The tetrapeptide HXGH in the 11 amino acid signature sequence in yIRS is His-Tyr-Gly-His and is numbered as amino acids $^{\#}$54-$^{\#}$57 while in bIRS this sequence is His-Ile-Gly-His and is numbered as amino acids $^{\#}$65-$^{\#}$68. For the KMSKS sequence, there is a perfect alignment between the two species; amino acids $^{\#}$602-$^{\#}$606.

The yIRS and bIRS bind the same substrates (isoleucine, ATP and tRNA), except that the $tRNA^{ile}$ is different for the two species. *E. coli* $tRNA^{ile}$ and *S. cerevisiae* $tRNA^{ile}$ share the same anticodons and the same conserved 3' CCA sequence found in the acceptor stems of all tRNAs. The sequence homology between the *E. coli* $tRNA^{ile}$ and yeast $tRNA^{ile}$, excluding the invariant and semi-invariant bases found in all tRNAs, is approximately 45%. The difference in tRNA sequences accounts for the different $tRNA^{ile}$ binding affinities observed in bIRS and yIRS. Yeast IleRS seems to have a broader specificity for tRNAs, capable of not only charging its own species $tRNA^{ile}$ but *E. coli* $tRNA^{ile}$ as well. The bIRS has a higher affinity for its $tRNA^{ile}$ and does not show significant charging activity with *S. cerevisiae* $tRNA^{ile}$ (60).

In order to determine the important structural elements involved in the recognition, binding and activation of substrates, mutant enzymes of IleRS can be genetically engineered. Using comparative sequence analysis as a guide, two types of mutant yeast isoleucyl-tRNA synthetase enzymes were created and used to study the relationship between structure and function.

In previous studies, C-terminal deletion mutants of class I aaRSs were used to find the minimal protein sequence required for aminoacylation activity and aminoadenylate formation. Among Class II enzymes, AlaRS requires only the first 385 amino acids of its N-terminal end to form the alanyladenylate (38). In TyrRS, a Class I enzyme of 419 residues in length in its monomeric state, C-terminal deletions of up to 100 amino acids have no affect on aminoadenylate activity (87). In MetRS (approximately 750 residues in length), which belongs to the same

subgroup as IleRS, deletions in the C-terminal domain of up to 200 amino acids do not affect the aminoadenylate activity of the enzyme (5). The latter two studies lend support to the idea that the N-terminal domains of Class I aaRSs, which contain the catalytic core of these enzymes, is probably functionally independent of the C-terminal domain, which is believed to be responsible for cognate tRNA recognition. Because of the differences in the size and the oligomeric state of IleRS in relation to TyrRS and MetRS, it was not clear whether large deletions of the C-terminal domain of IleRS would affect the formation of the aminoadenylate. A recent preliminary study has shown that the first 825 residues in yIRS and the first 640 residues in bIRS are required for aminoadenylate formation (18). Deletions of greater than 10 amino acids from the C-terminal end of yIRS resulted in loss of tRNA charging activity (18). It was hypothesized by Cramer *et al.* that the deletion of 250 residues from the carboxy terminus does not affect the three dimensional structure of the amino acid binding pocket. The C-terminal deletion mutants of yIRS may help determine the location the "editing" or "proof reading" site (25). Previous authors have hypothesised that the editing site is located within the nucleotide-binding fold, near or within the catalytic core of the enzyme (54). It is hypothesized that the editing mechanism consists of a four-step process that involves two initial binding steps and two proof reading steps (18). However, it has not been established which structural elements are important in the function of this editing site.

Many large proteins seem to be organised in a modular-type arrangement of structural and functional domains. Mild digestion of these proteins by trypsin can often yield stable fragments, thus providing a means to assess the individual activities of these substructures. This compartmentalisation of specific functions within a protein also provides a starting point for the design of chimeric enzymes with altered substrate specificity and/or catalytic function.

Chimeric enzymes are the products of fused genes. Chimeras are usually created from the fused gene fragments of genes that code for related proteins from the same species or for the same protein from different species. Chimeric enzymes can be designed in such a way that the

functional domains of related proteins can be swapped to yield a chimera that has an altered substrate specificity or catalytic activity (35). Chimeric enzymes can also be used to study the inter-relationship between structural and functional domains and the inter-changeability of these domains. There are a number of reports of inter-domain chimeric enzymes that combine the properties of both parental enzymes. For example, a chimera was created that has the N-terminal domain of the hepatic lipase and the C-terminal domain of lipoprotein lipase. From the results, it was proposed that the N-terminal domain of the chimera, which contained the active site of heptatic lipase, dictated the catalytic character of the chimera and the C-terminal domain of lipoprotein lipase dictated the preference for long-chained substrates (89). Another example of chimera with altered substrate specificity is a fusion of the extra-cellular domain of the insulin cell surface receptor and the transmembrane and intracellular domains of the epidermal growth factor (EGF) receptor. Both receptors have a highly conserved tryosine-specific protein kinase domain. It was found that the binding of insulin to the chimeric receptor stimulated tyrosine kinase activity from the EGF portion of the chimeric receptor. This suggests that these two receptors have a similar mechanism for signal transduction (63). Chimeric enzymes can also be useful in locating important structural/functional sites. For example, chimeric cDNAs of sheep and rat Na,K-ATPase α subunit were generated to determine the site responsible for oubain resistance in rat and mouse Na,K-ATPase α subunit. It was found that human and sheep Na,K-ATPase α subunits are more sensitive to oubain than the rat and mouse subunits. It was found that constructs containing the N-terminal domain of the rat enzyme and the C-terminal domain of the sheep enzyme conferred oubain-resistance whereas the reverse construct did not (59).

It has been suggested that the CP1 insertion sequence in the N-terminal nucleotide-binding domain is a good site for a junction in the design of a chimeric enzyme as it has been established that the N-terminal Rossmann fold can accommodate insertions without affecting the structure of the fold itself (83). Intra-domain chimeras of aminoacyl-tRNA synthetases have been constructed (81). These chimeric enzymes were composed of the N-terminal region of *E. coli* MetRS and the

C-terminal region of *E. coli* IleRS with junctions between the two fragments within the CP1 and CP2 insertion sequences. Of the five MetRS/IleRS chimeric enzymes designed by Starzyk *et al.*, only three were expressed *in vivo* and none were enzymatically active (81).

The similarity of yIRS and bIRS, with respect to their size, structure, function and similar preference for substrates should make it easy to create chimeric enzymes where the junction between the yIRS and bIRS portions of the chimera is least likely to affect the enzyme's overall structure and function. Chimeras of IleRS may help determine the important structural elements responsible for the recognition and binding of substrates and most importantly, to ascertain whether these structural elements are species specific. As previously mentioned, yIRS is known to charge both *S. cerevisiae* and *E. coli* $tRNAs^{ile}$ whereas bIRS is known to charge only *E. coli* $tRNA^{ile}$ (61). The differences in specificity may be due, in part, to certain recognition sites in the N-terminal and/or C-terminal domains for particular sites on the tRNA. In addtion, the differences in the sequences of *E. coli* $tRNA^{ile}$ and yeast $tRNA^{ile}$ may also contribute to the binding specificity of bIRS and yIRS. The creation of N-terminal yIRS/C-terminal bIRS chimeric enzymes (and vice versa) can help determine where the recognition sites are located. The study of chimeric enzymes may aid in the design of other chimeric enzymes with altered substrate specificity. This may lead to a technique that will allow the *in vivo* incorporation of novel amino acids into protein for the study of protein structure and function.

2. MATERIALS AND METHODS:

2.1 MATERIALS

All chemicals, enzymes, materials and supplies were of the highest grade that were commercially available and were purchased from the following suppliers: L-[U-^{14}C] isoleucine (313 mCi/mmol), L-[4,5-^{3}H] isoleucine (103 Ci/mmol), ECL Western Immunoblotting Detection Solutions, mouse IgG-horseradish peroxidase-linked whole antibody (from sheep), ECL molecular weight marker (biotinylated proteins) and streptavidin-horseradish peroxidase were purchased from Amersham. Restriction enzymes were purchased either from Bethesda Research Laboratories or New England Biolabs. T_4 DNA ligase and VentR polymerase were also purchased from Bethesda Research Laboratories. Mono Q column, Q Sepharose and deoxyribonucleotides were supplied by Pharmacia Biotech Inc. Hydroxylapatite, Macro-Prep HA, the Affi-Gel Hz Immunoaffinity Kit and the high and low molecular weight prestained protein standards were purchased from Bio-Rad. Protein-Pak Q 8HR AP-1 FPLC column was purchased from Waters/Millipore. The calf-intestinal phosphatase and ribonuclease T_1 were from Boehringer Mannheim. Oligonucleotides were purchased from the Institute of Molecular Biology and Biochemistry Services Lab at Simon Fraser University, Burnaby, B.C. Immobilon P was purchased from Millipore. Nitrocellulose filters (24 mm circles) were purchased from Hoefer Scientific Instruments. Cellulose dialysis tubing was supplied by Fischer Scientific. Comassie brilliant blue, bromophenol blue, xylene cyanol, thiamine (vitamin B_1), acrylamide, amberlite, N,N bis-acrylamide, agarose, sodium dodecyl sulphate, dithiothreitol, phenylmethylsulfonyl fluoride, ampicillin, tetracycline, bovine serum albumin, ovalbumin, Tween-20, 5-bromo-4-chloro-3-indoyl-β-D-galactose (X-Gal) isopropylthio-β-galactoside (IPTG), MOPS, Trizma base (Tris-Cl), o-phenylenediamine and ficoll were purchased from Sigma. L-isoleucine and β-mercaptoethanol was supplied by ICN. Bacteriological media, trypticase peptone and yeast extract was purchase from Merck. Goat-antimouse serum conjugated with horseradish peroxidase was purchased from Mandel Scientific and lithium

pseudomonate was donated by Smith-Kline Beecham Pharmaceuticals. All other chemicals were purchased from ACS or Sigma.

2.2 BUFFERS AND OTHER SOLUTIONS

Antibody Precipitation

100% Saturated Ammonium Sulphate (100% SAS) pH 7.5: 260 g $(NH_4)SO_4$ in 289 mL ddH_2O. pH to 7.5 with NH_4OH.

ELISA Solutions:

1X Phosphate Buffered Saline (1X PBS) pH 7.3: 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4 and adjust pH to 7.3 with NaOH.

ELISA Blocking Buffer (EB buffer): 1% (w/v) ovalbumin, 0.05% (v/v) Tween 20 in 1X PBS pH 7.3.

ELISA Dilution Buffer (ED buffer): 0.5% (w/v) ovalbumin, 0.05% (v/v) Tween 20 in 1X PBS pH 7.3.

ELISA Substrate: 10 mL 0.1 M $NaCH_3CO_2$, pH 6.0, 10 mg o-phenylenediamine, 10 µL 30 % H_2O_2.

Protein Assay Solutions:

Bradford Assay Dye Solution: 50 mg comassie blue, 25 mL ethanol (99.9%), 50 mL concentrated H_3PO_4, 425 mL H_2O. Filter with 3MM Whatmann paper before use.

Lowry Microtitre-Plate Assay "Working" Solution: 0.025% (w/v) $CuSO_4 \cdot H_2O$, 0.025% (w/v) potassium tartrate (from a 0.2% (w/v)$CuSO_4 \cdot H_2O$/0.2% (w/v) potassium tartrate stock solution), 2.5% (w/v) sodium carbonate (from a 20% (w/v) stock solution), 0.2 N NaOH (from a 0.8 N stock solution) and 2.5% (w/v) sodium dodecyl sulphate (SDS) (from a 10% (w/v) stock solution).

Sodium Dodecyl Sulphate (SDS) Polyacrylamide Gel Solutions:

Separating Buffer: 0.75 M Tris-Cl, pH 8.8, 0.2% (w/v) SDS

Stacking Buffer: 0.1458 M Tris-Cl, pH 6.8, 0.117% (w/v) SDS

Acrylamide Stock Solution: 30% (w/v) acrylamide, 0.8% (w/v) N,N-bis-acrylamide

SDS Protein Gel Loading Dye (5X): 7.5 mL 2.083 M Tris-Cl, pH 6.8, 11.0 mL β-mercaptoethanol, 5 g SDS. Add H_2O to 25 mL. Add glycerol to 50 mL. Lastly add 0.0025 g bromophenol blue.

SDS PAGE Running Buffer: 0.025 M Tris-Cl, pH 8.3, 0.1% (w/v) SDS, 0.192 M glycine

SDS PAGE Staining and Destaining Solutions:

Comassie Stain: 0.3% (w/v) comassie blue in fast destaining solution (see below).

Fast Destaining Solution: 50% (v/v) methanol, 10% (v/v) glacial acetic acid, 40% (v/v) ddH_2O

Slow Destaining Solution: 10% (v/v) glacial acetic acid, 90% (v/v) ddH_2O

Gel Drying Solution: 1:1 SDS PAGE Running buffer to glycerol

Western Blot Solutions and Reagents:

1X Transfer Buffer: 39 mM glycine, 48 mM Tris-Cl, 0.0375% (w/v) SDS

Tris-Buffered Saline (10X TBS): 0.2 M Tris-Cl, pH 7.4, 1.56 M NaCl

(Note: for ECL Technique, adjust pH to 7.6)

Western Blot Blocking Buffer (Blotto): 5% (w/v) skim milk powder, 0.05% (v/v) Tween 20 in 1X TBS, pH 7.4

(Note: ECL Blotto is 5% (w/v) skim milk powder, 0.1% (v/v) Tween 20 in 1X TBS, pH 7.6)

ECL Washing Buffer (For ECL Technique only): 1X TBS, pH 7.6, 0.1% (v/v) Tween 20

Developing Solution (old method only, not ECL): 6 mg 3,3-diaminobenzidine, 9 mL 50 mM Tris-Cl, pH 7.4, 1 mL 0.3% (w/v) $NiCl_2$ (dissolved in water), 10 µL 30% H_2O_2 (per membrane).

Western Blot Stripping Buffer: 62.5 mM Tris-Cl, 100 mM β-mercaptoethanol, 2% (w/v) SDS and adjust to pH 6.7

Plasmid Preparation Solutions:

Solution I: 25 mM Tris-Cl, pH 8.0, 50 mM glucose, 10 mM EDTA

-Autoclave.

-Add lysozyme to 4 mg/mL just before use.

Solution II: 0.2 N NaOH, 1% (w/v) SDS

-Prepare before use from stock solutions 0.4 N NaOH and 2 % (w/v) SDS (1:1).

Solution III: 60 mL 5 M KCH_3CO_2, 11.5 mL glacial acetic acid, 28.5 mL ddH_2O

Tris-EDTA Buffer pH 8.0 (TE 8.0): 10 mM Tris-Cl, pH 8.0, 1 mM EDTA

-Autoclave before use.

Restriction Enzyme Buffers:

NEBuffer 1 (1X): 10 mM Bis Tris Propane-HCl, 10 mM $MgCl_2$, 1 mM dithiothreitol (DTT) at pH 7.0 at 25°C.

NEBuffer 2 (1X): 10 mM Tris-Cl, 10 mM $MgCl_2$, 50 mM NaCl, 1 mM DTT (pH 7.9 at 25°C).

NEBuffer 3 (1X): 50 mM Tris-Cl, 10 mM $MgCl_2$, 100 mM NaCl, 1 mM DTT (pH 7.9 at 25°C).

NEBuffer 4 (1X): 20 mM Tris-acetate, 10 mM magnesium acetate, 50 mM potassium acetate, 1 mM DTT (pH 7.9 at 25°C).

NEBuffer Nsi I (1X): 10 mM Tris-Cl, 10 mM $MgCl_2$, 100 mM NaCl, 1 mM DTT (pH 8.4 at 25°C).

REact 2 (1X): 50 mM Tris-Cl, pH 8.0, 10 mM $MgCl_2$, 50 mM NaCl.

REact 3 (1X): 50 mM Tris-Cl, pH 8.0, 10 mM $MgCl_2$, 100 mM NaCl.

REact 4 (1X): 20 mM Tris-Cl, pH 7.4, 5 mM $MgCl_2$, 50 mM KCl.

Modification Enzyme Buffers:

Calf Intestinal Alkaline Phosphatase Phosphorylation Buffer (CIP buffer) (1X): 50 mM Tris-Cl, pH 8.5, 0.1 mM EDTA.

T_4 Ligase Buffer (5X): 0.25 M Tris-Cl , pH 7.6, 50 mM $MgCl_2$, 5 mM ATP, 5 mM DTT, 25% (w/v) polyethylene glycol-8000.

VentR DNA Polymerase Reaction Buffer (1X): 20 mM Tris-Cl (pH 8.8 at 25 °C), 10 mM KCl, 10 mM $(NH_4)SO_4$, 2 mM $MgSO_4$, 0.1% Triton X-100, dNTPs, DNA.

Agarose Gel Running Buffer (50X TAE stock): 242 g Tris-Cl, pH 8.5, 57.1 mL glacial acetic acid, 37.2 g $Na_2EDTA{\cdot}2H_20$ (at a final concentration of 2 mM).
-Make up to 1 L with ddH_2O.

Ethidium Bromide Stock Solution (1000X): 50 mg ethidium bromide, 100 mL ddH_2O

Agarose Gel Loading Dye: 20% Ficoll 400, 0.1 M Na_2EDTA, pH 8.0, 1.0% (w/v) SDS, 0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol

Competent Cell Preparation Buffers:

TFBI: 30 mM KCH_3CO_2, 50 mM $MnCl_2$, 100 mM KCl, 10 mM $CaCl_2$, 15% glycerol. Adjust to pH 7.0 and filter sterilize.

TFBII: 10 mM Na-MOPS, pH 7.5, 75 mM $CaCl_2$, 10 mM KCl, 15% glycerol. Adjust to pH 7.0 and filter sterilize.

Column Chromatography Buffers:

Immunoaffinity Column Kit 1X Coupling Buffer (CB buffer): 100 mM $NaCH_3CO_2$, pH 5.5, 150 mM NaCl.

Lysis Buffer: 50 mM Tris-Cl, pH 8.0, 25 % (w/v) sucrose, 1 mM EGTA, 0.5 mM PMSF

Buffer A: 20 mM Tris-Cl, 20 mM NaCl, pH 8.0

Buffer B: 20 mM Tris-Cl, 400 mM NaCl, pH 8.0

Buffer C: 50 mM potassium phosphate, pH 7.0, 50 mM KCl

Buffer D: 50 mM potassium phosphate, pH 7.0, 500 mM KCl

Buffer E: 50 mM potassium phosphate, pH 7.0, 50 mM KCl, 0.5 mM DTT, 0.5 mM PMSF

Buffer F: 50 mM potassium phosphate, pH 7.0, 1.0 M KCl, 0.5 mM DTT, 0.5 mM PMSF

Buffer G: 10 mM potassium phosphate, pH 7.0, 25% (v/v) glycerol, 1 mM DTT, 0.5 mM PMSF

Buffer H: 400 mM potassium phosphate, pH 7.0, 25% (v/v) glycerol, 1 mM DTT, 0.5 mM PMSF

Buffer I: 50 mM potassium phosphate, pH 7.0, 1 mM DTT, 0.5 mM PMSF

tRNA Charging Assay Solutions:

5X tRNA Charging Buffer: 720 mM Tris-Cl, pH 7.8, 50 mM β-mercaptoethanol, 0.1 mM PMSF, 50 mM $MgCl_2$

ATP Stock Solution: 100 mM ATP, 100 mM $MgCl_2$, pH 8.0

Solution A:

200 µL 5X tRNA Charging Buffer

100 µL 100 mM ATP·Mg^{2+}

2 µL 1 unit/µL inorganic pyrophosphatase

40 µL ddH_2O

Active-Site Titration Assay:

5X A.S.T. Buffer: 65 mM Tris-Cl, pH 5.87, 50 mM $MgCl_2$

1X A.S.T. Buffer: 30 mL 5X A.S.T. Buffer, 150 µL 100 mM PMSF, 105 µL β-mercaptoethanol and make up to 150 mL.

1X A.S.T. Reaction Solution:

(to 1 mL 5X A.S.T. Buffer, add 5 µL 100 mM PMSF, 3.5 µL β-mercaptoethanol).

30 µL 5X A.S.T. Buffer from 1 mL 5X A.S.T. Buffer listed above

10 µL ddH_2O

5 µL 154.22 µM ^{14}C-labeled isoleucine (Amersham)

1.5 µL 500 µM isoleucine

3 µL 100 mM ATP

0.5 µL 1 unit/µL pyrophosphatase

total volume 50 µL

2.3 BACTERIOLOGICAL MEDIA, STRAINS AND METHODS

2.3.1. Media

- TY/Mg Broth: 20 g tryptone, 5 g yeast extract, 5 g NaCl, 10 mM $MgSO_4$ per L. $MgSO_4$ added from 1 M filter sterilized stock.

- 5X Minimal Media (MM) Salts: 52.5 g K_2HPO_4, 22.5 g KH_2PO_4, 5 g $(NH_4)_2SO_4$, 2.5 g $Na_3C_6H_5O_7 \cdot 2H_2O$ and make up to 1 L with ddH_2O.

- 1X Minimal Media Broth (MM): 10 mL 5X minimal media salts in 39.5 mL ddH_2O

autoclave at 121°C for 20 minutes, then cool to 60°C.

add 0.5 mL 20% (w/v) glucose (previously autoclaved)

50 µL 20% (w/v) $MgSO_4$ (previously autoclaved)

25 µL 1% (w/v) Vitamin B_1 (filter sterilized)

- 1X MM/amp Broth: 1X MM broth supplemented with ampicillin (filter sterilized) to a final concentration of 100 µg/mL

- 1X Minimal Media Plates (MM plates):

100 mL 5X minimal media salts, autoclaved separately from agar solution.

7.5 g agar in 400 mL ddH_2O, autoclaved separately from minimal media salts.

cool both solutions to 60°C and mix.

add 5 mL 20% (w/v) glucose (previously autoclaved)

0.5 mL 20% (w/v) $MgSO_4$ (previously autoclaved)

250 µL 1% (w/v) Vitamin B_1 (filter sterilized)

- 1X MM Plates/amp: 1X MM plates supplemented with ampicillin (filter sterilized) to a final concentration of 200 µg/mL prior to pouring plates.

- 2X TY Plates: 10 g tryptone, 5 g yeast extract, 5 g NaCl, 15 g agar per L and autoclave at 121°C for 20 minutes.

- 2X TY/amp Plates: 2X TY Plates supplemented with ampicillin (filter sterilized) to a final concentration of 200 µg/mL prior to pouring plates.

- TY Plates: 10 g tryptone, 5 g yeast extract, 10 g NaCl, 7.5 g agar per L.

- TY/Tet Plates: TY plates supplemented with tetracycline to a final concentration of 50 µg/mL.

- 2X TY Broth: 16 g tryptone, 10 g yeast extract, 5 g NaCl per L.

- 2X TY/amp Broth: 2X TY Broth supplemented with ampicillin (filter sterilized) to a final concentration of 100 µg/mL.

- TY Broth: 10 g typtone, 5 g yeast extract, 10 g NaCl per L.

- TY/Tet Broth: TY broth supplemented with tetracycline to a final concentration of 50 µg/mL.

2.3.2 Bacterial Strains

- 2.3.2.1 *Escherichia coli* strain DH5α

Genotype: *sup*E44 Δ*lac*U169 (ϕ80 *lac*ZΔM15) *hsd*R17 *rec*A1 *end*A1 *gyr*A96 *thi*-1 *rel*A1

Growth Conditions: *E. coli* strain DH5α (70) was maintained on 2X TY plates and grown in 2X TY liquid broth at 37°C.

Relevant Characteristics: Used for all routine transformations and ligations unless otherwise stated.

- 2.3.2.2 *Escherichia coli* strain TG2

Genotype: *sup*E *hsd*Δ5 *thi* Δ (*lac-pro*AB) Δ (*srl-rec*A) 306::Tn 10 (*tet*^{r}) F' [*tra*D36 *pro*AB$^+$ *lac*I^q *lac*ZΔM15]

Growth Conditions: *E. coli* strain TG2 (70) was maintained on minimal media plates and grown either in minimal media broth or 2X TY liquid media broth at 37°C.

Relevant Characteristics: Repression of recombinant gene expression by a lacI^q repressor gene from the host with an IPTG inducible promoter. Used as host for all pKK223-3 derived plasmids.

- 2.3.2.3 *Escherichia coli* strain QY13

Genotype: F^- $lac_{am}trp_{am}$BB'*bio*-256 N^+ *c*1857 ΔH Sm^r *rec*A

Growth Conditions: *E. coli* strain QY13 (56) was maintained on 2X TY plates and grown in 2X TY liquid broth at 30°C.

Relevant Characteristics: Used only to grow up pLmp10 plasmids containing the recombinant yIRS 3' deletion genes.

2.3.3 Preparation of Competent Cells

2.3.3.1 Preparation of Competent *E. coli* Cells (Strains DH5α and QY13):

A test tube containing 2 mL of TY/Mg broth was inoculated with a single *E. coli* colony from a fresh plate (the plate was grown for 16-20 hours at the appropriate temperature of 37°C or 30°C) and was grown to a cell density of OD_{600} = 0.5 (at 37°C or 30°C) on a rotating shaker. The 2 mL culture was then used to inoculate 20 mL of TY/Mg broth (pre-warmed to 37°C or 30°C) in a 250 mL flask and grown to a cell density of OD_{600}=0.5 with vigorous shaking (300 cycles/minute on a rotary shaker). The 20 mL culture was then added to 200 mL of pre-warmed (at 37°C or 30°C) TY/Mg broth in a 1 L flask on a rotary shaker and grown to until the cell density was OD_{600}=0.6. The culture was chilled on ice and aseptically transferred to pre-cooled sterile 50 mL polypropylene tubes. The cells were centrifuged at 4,000 r.p.m. in a Sorvall SS34 rotor at 4°C for 5 minutes. All subsequent steps were performed at 4°C, aseptically. The media was decanted and the cell pellet was gently resuspended in 40 mL of TFBI buffer. The resuspended cells were centrifuged again at 4,000 r.p.m. for 5 minutes. The TFBI buffer was decanted and the cell pellet was resuspended in 10 mL of TBFII buffer. The resuspended cells were divided into approximately equal aliquots in 1.5 mL Eppendorf tubes. The competent cells were then frozen on dry ice and stored at -80°C.

2.3.3.2 Preparation of Competent *E. coli* Cells (Strain TG2):

The procedure for the preparation of competent *E. coli* strain TG2 cells is identical to the procedure outlined in section 2.3.3.1 except for the media used. *E. coli* TG2 cells were grown on TY/Tet plates overnight and a single colony was used to inoculate 2 mL TY/Tet broth. In the place of TY/Mg broth (outlined in section 2.3.3.1), TY/Tet broth was used in the preparation of competent *E. coli* TG2 cells. Cultures were grown at 37°C.

2.3.4 Transformation of *E. coli* Strains

2.3.4.1 Transformation of *E. coli* DH5α Strain

Approximately 1 to 3 µL of plasmid was added to 50 µL of previously thawed competent DH5α cells and incubated on ice for 30 minutes. The cells were then heat shocked at 37°C for 1 minute, followed by re-incubation on ice for another 1 minute. An aliquot of 450 µL of 2X TY broth was added to the cells and incubated at 37°C for 1 hour on a rotary shaker. After incubation, 10 µL and 50 µL aliquots of the cells were spread-plated on 2X TY/Amp plates and grown at 37°C overnight.

For a ligation experiment, 12.5 µL of the diluted ligation mixture was added to 120 µL of thawed competent cells and transformed as described above. After the 1 hour incubation at 37°C, a 1 mL aliquot of the culture was spread-plated on 2X TY/Amp plates at grown at 37°C overnight.

2.3.4.2 Transformation of *E. coli* TG2 Strain

The procedure for the transformation of *E. coli* strain TG2 cells is identical to the procedure described for DH5α except for the following: After the 1 hour incubation at 37°C, the cells were spread-plated in 10 µL, 50 µL, and 75 µL aliquots on 1X MM/Amp plates and grown for 24-36 hours at 37°C.

2.3.4.3 Transformation of *E. coli* QY13 Strain

Aliquots of 8 to 10 µL of plasmid (pLcII-Hinc II, pLcII-Cla I, pLcII-Eco RI, pLcII-Kpn I and pLcII-Sca I) were added to 0.5 mL of previously thawed competent QY13 cells and incubated on ice for 30 minutes. The cells were heat shocked at 30°C for 1 minute, then re-incubated on ice for 1 minute. The cells were then added to 2 mL of 2X TY broth and the culture was grown at 30°C for 1 hour. Transformed cells were spread-plated in 200 µL and 750 µL aliquots on 2X TY/Amp plates and grown overnight at 37°C.

2.4 PLASMIDS

2.4.1 Plasmids Used

Note: Plasmid Maps of all the plasmids used are in Appendix A.

2.4.1.1 pKK223-3 (Expression Vector)

The plasmid pKK223-3 (11) is an expression vector containing a *tac* promoter which drives the expression of recombinant genes. The plasmid also contains an amp^r gene.

2.4.1.2 pKR3 (yIRS Cloning Vector)

The plasmid pKR3 was constructed by Racher *et al.* (61) and is derived from the following: plasmid pZG100 (M5) contains the entire coding region of the *Saccharomyces cerevisiae* isoleucyl-tRNA synthetase (yIRS) gene as well as the 5' and 3' non-coding regions. A fragment of the 5' non-coding region of pZG100 was altered by site-directed mutagenesis to

introduce an efficient *E. coli* ribosome-binding site (61). This fragment and another fragment containing the entire coding region of the yIRS gene from pZG100 were recombined in pUC18 to reconstruct the yIRS gene with the altered 5' consensus ribosome-binding site. This pUC18 plasmid with the inserted yIRS gene was named pKR3.

2.4.2.3 pKR4 (yIRS Expression Vector)

The reconstructed yIRS gene from pKR3 was subsequently subcloned into pKK223-3 by Racher *et al.* (61) to create the expression plasmid pKR4.

2.4.1.4 pMT521 (bIRS Expression Vector)

The plasmid pMT521 was constructed by Webster *et al.* (88) and was obtained from P. Schimmel of M.I.T. pMT521 is the expression vector for the *E. coli* isoleucyl-tRNA synthetase (bIRS) which is expressed under its own promoter.

2.4.1.5 pKR5 (yeast/*E. coli* IRS Chimera Expression Vector)

The plasmid pKR5 is a chimeric plasmid constructed by Racher (60) from pKR4 and pMT521. It contains the 5' non-coding and coding region of the yIRS gene from pKR4 (bases #1-#711) and the 3' coding region of the bIRS gene from pMT521 (bases #693-#2917). Construction of the plasmid pKR5 were as follows: pMT521 was cleaved at the Hpa I and Xba I sites and a 2.8 kilobase (kb) fragment containing bases #693-#2917 of the bIRS gene and 701 base pairs (bps) of unknown sequence 3' to the stop codon of the bIRS gene was isolated. pKR4 was also cut at the Hpa I and Xba I sites. A 5.4 kb fragment containing the entire regulatory region of pKR4, the modified 5' non-coding region with the consensus ribosome-binding sequence and bases #1-#711 of the yIRS coding sequence was isolated and ligated to the 2.8 kb fragment, thus creating the plasmid pKR5. The chimeric gene, designated Hpa I/Hpa I, is expressed under the control of a *tac* promoter.

2.4.1.6 pKR7 (yeast/*E. coli* IRS Chimera Expression Vector)

The chimeric expression plasmid pKR7 was constructed by Racher (60) and was derived from the following: pMT521 was initially cleaved at the Sac II site and blunt ended, followed by cleavage with Xba I. A 1.6 kb fragment containing bases #1699-#2917 of the bIRS gene and 701 bps of unknown sequence 3' to the stop codon of the bIRS gene was isolated. pKR4 was initially cut by Afl III, blunted ended and then cut at the Xba I site to give a 6.3 kb fragment containing the entire regulatory region of pKR4, the 5' consensus sequence and bases #1-#1715 of the yIRS gene. The 6.3 kb and 1.6 kb fragments were ligated to generate pKR7. The chimeric gene was designated Afl III/Sac II and is expressed under the control of a *tac* promoter.

2.4.1.7 pKR8 (*E. coli*/yeast IRS Chimera Expression Vector)

pKR8 is the expression plasmid containing a portion of the 5' coding region of the bIRS gene and a portion of the 3' coding region of the yIRS gene. This plasmid was constructed by Racher (60) as follows: pMT521 was cleaved at the Bgl II site, blunt ended and later cleaved at the Bam HI site. A 500 bp fragment of the bIRS gene was excised and the 8.4 kb vector fragment was isolated. pKR4 was cut at the Pvu II site, blunt ended, then cut at the Bam HI site. A 2.6 kb fragment from this double digest, containing bases #610-#3216 of the yIRS gene, was subcloned into the 8.4 kb vector fragment to create pKR8. The chimeric gene was designated Bgl II/Pvu II and is expression under the same promoter as the bIRS gene in pMT521.

2.4.1.8 pKR9 (*E. coli*/yeast IRS Chimera Expression Vector)

Like pKR8, the expression plasmid pKR9 contains a chimeric gene of isoleucyl-tRNA synthetase comprised of the bIRS gene from base #1-#2381 and the yIRS gene from base #2493-#3216. pKR9 was constructed by Racher (60) as follows: pMT521 was cut with Stu I, blunt ended and then cut at the Xba I site to excise a 1 kb fragment of the bIRS gene (including the 3' 701 bps of unknown sequence) from the plasmid. An 8 kb fragment of the plasmid pMT521, containing the promoter of the bIRS gene and the bIRS gene from base #1-#2381, was isolated. pKR4 was cleaved at the Cla I site, blunt ended and then cleaved at the Xba I site. A 0.6 kb fragment of the yIRS gene from base #2493-#3216 was isolated and subcloned into the 8 kb vector fragment of pMT521. This created the plasmid pKR9 with the chimeric gene designated Stu I/Cla I.

2.4.1.9 pEND (Cloning Vector for C-terminal Deletion Genes of yIRS)

The plasmid pEND is a derivative of the plasmid pUC19 (39) with an inserted termination sequence engineered into pUC19's poly-linker region. This termination sequence contains stop codons in all three reading frames. For complete cloning procedure, see Appendix B.

2.4.1.10 pLmp10 (Expression Vector)

The expression vector pLmp10 (62) is driven by a promoter from bacteriophage λ. This expression plasmid was used to express the yIRS C-terminal deletion genes (see section 2.4.1.12).

2.4.1.11 pEND-ITS (Cloning Vector)

The plasmid, pEND-ITS, was used as an intermediate for the construction of four of the five 3' deletion mutants of the yIRS gene and was derived from the plasmids pEND and pKR3. Construction of pEND-ITS is shown in Appendix B.

2.4.1.12 pLcII-Hinc II, pLcII-Cla I, pLcII-Eco RI, pLcII-Kpn I and pLcII-Sca I (yIRS C-terminal Deletion Protein Expression Vectors)

The five yIRS C-terminal deletion constructs were constructed by S. Sidhu (76). Five 3' deletions of various lengths of the yIRS gene were originally engineered into the pEND vector (see section 2.4.1.11) and subsequently subcloned as a "cassette" into pLmp10. For complete cloning procedures of the yIRS C-terminal deletion constructs, see Appendix B.

2.4.1.13 pKR15Q (Expression Vector)

The plasmid pKR15Q was constructed by Racher (60) to constitutively over express the lac I gene. pKR15Q was derived from plasmids pKK223-3 and pMal c (47) and was engineered as follows:

A pair of primers were designed to PCR amplify the lac I gene and the *ptac* promoter from the plasmid pMal c. The 3' primer was designed to introduce a Stu I restriction site at the 5' end of the of lac I gene and a single base pair from of C to T at base #4 of the lac I promoter. The single base pair substitution causes the lac I gene to be transcribed at approximately 10 times the level as the normal lac I promoter. This mutated lac I gene was henceforth called lac Iq.

pMal c native sequence 5'~ ^{10}TCGAATGGCGCAAAACCT^{27}~3'
3' primer sequence 3'~TCCGGAGCTTACCACGTTTTGGA
Stu I

The PCR product, a 1.5 kb fragment, was then cloned into pUC18 (70) to create the intermediate plasmid placI^{q}.

The plasmid pKK223-3 was digested with Pvu II and Eco RI and the desired 2.6 kb fragment containing the ori, amp^{r}, rrn B and 5S genes was isolated. placI^{q} was cleaved at the Bam HI and Eco RI sites and the lacI^{q} gene was isolated. The lacI^{q} fragment was then ligated to the 2.6 kb fragment from pKK223-3 to create the expression plasmid pKR15Q.

2.4.1.14 pKR16Q (yIRS Expression Vector)

The expression plasmid pKR16Q contains the entire coding region of the yIRS gene as well as the altered 5' non-coding consensus ribosome-binding sequence (see section 2.4.1.2). pKR16Q was constructed by Racher (60) in the following manner: pKR15Q was digested with Hind III and blunted ended (pKR15Q/Hind III/blunt). pKR3 was cleaved at the Hind III and Sac I sites and blunted ended. The 3.2 kb yIRS gene was isolated and subcloned into pKR15Q/Hind III/blunt to create pKR16Q.

2.4.1.15 pKR18Q (yIRS Expression Vector)

The plasmid pKR18Q (60) is virtually identical to plasmid pKR16Q except for an alteration of the restriction sites in the poly-linker region 3' to the yIRS gene. In pKR18Q, the Bam HI, Sma I and Kpn I sites were removed and replaced with a Bgl II site and a Sac I site. The replacement of the restriction sites was achieved as follows: The primers, Kpn I/for and ARF were used to PCR amplify a 1.5 kb fragment of the C-terminal end of the yIRS gene in pKR4. The primer ARF removed the Bam HI, Sma I and Kpn I sites and replaced them with Bgl II and Sac I sites. The 1.5 kb PCR fragment was ligated to pKR15Q/Hind III/blunt (see section 2.4.1.18) and the construct in the correct orientation was dubbed "X5". "X5" was subsequently cleaved at the Nde I and Kpn I sites and a 3.85 kb fragment was recovered. pKR16Q was digested with Nde I and Kpn I and a 3.5 kb fragment containing the lacI^{q}, ptac

and the yIRS gene up to the Kpn I site was isolated. The yIRS gene was re-constructed by ligating the 3.85 kb fragment with 3.5 kb fragment to create pKR18Q.

2.4.2 Small Scale Plasmid Preparation

Plasmids were prepared from 2 mL 2X TY/Amp cultures which were inoculated with a single bacterial colony and were grown at the appropriate temperature (30°C or 37°C) for 8-18 hours. Each culture was poured into an eppendorf tube and centrifuged at 13,000 r.p.m. for 1-2 minutes in a Baxter Biofuge 15 mircocentrifuge. The pellet was then resuspended in 100 µL of Solution I supplemented with lysozyme (to final concentration of 4 mg/mL) and incubated at room temperature for 10 minutes. A 200 µL aliquot of Solution II was added and then mixed by inverting the eppendorf tubes. The mixture was incubated a further 5 minutes at room temperature until the solution was transparent. 150 µL of Solution III was then added and mixed again by inverting. After 5 minutes of incubation at room temperature, the mixture was centrifuged for 5 minutes and the supernatant was transferred to a fresh eppendorf tube. To the supernatant, 200 µL of phenol and 100 µL of chloroform was added and finger vortexed. The mixture was centrifuged for 2 minutes and the top layer (supernatant) was transferred to another fresh eppendorf tube. The eppendorf tube was then filled with 95% ethanol, inverted to mix and incubated for 10 minutes at room temperature. After incubation, the ethanol solution was centrifuged for 10 minutes to precipitate the DNA/RNA. The supernatant was removed using a drawn out pipette and the DNA/RNA pellet was allowed to air dry or dry using a Speed-Vac.

The DNA/RNA pellet was resuspended in 50 µL of TE 8.0 supplemented with DNase-free pancreatic RNase A and RNase T_1 (1 µL of each in 50 µL of TE 8.0; RNase A = 10 mg/mL stock; RNase T_1 from Boehringer Mannheim at 100 units/µL). The RNA in the DNA/RNA mixture was allowed to digest for 1 hour at 37°C. The DNA obtained was suitable for restriction enzyme digests and transformations.

2.5 CLONING PROCEDURES

2.5.1 Size Estimation of DNA by Agarose Gel Electrophoresis

Linearized DNA fragments were separated by size on 0.8% (w/v) or 1.0% (w/v) agarose gels by electrophoresis. The agarose gels were supplemented with 0.5 mg/mL ethidium bromide (EtBr). Agarose gels with greater than 1% w/v agarose were used to separate small DNA fragments. The agarose gels were prepared using 1X TAE buffer and electrophoresis was carried out in a horizontal chamber using 1X TAE buffer as the running buffer. Large gels (80 mL) were maintained at 110 volts constant voltage and mini-gels (30 mL) were ran at 70 volts constant voltage. DNA fragment sizes were estimated by comparison against a standard 1 kb ladder (BRL) or by comparison with Cla I digests of bacteriophage λ DNA. DNA fragments were visualized by fluorescence of the DNA-EtBr complex under ultraviolet (UV) light.

2.5.2 DNA Modifications

2.5.2.1 Restriction Digests

All restriction digests were performed as recommended by the manufacturers. In the instances where double digests with two different restriction enzymes in two different reaction buffers were required, the plasmid DNA was digested according to one of the two following methods: 1) Plasmid DNA was digested with both restriction enzymes at the same time in one of the two recommended reaction buffers (Note: this method was used when the reaction buffers were similar in composition and the activities of both enzymes in the selected reaction buffer were tested beforehand on a control). 2) The plasmid DNA was digested one enzyme at a time. The DNA digested by the first enzyme was purified by a combination of phenol/chloroform extractions (3:1), followed by a chloroform only extraction and DNA precipitation with ethanol and 3 M sodium acetate , pH 6.0 (25:1). The DNA pellet was resuspended in the 1X reaction buffer of the subsequent restriction enzyme digest.

2.5.2.2 Blunt-Ending by T4 DNA Polymerase

The adhesive ends of DNA fragments were made blunt by the enzyme T4 polymerase by filling in the adhesive ends using the 5' to 3' polymerase activity of T4 polymerase.

Ethanol precipitated DNA fragments, which were previously cleaved with restriction enzymes and purified, were solublized in 43.5 µL of ddH_2O. The reaction mixture was made up to 50 µL with 5 µL of 10X T4 polymerase reaction buffer, 1 µL of 25 mM dNTP's and 0.5 µL of T4 polymerase. The reaction solution was incubated at 37°C for 10 minutes and the DNA fragments purified by phenol/chloroform extraction and ethanol/NaOAc precipitation.

2.5.2.3 Dephosphorylation of Plasmid

The 5' phosphate of linearized plasmid DNA was removed by calf intestinal alkaline phosphatase (CIP) to prevent the re-circularisation of the plasmid DNA. After digestion of the plasmid DNA with the appropriate restriction enzymes, the total volume of the reaction solution was doubled with 2X CIP reaction buffer (to a final concentration of 1X CIP reaction buffer) and 2 µL of CIP was added (from a stock concentration of 1 unit/µL). The mixture was incubated at 50°C for 30-60 minutes. The reaction was stopped by either purification of the linearized plasmid DNA (by phenol/chloroform extraction and precipitation with ethanol and sodium acetate as described in section 2.5.2.1) or by freezing the mixture overnight at -20°C before purification of the digested plasmid. The purified, linearized plasmid DNA was subsequently separated by electrophoresis and gel extracted (see section 2.5.3).

2.5.2.4 Ligation of DNA Fragments

Linearized and dephosphorylated plasmid DNA were ligated by the enzyme T_4 DNA ligase. 1 µL samples of the linearized insert DNA and dephosphorylated plasmid DNA were first separated on an 0.8 % (w/v) thin agarose mini-gel (15 mL) and visualized under UV light to estimate the relative ratio between the vector and insert. For most cases, the vector to insert ratio used in a ligation was 1:4. The ligation mixture was set up to a total volume of 5 µL with the addition of the vector and insert (and ddH_2O if required to make up to volume). The ligation mixture was incubated overnight at 16°C. After incubation, the volume of the ligation mixture was increased to 25 µL with ddH_2O. 12.5 µL of this diluted ligation mixture was added to 120 µL of thawed competent DH5α cells for transformation as described in section 2.3.4.

2.5.3 Purification of DNA Fragments by Agarose Gel Extraction

Isolation of DNA fragments from restriction digests for ligation was achieved by extracting the desired DNA fragment from agarose gels. Plasmid DNA cleaved with the appropriate restriction enzymes was purified and precipitated as described in section 2.5.2.1. The precipitated DNA was resuspended in 15 µL of TE8.0 and an 0.8% (w/v) or 1% (w/v) agarose gel supplemented with EtBR was prepared. To the resuspended DNA, 1.5 µL of agarose gel loading dye was added. A 3 µL aliquot of the DNA sample was loaded into a well as the "sacrifice lane" which was used to visualize the position of the DNA fragments over UV light. This was used to prevent irradiation of the DNA to be used in the cloning step. The rest of the sample was loaded in an adjacent well. The agarose gel was electrophoresed at 70 volts constant voltage to separate the DNA fragments. After electrophoresis, the 1 kb standard DNA ladder lane and the "sacrifice lane" were cut from the gel and visualized under UV light to determine the location of the desired fragment(s) in the "sacrifice lane". The location of the desire fragment(s) was marked and the "sacrifice lane" was removed from the UV light. The "sacrifice" lane was then lined up against the sample lane and the desired fragment(s) was cut from the gel.

The DNA fragment was recovered from the agarose slab by centrifugation through a "home-made" spin column. The "home-made" spin column consisted of a small 0.5 mL eppendorf tube with a small hole punched at the bottom of the tube. This small eppendorf tube was then fitted within a 1.5 mL Eppendorf which would collect the liquid containing the DNA. A P1000 (blue) pipette tip was cut in half and the lower portion of the pipette tip was stuffed with sterilized glass wool. This pipette tip was then placed within the small eppendorf tube mentioned above. The agarose slab containing the DNA fragment was placed in the stuffed P1000 pipette tip and the entire apparatus was centrifuged at 6,000 r.p.m. for 10 minutes in a Baxter Biofuge 15 mircocentrifuge. The recovered DNA solution was purified by phenol/chloroform extraction, followed by ethanol precipitation. The DNA pellet was resuspended in 15 µL of TE8.0 for ligation (see section 2.5.2.4).

2.5.4 Assembly of Expression Vector Constructs

Although the yIRS 3' deletion mutant genes in the pLcII vector constructs expressed and produced mutant protein, the level of protein expression was deemed insufficient for subsequent purification and analysis. In order to achieve higher expression of the mutant proteins, the yIRS 3' deletion mutant genes were cloned into either pKR16Q or pKR18Q. Likewise for the expression plasmid pKR7. While the level of protein expression was adequate for Western Immunoblots of crude protein preparations, it was not sufficient for large-scale purification and kinetic analysis. Thus, it was hoped that by cloning the Afl III/Sca II chimeric gene into pKR16Q, a higher level of protein expression could be achieved.

2.5.4.1 Assembly of the Plasmids pGC3Q, pGC4Q and pGC5Q

The Eco RI, Kpn I and Sca I 3' deletion mutant genes in plasmids pLcII-Eco RI, pLcII-Kpn I and pLcII-Sca I, respectively, were cloned into the plasmid pKR16Q in the following manner:

The plasmids pLcII-Eco RI, pLcII-Kpn I, pLcII-Sca I and pKR16Q were initially cleaved with the restriction enzyme Pml I. The linearized plasmids were purified by phenol/chloroform extraction and ethanol precipitation as described in section 2.5.2.1 and cut again with Xba I. The pKR16Q fragments were then subjected to dephosphorylation by CIP (see section 2.8.2.3) and a 4.4 kb fragment from pKR16Q (vector fragment) was subsequently isolated. From the Pml I/Xba I double digests of pLcII-Eco RI, pLcII-Kpn I and pLcII-Sca I, a 2.0 kb fragment from pLcII-Eco RI (Eco RI fragment), a 1.5 kb fragment from pLcII-Kpn I (Kpn I fragment), a 0.9 kb fragment from pLcII-Sca I (Sca I fragment) were recovered by gel extraction (see section 2.5.3). The Eco RI, Kpn I and Sca I fragments were then cloned separately into the vector fragment, thereby creating the pGC3Q, pGC4 and pGC5, respectively. The new constructs were tested by restriction enzyme digests to confirm that the new constructs contained the desired inserts.

2.5.4.2 Assembly of pGC1Q and pGC2Q

The Hinc II and Cla I 3' deletion mutant genes could not be cloned into pKR16Q in the same manner as the other three 3' deletion mutant genes because of a Xba I site in the yIRS gene at a position 5' to the Cla I site. Thus the Hinc II and Cla I 3' deletion mutant genes were cloned into the pKR18Q expression vector which has the altered poly-linker restriction sites.

The plasmids pLcII-Hinc II, pLcII-Cla I and pKR18Q were cleaved initially with Pml I. Then, the linearized pLcII-Hinc II and pLcII-Cla I plasmids were digested with Bam HI and Bgl I. The latter digest with Bgl I was used to cleave the vector fragment so that the gene fragment and the vector fragment can be distinguished by size on an agarose gel. A digest of pLcII-Hinc

II with Pml I and Bam HI alone would have resulted in a gene fragment and vector fragment of roughly equal size (2745 bp and 2716 bp, respectively) that could not be resolved on an agarose gel. From these triple enzyme digests, a 2.7 kb Hinc II gene fragment and a 2.2 kb Cla I gene fragment were isolated.

The linearized pKR18Q plasmid was subjected to a second digest with Bgl I. This resulted in a 4.4 kb vector fragment which was subsequently isolated. The Hinc II and Cla I fragments were then individually cloned into the pKR18Q vector fragment to generate pGC1Q and pGC2Q, respectively. The new constructs were tested by restriction enzyme digests to confirm the presence of the desired inserts.

2.5.4.3 Assembly of pGC7Q

The plasmids pKR7 and pKR16Q were digested with Afl II and Xba I. A 2.3 kb fragment of the Afl III/Sac II chimeric gene from pKR7 and a 4.7 kb vector fragment of pKR16Q were isolated by gel extraction. The 2.3 kb fragment was ligated with the 4.7 kb vector fragment to create pGC7Q.

2.6 DESIGNING A CHIMERIC ENZYME

Previously, the chimeric isoleucyl-tRNA synthetase enzymes designed by Racher (60) were constructed based on convenient restriction sites on the yIRS and bIRS genes. These sites were used to join the yIRS and bIRS gene fragments to form the chimeric gene, hence the names given to the chimeric genes were based on the restriction sites at which the yIRS and bIRS fragments were joined. All of these bIRS/yIRS chimeric enzymes, as it turned out, are ***intra***-domain chimeras where the junction between the yIRS and bIRS gene fragments are within either the N-terminal or the C-terminal domain.

An ***inter***-domain chimeric isoleucyl-tRNA synthetase composed of the N-terminal domain of yIRS and the C-terminal domain of bIRS was proposed. This chimera was designed and constructed as follows:

2.6.1 Designing Oligonucleotide Primers

A unique Nsi I site, located at base #1872 of the yIRS gene, was chosen as the point of junction between the yIRS and bIRS gene fragments because a Nsi I restriction site can be easily engineered into a corresponding region of the bIRS gene. The introduction of an Nsi I site into the bIRS gene would not disrupt the amino acid sequence by either introducing a stop signal and/or changing the amino acid sequence to a great extent. The following oligonucleotide primers were designed to introduce the Nsi I restriction site into the 5' end of the bIRS fragment to be used for create the inter-domain chimera. A Sac I site was also introduced to after the stop codon of the bIRS fragment. This was to allow the easy cloning of the bIRS fragment into expression vectors.

bIRS Primers:

5' primer

gly ala asp **ala trp** arg leu
21 mer 5'-GGCGCGGATGCATTGCGTCTG-3'
Nsi I

3' primer

24 mer 5'-GAGCTCTCAGGCAAACTTACGTTT-3'
Sac I

The oligonucleotides were synthesized on an Applied Biosystems 329 DNA/RNA Synthesizer at the Institute of Molecular Biology and Biochemistry (S.F.U.).

2.6.2 Amplification of DNA Fragments by the Polymerase Chain Reaction

The Polymerase Chain Reaction (PCR) was used to generate the bIRS DNA fragment that would code for the C-terminal domain of the inter-domain chimera. This bIRS DNA fragment would also contain the new restriction sites as dictated by the oligonucleotide primers (see section 2.6.1). The plasmid pMT521 was used as the template to generate the bIRS DNA fragment.

After PCR, 5 μL of each PCR mixture was loaded onto a 1% (w/v) agarose gel, supplemented with EtBr, and the PCR fragments were separated by electrophoresis. The gel was visualized under UV light to confirm the presence of a fragment corresponding to the desired PCR fragment.

2.6.3 Purification of PCR fragments, Subcloning and Screening

The PCR mixture was loaded onto a 1% (w/v) agarose gel for gel purification. The desired 1 kb bIRS fragment gel extracted, purified and ethanol precipitated (see section 2.5.2.1). The DNA pellet was then resuspended in 15 μL of TE8.0.

The 1 kb bIRS fragment was inserted into the Sma I site of pUC18. The pUC18ΔbIRS construct was then transformed into competent *E. coli* strain DH5α cells and spread in 10 μL, 100 μL, 250 μL and 850 μL aliquots onto 2X TY plates that were previously prepared with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) and Isopropyl β-D-thiogalacto-pyranoside (IPTG). Bacterial colonies were screened by α-complementation (71). Colonies with the insert into the Sma I set lack the ability to cleave X-gal and thus remains white. Colonies without the insert in the Sma I site of pUC18 retains the ability to cleave X-gal and would be blue in colour. Plasmid was prepared from some of the white colonies and with the aid of restriction digest tests, the orientations of the insert in pUC18 was determined. The construct in the desired orientation was designated pUC18ΔbIRSA.

2.6.4 Assembly of pGC6Q

The plasmid pKR18Q was cleaved at the Nsi I site and the Sst I site. A 1.3 kb fragment of the yIRS gene from base #1873-#3216, plus the Xba I site in the poly-linker was excised, and the 6 kb vector fragment, which includes the yIRS fragment of the inter-domain chimera, was isolated. pUC18ΔbIRSA was digested with Nsi I and Sst I to obtain the sticky ends, the 1 kb fragment of the bIRS gene was ligated to the 6 kb vector fragment to create pGC6Q. The inter-domain chimeric gene was designated the Nsi I Chimera.

2.7 PROTEIN ANALYSIS

2.7.1 Separation of Proteins by Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Proteins were separated by electrophoresis on either 9% (w/v), 12% (w/v) or 15% (w/v) Mini-PROTEAN II (Bio-Rad) polyacrylamide gels. The lower percentage gels were used to separate proteins of high molecular weight. Protein samples were first denatured in 1X SDS-Loading Dye (final concentration) for 5 minutes at 98°C, followed by centrifugation at 13,000 r.p.m. for 30 seconds in a Baxter Biofuge 15 mircocentrifuge. The denatured protein samples were then loaded into the wells of the polyacrylamide gel and electrophoresed at 200 volts constant voltage for 45-60 minutes, using the SDS-buffer system developed by Laemmeli (42).

After electrophoresis, the gels were stained in Comassie Blue stain for 20 minutes with gentle shaking. This was followed by 2X 15 minutes destaining in Fast Destaining Solution and subsequently, 2X 10 minutes destaining in Slow Destaining Solution until background colour was removed.

The resulting gels were then scanned using an Apple OneScanner desktop scanner and the program OFOTO (Light Source Inc.).

2.7.2 Determination of Protein Concentration

2.7.2.1 The Bradford Assay

Protein samples were prepared in aliquots of 10 µL, 25 µL, 50 µL and 75 µL and diluted to a final volume of 100 µL with ddH_2O. A 1 mL aliquot of the Bradford Dye Solution (9) was added to each sample and the mixture was incubated in a water bath at 37°C for 20 minutes. After incubation, the absorbance of each sample was measured at the wavelength of 595 nm using a spectrophotometer. A known concentration of bovine serum albumin (range of 0-100 µg) was used to generate the standard curve.

2.7.2.2 Lowry Microtitre-Plate Assay

The protein concentration of small protein samples were determined using a reduced volume version of the Lowry Assay (45). The Lowry Microtitre-Plate Assay was developed by H. Sweet (84) and utilizes microtitre plate. A bovine serum albumin solution of known concentration was used to plot the standard curve (range 0-45 µg). Known aliquots of the protein samples were diluted to a total volume of 60 µL in the microtitre plate wells with ddH_2O. A 60 µL aliquot of "Working" solution (see section 2.2 under Protein Assay Solutions) was added to each protein sample and mixed with a toothpick. After a 10 minute incubation at room temperature, 30 µL of 20% (v/v) Folin reagent was added, mixed and incubated for another 30 minutes at room temperature for colour development. The absorbance of each well was measured in an ELISA plate reader (Bio-Tek Instruments model Ceres 900 HDSi) which was set at the wavelength of 490 nm.

2.8 PROTEIN EXPRESSION

2.8.1 Protein Expression of pKR16Q and pKR18Q Derived Constructs

Small scale test expressions were performed on the yIRS 3' deletion genes, the Nsi I chimeric gene and the Afl III/Sac II chimeric gene in the constructs pGC1Q, pGC2Q, pGC3Q, pGC4Q, pGC5Q, pGC6Q and pGC7Q, respectively. Test expressions were carried out in 2 mL 2X TY/Amp liquid cultures inoculated with a freshly grown *E. coli* DH5α strain colony which were transformed with one of the aforementioned constructs. After inoculation, the 2 mL 2X TY/Amp culture was grown at 37°C on a rotary shaker for 5 hours or until the OD_{600}=0.8. The cultures were then induced for protein expression by the addition of IPTG to a final concentration of 0.4 mM and grown for another 3 hours at 37°C.

The cells were harvested by centrifugation (13,000 r.p.m. for 2 minutes in a Baxter Biofuge 15 mircocentrifuge) and the pellet was lysed in 100 μL of TE8.0 supplemented with lysozyme (final concentration of lysozyme was 2 mg/mL). The cells were incubated on ice for 30 minutes and subsequently sonicated (Fisher Sonic-Dismembranator model 300) with a small probe for 20 seconds. The lysed cells were centrifuged again at 13,000 r.p.m. at 4°C for 15 minutes to pellet the cellular debris. The supernatant, containing the solublized protein, was removed to a fresh tube and stored on ice.

2.8.2 Test Expression of pMT521 Derived Constructs

Small scale test protein expressions were also performed on the pKR8 and pKR9 expression vectors which contain the Bgl II/Pvu II and Stu I/Cla I chimeric genes, respectively. The protocol for test expression is the same as the protocol outlined in section 2.8.1.

2.8.3 Test Expression of pKK223-3 Derived Constructs.

For the pKK223-3 derived expression vectors, pKR5 and pKR7, the protocol for small scale test expression is slightly different than the protocol outlined in section 2.8.1. The constructs pKR5 and pKR7 were separately transformed into *E. coli* strain TG2 (see section 2.3.4.2) and grown on 1X MM/Amp plates. A freshly grown colony was used to inoculate a 2 mL 1X MM/Amp liquid broth and the culture was grown at 37°C on a rotary shaker for 8-10 hours or until the OD_{600}=0.8. The cultures were then induced with IPTG (final concentration 0.4 mM) and grown for another 3 hours at 37°C.

The isolation of protein from the cells is the same as the procedure outlined in section 2.8.1.

2.8.4 Large Scale Expression of pGC6Q and pGC7Q

Large scale protein expression of constructs pGC6Q and pGC7Q were also performed using the fermentation apparatus. The procedure for the large scale expression of pGC6Q and PGC7Q is the same as the procedure outlined in section 2.8.4.1 except for the following differences: pGC6Q and pGC7Q were transformed into *E. coli* strain DH5α and grown on 2X TY/Amp plates. A freshly grown colony was used to inoculate a 2 mL culture tube of 2X TY/Amp broth and the culture was grown for 6-8 hours at 37°C on a rotary shaker. A 0.5 mL aliquot of the 2 mL culture was used to inoculate 200 mL of (pre-warmed to 37°C) 2X TY/Amp broth in a 500 mL culture flask and grown overnight at 37°C.

The fermentation chamber was filled with 15 L of 2X TY and autoclaved at 121°C for 20 minutes. After the media is cooled to 37°C, 7.5 mL of filter sterilized ampicillin (200 mg/mL) was added aseptically to the 2X TY media. The 15 L of 2X TY/Amp was inoculated with the 200 mL culture and grown at 37°C until OD_{600}=0.8. The culture was then induced for

another 3 hours with the addition of 7.5 mL of 0.8 M IPTG. The cells were harvested in the same manner as described in section 2.8.4.1.

2.9 PSEUDOMONIC ACID A INHIBITION OF CELL GROWTH

2.9.1 Transformation into TG2

Plasmids pGC1Q, pGC2Q, pGC3Q, pGC4Q, pGC5Q, pGC6Q, pKR5, pKR7, pKR8, pKR9 and control plasmids pKK223-3, pKR4, pMT521, pKR15Q, pKR16Q and pKR18Q were transformed in the *E. coli* strain TG2 for *in vivo* inhibition studies using the competitive inhibitor, pseudomonic acid A (61) (see section 2.3.4.2 for transformation procedure).

2.9.2 *In Vivo* Protein Expression

Small scale *in vivo* protein expression were performed for each of the plasmids listed in section 2.9.1. *In vivo* protein expression was carried out as described in section 2.8.3.

2.9.3 Preparation of Media Plates Supplemented with Pseudomonic Acid A

Pseudomonic Acid A (Smith-Kline Beecham Pharmaceuticals) was freshly prepared in a 50% methanol stock solution at a concentration of 25 mg/mL and kept at 4°C.

Minimal Media (1X MM) plates were prepared as described in section 2.3.1 and were supplemented with ampicillin (to a final concentration of 200 μg/mL) and varying concentrations of pseudomonic acid A (from stock solution) prior to pouring the plates. The 1X MM/Amp-PA plates were allowed to solidify and dry in a laminar flow hood for 45-60 minutes

2.9.4 Plating of Cells Harbouring the IRS Mutant Constructs

An hour after induction with IPTG, the cell density of the 1X MM/Amp cultures was determined by spectrophotometry (at the wavelength of 600 nm). Each culture was serially diluted to $5x10^3$ colony forming units per milliliter (cfu/mL). Aliquots of 100 µL of the diluted culture were spread-plated on 1X MM/Amp-PA plates in duplicates. The plates were incubated at 37°C for 2-4 days. Colony growth and the size of the colonies were recorded on day 2, day 3 and day 4.

2.10 PREPARATION OF ANTIBODIES AND IMMUNOCHEMICAL TECHNIQUES

Mouse hybridomas producing monoclonal antibodies against yIRS and bIRS were created by Kalmar (39) as described in Chapter 6 of Antibodies- A Laboratory Manual (33). The hybridomas were frozen for long term storage with liquid nitrogen at -185°C.

2.10.1 Production of Monoclonal Antibodies From Hybridoma Cells

2.10.1.1 Growing Hybridoma Cells by Tissue Culture

Hybridoma cells, which had been frozen in liquid nitrogen, were thawed and diluted ten-fold in Dulbecco's Modified Eagle (DME) media. The diluted hybridoma cell suspension was centrifuged at 10,000g for 5 minutes to pellet the cells. The supernatant was carefully removed and the cell pellet was subsequently resuspended in 1 mL DME media (The procedure for culturing hybridomas is described in detail in Chapter 7 of Antibodies- A Laboratory Manual (33)).

Tissue culture plates were prepared with 4 mL DME media and 1 mL 20% (v/v) Fetal Calf Serum (FCS). The resuspended hybridoma cells were added to the tissue culture plate and incubated at 37°C in a 5% CO_2 incubator. The cells were periodically checked and split every 2-3 days into fresh media supplemented with FCS (to a final concentration of 4% FCS).

Antibody titre was checked by enzyme-linked immunoabsorbent assays (ELISA) (see section 2.10.2).

2.10.1.2 Production of Ascites Fluid in Mice

Six to eight week old mice were injected with 0.5 mL pristane (2,6,10,14-tetramethylpentadecane) one to two weeks prior to injection with hybridoma cells. Tissue culture prepared hybridoma cells were centrifuged at 10,000g for 5 minutes to pellet the cells and the cells were resuspended in 2 mL DME media. Each mouse was injected subcutaneously with roughly 10^6 cells in a volume of 0.5 mL. Tumor growth was monitored over a period of 3 weeks.

2.10.1.3 Harvesting Ascites Fluid

Ascites fluid was collected over a period of two days and the mice were sacrificed on the third day to recover as much fluid as possible. The recovered fluid was centrifuged at 200g for 10 minutes to pellet the cells. The supernatant was transferred to a fresh tube and centrifuged at 10,000g for 15 minutes, producing two layers, an upper fatty layer and a lower layer containing the ascites fluid. The clear supernatant was recovered by piercing the fatty layer with a Pasteur pipette and drawing off the clear supernatant. The clear ascites fluid was stored in 50% (v/v) ultra-pure glycerol at -20°C. (Chapter 7, page 274 of Antibodies- A Laboratory Manual (33)).

2.10.2 ENZYME-LINKED IMMUNOABSORBENT ASSAY (ELISA)

ELISAs were used to test for antigen-binding activity in the antibody samples and to monitor the purification of the antibody samples by Fast Protein Liquid Chromatography (FPLC) (see section 2.10.4). Each well of the ELISA microtitre plate was coated with approximately 1 µg of purified yIRS (from a ED Buffer (see section 2.2 under ELISA solutions)-diluted stock solution) in a 100 µL volume and incubated either at 37°C for 2 hours or overnight at 4°C. The plates were then washed exhaustively with distilled water and

subsequently, each well was blocked with 200 µL of EB Buffer. The plates were incubated at 37°C for 2 hours, followed by washing the plates with distilled water. Then, 100 µL of diluted primary antibody (stock ascites fluid diluted 1000X in ED Buffer) was added to each well and the plates were allowed to incubated at 37°C for another 2 hours. After washing out the plates again to get rid of any unbound antibody, 100 µL of diluted goat-antimouse peroxidase antibody (source) (20 µL of stock diluted to 20 mL with ED Buffer) was added to each well and incubated for 2 hours at 37°C. After incubation and washing of the plates, 100 µL of ELISA substrate was added to each well and the plates were again incubated at 37°C until colour developed. The reaction was stopped with the addition of 50 µL of 1 M H_2SO_4 to each well and the plate was read on an ELISA plate reader set at 590 nm.

2.10.3 WESTERN IMMUNOBLOTTING OF PROTEINS

Two methods for the Western Immunoblotting of proteins were employed, each using a different method of detection of the antibody-bound protein. One method employs the use of 3,3-diaminobenzidine and nickel chloride as substrates and the other method uses a chemi-luminescent substrate by Amersham (ECL Method).

Proteins samples for both methods of detection were prepared in the same manner. The protein samples were first electrophoresed on either 9% or 12% SDS polyacrylamide gels (see section 2.7.1). The separated proteins on the resulting gel were blotted onto a Immobilon-P membrane (6.5 cm x 9.5 cm) which was previously soaked in methanol. The polyacrylamide gel and the membrane were sandwiched between the cathode and the anode plates of a LKB multipore transfer apparatus or a Pharmacia Nova Blot apparatus in the following order going from the cathode to anode: 2 pieces of 3MM Whatmann paper (6.5 cm x 9.5 cm and previously soaked in Transfer Buffer), immobilon P membrane, polyacrylamide gel, 2 pieces of 3 MM Whatmann paper (6.5 cm x 9.5 cm and previously soaked in Transfer Buffer) and the anode

plate. Electroelution was performed at 400 mA constant current for 2 hours (Note: for the ECL method, proteins were transferred at 250 mA constant current for 2 hours).

2.10.3.1 Using 3,3-Diaminobenzidine and Nickel Chloride as Substrate

Protein blotted membranes were blocked in Western Blot Blocking Buffer (Blotto) for 2 hours at room temperature or overnight at 4°C on a rotary shaker. Afterwards, the Blotto was drained and diluted 1° antibody was added (1° antibody from mouse ascites fluid was diluted 1000-fold in Blotto) to the membrane. The membrane was incubated with the 1° antibody for 2 hours at room temperature on a rotary shaker. Any unbound antibody was washed from the membrane with Blotto in three washes for 10 minutes each. The washed membrane was then incubated with 1000-fold diluted goat-antimouse peroxidase (diluted in Blotto) for another 2 hours at room temperature. After 2° antibody incubation, the following series of washes were performed: two washes at 5 minutes each with Blotto, two washes at 5 minutes each with 1X TBS-Tween and lastly two washes at 10 minutes each with 1X TBS. The washed membrane was then soaked in Developing solution (see section 2.2 under Western under Western Blot Solutions) until protein bands colourize. The reaction was stopped by washing the membrane in ddH_2O.

2.10.3.2 Using the Amersham ECL Method

The blotted immobilon P membranes were blocked in ECL Blotto for 2 hours at room temperature or overnight at 4°C on a rotary shaker. After draining the ECL Blotto from the membranes, 1° antibody, diluted 5000-fold in ECL Blotto, was added to the membrane and was allowed to incubate at room temperature for 1 hour with gentle shaking. The membranes were then washed in the following sequence: two times at 5 minutes each with 1X TBS-Tween Buffer and once for 15 minutes with 1X TBS-Tween Buffer. Secondary antibody (goat-antimouse peroxidase) supplied by Amersham (catalog number here) was diluted 5000-fold in 1X TBS-Tween Buffer and the mixture was added to the membrane. After another hour of incubation at

room temperature with the 2° antibody, the following wash sequence was used to remove any unbound antibody: Two washes at 5 minutes each with 1X TBS-Tween, followed by two washes at 10 minutes each with 1X TBS-Tween. The membranes were subsequently drained of buffer and developed using the Amersham detection solution in the following manner: the membrane was soaked for 1 minute in at mixture of 2.5 mL Solution A and 2.5 mL of Solution B. The membrane was then wrapped in Saran Wrap and developed with PDB-1 (Kodak) film for exposures of 1-30 seconds.

2.10.4 PURIFICATION OF MONOCLONAL ANTIBODY

The monoclonal antibodies were purified from ascites fluid by a combination of ammonium sulphate precipitation and anion-exchange chromatography (4). The ascites fluid was first dialyzed, using a Spectropor 2 dialysis membrane with a molecular weight cut-off at 12500-14000, for 24 hours with three changes of Buffer A to remove the glycerol. The dialysed ascites fluid was subjected to 50% (from a 100% saturated stock solution) ammonium sulphate precipitation over a period of 2-4 hours on ice with constant mixing. The precipitated protein was recovered by centrifugation at 12,000 r.p.m. (Sorvall SS34 rotor) for 20 minutes. The protein pellet was washed two times with 50% ammonium sulfate, centrifuging at 12,0000 r.p.m. for 20 minutes after each wash. The pellet was later resuspended in 5 mL of Buffer A and protein solution dialysed, using a Spectropor 2 dialysis membrane, in Buffer A for 24-36 hours with 3-4 changes of Buffer A. The dialysed protein solution was then loaded onto a Pharmacia HR/10 Mono Q column for Fast Protein Liquid Chromatography (FPLC) purification. The proteins were eluted with a linear gradient from 20 mM NaCl to 400 mM NaCl. The main antibody peak was eluted at approximately 15% Buffer B (80 mM NaCl). The antibody peak was subsequently concentrated using a Centricon 10 filter and stored at 4°C until use.

2.10.5 Immuno-Affinity Column Chromatography

2.10.5.1 Construction of Immuno-Affinity Column

An immuno-affinity column was constructed for use as a tertiary step for the purification of mutant IRS proteins. The monoclonal antibody Y12 was purified as described in section 2.10.4 and coupled to a hydrazine-activated agarose matrix (Affi-Gel Hz) purchased from Bio-Rad. The procedure (as described by Bio-Rad) (8) for the coupling of the antibody to the column matrix is described in detail in Appendix C.

2.10.5.2 Test Separation of Wild-Type Yeast and *E. coli* Isoleucyl-tRNA Synthetases

In order to test the specificity of the Y12 immuno-affinity column, a sample of containing an equal molar concentration of purified yeast and *E. coli* IRS was prepared. The protein mixture was applied to the column, drained into the matrix and 1 mL of Buffer C was added to top off the column bed. The proteins were allowed to incubate with the matrix for 30 minutes at room temperature. Unbound protein was washed from the column with 3 column volumes of Buffer C and the elutent was collected in 1 mL fractions and stored on ice. Bound protein was eluted using Buffer D and 1 mL fractions were collected and stored on ice. Lastly, the immuno-affinity column was washed with 1 column volume of glycine-HCl (pH 3.0) and 1 mL fractions were collected.

To determine the protein content of each of the fractions, 0.5 mL of each fraction eluted from the column was used for western immuno-blot analysis and 0.5 mL was used for aminoacylation assays (see section 2.12.1). Because of the low concentration of protein in each fraction, the protein samples used for western immuno-blot analysis were concentrated by precipitating the protein with 10% w/v trichloroacetic acid (final concentration) on ice for 30 minutes. Precipitated protein was centrifuged at 40,000 r.p.m. in an Ultra-centrifuge for 30 minutes at 4°C. The supernatant was removed and the protein pellet was resuspended and washed with acetone. The sample was spun again to pellet the protein at 40,000 r.p.m. for 15

minutes at 4°C. The acetone was removed and the pellet was allowed to dry. The dried protein pellet was later resuspended in 20 µL ddH20 and 20 µL SDS loading dye and separated on a 12% SDS acrylamide gel (see section 2.7.1 for procedure). The separated proteins were transferred onto Immobilon-P membranes and probed with monoclonal antibody Y12 (see section 2.10.3).

2.11 PURIFICATION OF MUTANT ISOLEUCYL-tRNA SYNTHETASE ENZYMES

2.11.1 Lysis of Cell Pellet

Frozen cell pellets from a 7.5 L fermentor load of *E. coli* cells expressing the mutant protein were thawed and resuspended firstly in 150 mL of Buffer E to wash the last traces of growth media from the cells. The cells were pelleted again by centrifugation at 8,000 r.p.m. (Sorvall GS3 rotor) for 15 minutes at 4°C. The cell pellet was then resuspended in 250 mL lysis buffer supplemented with lysozyme (at a final concentration of 2.2 mg/mL) and incubated on ice for 30 minutes. After the incubation, PMSF was added to the lysis mixture to a final concentration of 0.5 mM. The mixture was then sonicated with 3X 20 second bursts at maximum power from a probe sonicator (Fischer Sonic-Dismembranator) fitted with a large sized probe. $MgCl_2$, $MnCl_2$ and Deoxyribonuclease I was subsequently added to the lysis mixture to a final concentration of 2.2 mM, 1.1 mM and 11 µg/mL, respectively and the mixture was incubated on ice for another 30 minutes. Cell debris was removed from the lysate by centrifugation at 15,000 r.p.m. (Sorvall SS34 rotor) for 30 minutes at 4°C. The supernatant was pooled and stored on ice prior to purification.

2.11.2 Primary Purification of Isoleucyl-tRNA Synthetase Mutants by Anion-Exchange Chromatography

All subsequent procedures were performed at 4°C. The purification of mutant protein from *E. coli* strain TG2 or DH5α by anion-exchange chromatography is a modification of the procedure described for the purification of recombinant yeast isoleucyl-tRNA synthetase from *E. coli* strain TG2 (61). An anion-exchange column (Pharmacia LKB Biotechnology Inc., 2.6 x 31 cm) was packed with Q Sepharose and washed with Buffer E. The cell lysate was applied to the column at the flow rate of 3 mL/min. using a peristaltic pump. After the sample was loaded, the column was washed with 500 mL of Buffer E to remove any unbound protein from the column. The mutant protein was eluted in 15 mL fractions using a 2 L linear gradient from 50 mM KCl to 1 M KCl (Buffer F) over a period of 12 hours at a flow rate of 180 mL/hour. The elution profile was monitored by a UV. detector and a chart recorder. The eluted protein was collected in 15 mL fractions and fractions containing mutant protein were identified by SDS-PAGE (as identified by the mutant protein's characteristic mobility) and western-immunoblotting. Typically, the mutant proteins were eluted between (300 mM - 500 mM KCl).

2.11.3 Secondary Purification of Isoleucyl-tRNA Synthetase Mutants by Affinity Chromatography.

Fractions containing mutant protein were pooled and diluted by 2 fold prior to loading onto a hydroxylapatite column (2.5 X 20 cm) (Bio-Rad Macroprep HA) that has been washed with Buffer G. Purification of mutant protein using the hydroxylapatite column was performed at room temperature and the protein fractions were collected at 4°C. After loading the protein sample, the column was washed with 100 mL of Buffer G. The mutant protein was eluted from the column with a 1 L linear gradient from 10 mM potassium phosphate to 400 mM potassium phosphate (Buffer H). Fractions were again screened for mutant protein by SDS-PAGE and

western-immunoblotting. The mutant protein typically eluted between 400 mM potassium phosphate. Fractions containing the mutant protein were pooled and concentrated in an Amicon stirred ultra-filtration cell fitted with an XM50 Diaflo-ultrafiltration membrane. During the concentration process, the buffer was also exchanged with Buffer I. The concentrated protein was adjusted to 50% w/v glycerol (ultra-pure) and stored at -20°C.

2.12 AMINOACYLATION ASSAYS

2.12.1 Standard Assay Procedure Using

2.12.1.1. Using ^{14}C-Labeled Isoleucine

Aminoacylation activities (61) of crude and purified mutant IRS samples were measured in standard reaction Solution A (144 mM Tris-Cl, pH 7.8, 10 mM β-mercaptoethanol, 0.1 mM PMSF and 10 mM $MgCl_2$) with 10 mM ATP·Mg^{2+}, 6.2 μM ^{14}C-labeled isoleucine (Amersham), 16 μM isoleucine (unless otherwise stated), 160 μM yeast tRNA or 160 μM *E. coli* tRNA and 0.002 units/μL inorganic pyrophosphatase.

Reaction volumes were 25 μL and the reaction was initiated with the addition of enzyme. The reaction mixture was incubated at 37°C for 20 minutes (unless otherwise stated). Aliquots of 20 μL were taken from the reaction mixture after incubation and quenched in 3 mL ice-cold 5% w/v trichloroacetic acid (TCA). The quenched sample was filtered through Whatmann GF/C glass fiber filters and the filter was washed with three times with 3 mL 5% TCA, followed by one wash with 1 mL 95% ethanol. The filter was dried under a heat lamp and counted in a Beckmann LS3801 scintillation counter.

2.12.1.2 Using ^{3}H-labeled Isoleucine

The ^{3}H-labeled isoleucine (supplied by Amersham) was too dilute (10 μM) for the aminoacylation assay. Thus the ^{3}H-labeled isoleucine was concentrated 5X by evaporation using a Speed-Vac. The concentration of the ^{3}H-label isoleucine concentrate was determined by spotting 1 μL onto a Whatmann GF/C filter and counting the filter in the scintillation counter. From the dpm and the known specific activity of the ^{3}H-labeled isoleucine, the concentration can be calculated.

Aminoacylation activities of purified samples were measured in standard reaction Solution A with 10 mM ATP·Mg^{2+}, 5 μM ^{3}H-labeled isoleucine (Amersham), 15 μM isoleucine (unless otherwise stated), 160 μM yeast tRNA or 160 μM *E. coli* tRNA and 0.002 units/μL inorganic pyrophosphatase.

The reaction was initiated with the addition of enzyme and the mixture was incubated at 37°C for 20 minutes. The reaction was quenched and filtered as described in section 2.12.1.1. The filters were then washed exhaustively with 4X 3 mL ice cold 5% w/v TCA and 2X 2 mL 95% ethanol. The filters were dried and counted as stated in section 2.12.1.1.

2.12.2 Aminoacylation Assays Using the Competitive Inhibitor Pseudomonic Acid A

Pseudomonic acid A was added to the reaction in the place of ddH_2O to a final concentration of 1 μM in a 25 μL reaction volume. Pseudomonic acid A was freshly prepared in a 50% v/v methanol solution at a concentration of 50 μM.

Assays were performed in the same manner as described in section 2.12.1.1.

2.12.3 Assaying the Effect of Monoclonal Antibodies on Aminoacylation Activities OF yIRS and bIRS.

2.12.3.1 The Effect of Pre-Incubation Between Antibody and Enzyme on Aminoacylation Activities

Prior to assay, the concentration of purified yeast IRS and *E. coli* IRS were determined by Microtitre Lowry Protein Assay (see section 2.7.2.2). The enzymes were pre-incubated with the FPLC purified monoclonal antibody, on ice, for varying periods of time at enzyme to antibody ratios of 1:1000 and 1:100 (yeast IRS and *E. coli* concentrations were constant at 7.8 nM and 20 nM respectively). Aminoacylation activities were measured in standard reaction Solution A with 10 mM ATP-Mg^{2+}, 6.2 μM ^{14}C-labeled isoleucine (Amersham), 16 μM isoleucine (unless otherwise stated), 160 μM yeast tRNA for yeast IRS or 160 μM *E. coli* tRNA for *E. coli* IRS and 0.002 units/μL inorganic pyrophosphatase.

Reaction volumes were 25 μL and the reaction was initiated with the addition of the pre-incubated enzyme-antibody mixture. The reaction was incubated at 30°C for 5 minutes. A 20 μL aliquot was taken from the reaction mixture and quenched with 3 mL ice cold 5% w/v TCA. The quenched sample was filtered through Whatmann GF/C filter and washed as described in section 2.12.1.1.

2.12.3.2 The Effect of Varying Concentrations of Monoclonal Antibody on Aminoacylation Activities.

The purified monoclonal antibodies were serially diluted in 10 fold increments up a 10^7X dilution. The enzyme was pre-incubated with varying concentrations of monoclonal antibody, on ice, for 15 minutes prior to activity assay. Aminoacylation activities were measured as described in section 2.12.3.1. The yeast IRS and *E. coli* IRS concentrations in this assay were held constant at 10 nM and 20 nM respectively.

2.13 AMINOADENYLATION ASSAYS (Active-Site Titration)

2.13.1 Aminoadenylation Assay - Standard Procedure

Aminoadenylation activities (M19) were measured in Standard AST reaction buffer (15 mM bisTris, pH 5.87, 10 mM $MgCl_2$, 0.1 mM PMSF and 30 mM β-mercaptoethanol) with 2 mM ATP·Mg^{2+}, 5 μM ^{14}C-labeled isoleucine (or ^{3}H-labeled isoleucine), 5 μM isoleucine and 0.003 units/μL inorganic pyrophosphatase.

Final reaction volume was 30 μL. The reaction was initiated with the addition of the enzyme sample. The reaction was allowed to proceed at room temperature (24°C) for 3 minutes before replacing the reaction tube back on ice. An 27 μL aliquot of the reaction mixture was cold-filtered through a nitrocellulose membrane and rinsed 2X with 2 mL cold standard AST buffer. Filters were allowed to dry and then were counted in the Beckmann LS3801 scintillation counter.

2.13.2 Aminoadenylation Assay Performed in the Presence of Pseudomonic Acid A

Pseudomonic Acid A was added to the final aminoadenylation assay reaction mixture (in the place of ddH_2O) to a final concentration of 0.3 μM. Reactions were performed as described in section 2.13.1.

2.13.3 Assaying the Effect of Monoclonal Antibodies to yIRS and bIRS on Aminoadenylation Activities.

2.13.3.1 The Effect of Pre-Incubation Between Antibody and Enzyme on Aminoadenylation Activities

Prior to assaying aminoadenylate activity, the enzyme (yIRS or bIRS) was pre-incubated with the purified monoclonal antibody for 30 minutes on ice at a enzyme to antibody ratio of 1:10. After pre-incubation, the enzyme-antibody mixture was assayed for aminoadenylate activity as described in the standard procedure (section 12.13.1).

2.13.3.2 The Effect of Varying Concentrations of Monoclonal Antibody on Aminoadenylation Activities.

The enzyme (yIRS or bIRS) was pre-incubated with varying concentrations of purified monoclonal antibody for 15 minutes on ice as described in section 12.12.3.2. Then the enzyme-antibody mixture was assayed for aminoadenylation activity as described in the standard procedure (section 2.13.1)

3. RESULTS:

3.1 CLONING SUMMARY

The C-terminal deletion genes of yeast isoleucyl-tRNA synthetase were originally designed and cloned into the expression vector pLmp10 by S. Sidhu (76). These C-terminal deletion genes were subsequently subcloned as a "cassette" into the *E. coli* expression vectors pKR16Q and pKR18Q (60) to improve the level of mutant protein expression. The cloning summaries of the five C-terminal deletion genes are depicted in Figures 4 and 5.

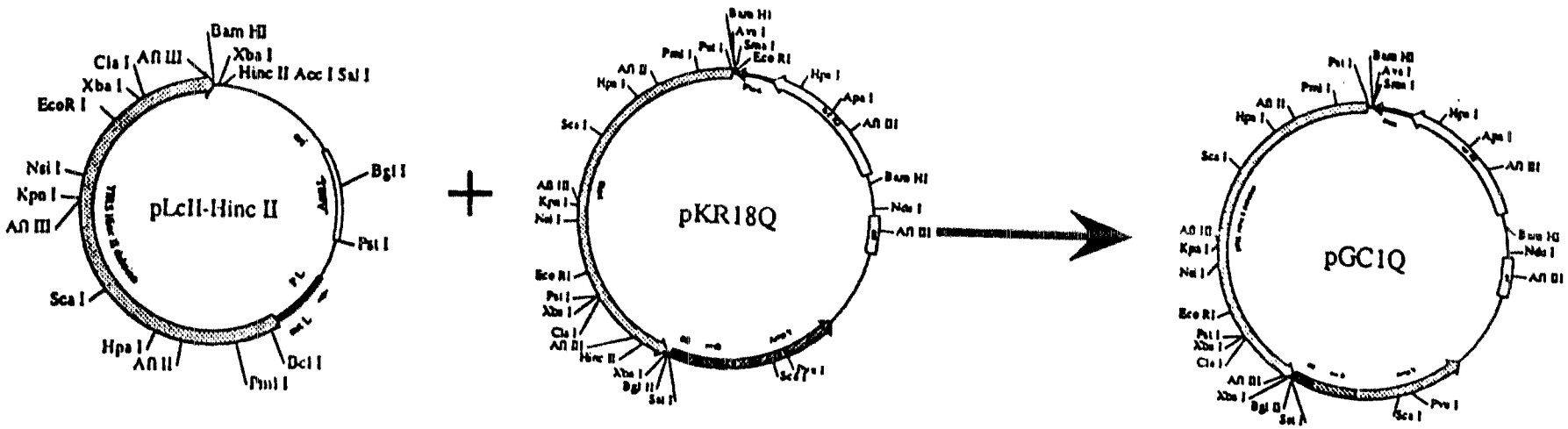
Site-directed mutagenesis and PCR were used to introduce two novel restriction sites in the bIRS gene. A Nsi I restriction endonuclease site was inserted at base #1873 of the bIRS gene and a Sst I restriction site was inserted after the stop codon of the bIRS gene at base #2828. A 953 bp fragment of the C-terminal domain of the bIRS gene was then amplified and cloned into pKR18Q to create a chimeric construct called pGC6Q. The cloning scheme of pGC6Q is illustrated in Figure 6.

The gene of the Afl III/Sac II chimeric IleRS was subcloned as a "cassette" into the *E. coli* expression plasmid pKR16Q to improve the level of expression of this chimera. The cloning scheme is shown in Figure 7.

All final expression constructs were confirmed by restriction endonuclease digests.

Figure 4: Cloning Scheme of the Hinc II and Cla I yIRS C-terminal Deletion Genes. A diagram outlining the subcloning of **A**: the Hinc II and **B**: the Cla I C-terminal deletion genes from the pLmp10 vector into pKR18Q. Please refer to section 2.5.4.2 for a more complete description of the cloning steps.

A



B

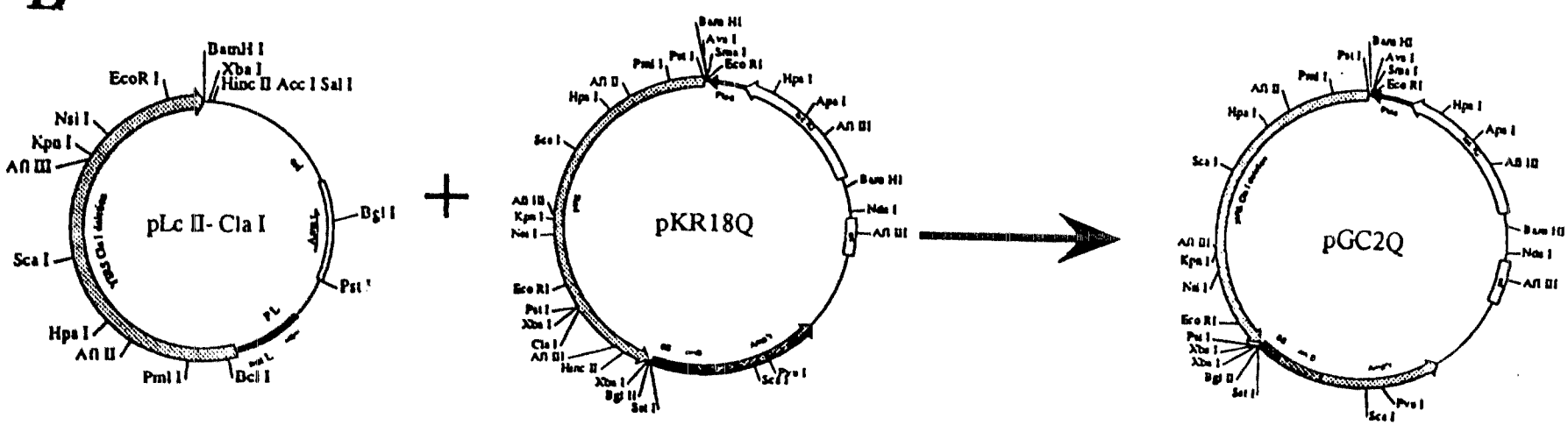
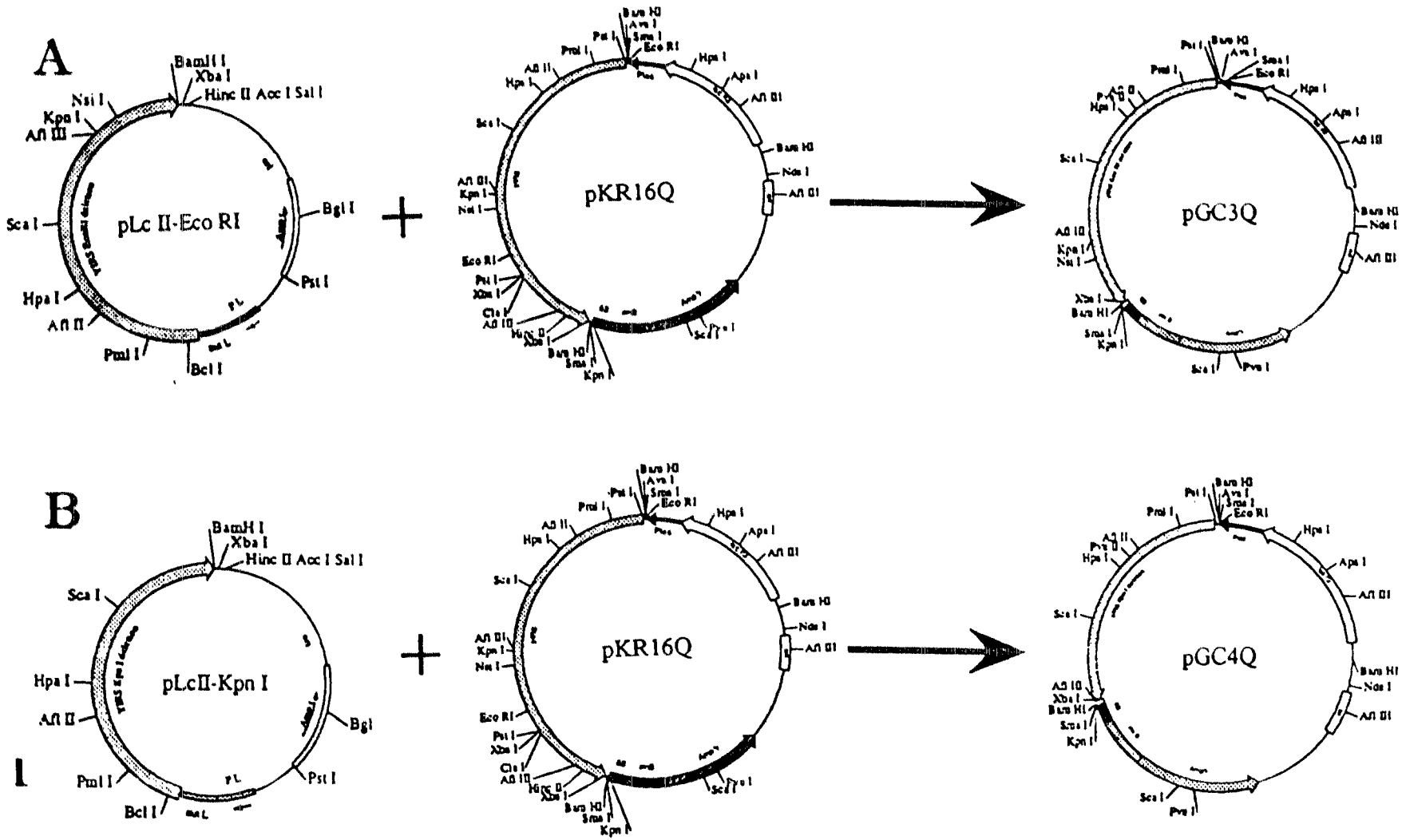


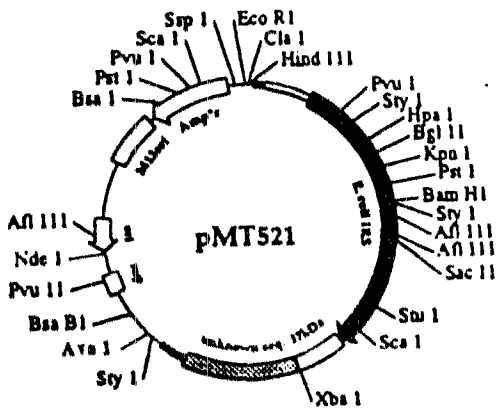
Figure 5: Cloning Scheme of the Eco RI, Kpn I and Sca I yIRS C-terminal Deletion Genes. A diagrammatic representation of the subcloning of **A**: the Eco RI, **B**: the Kpn I and **C**: the Sca I C-terminal deletion genes from the plasmid, pLmp10, into the plasmid pKR16Q. Please see section 2.5.4.1 for a more detailed outline of the cloning procedure.


A
BamH I
Xba I
Hinc II Acc I Sal I
Nsi I
Kpn I
Afl III
Sca I
pLc II-Eco RI
Bgl I
Pst I
Hpa I
Afl II
Pml I
Bcl I
pKR16Q
pGC3Q
B
BamH I
Xba I
Hinc II Acc I Sal I
Sca I
pLcII-Kpn I
Hpa I
Afl II
Pml I
Bcl I
Pst I
pKR16Q
pGC4Q

65b

C

Figure 6: Cloning Scheme of the Nsi I Chimeric IleRS Enzyme. A diagram outlining the creation of the IleRS chimeric gene called the Nsi I Chimera. The C-terminal domain of the bIRS fragment was modified by site-directed mutagenesis to introduce two novel restriction sites. This bIRS fragment was finally subcloned into pKR18Q to yield the plasmid pGC6Q. Please refer to section 2.6 for the complete cloning procedure.



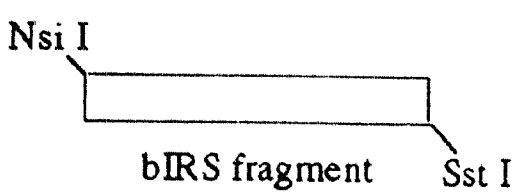
PCR Primers:

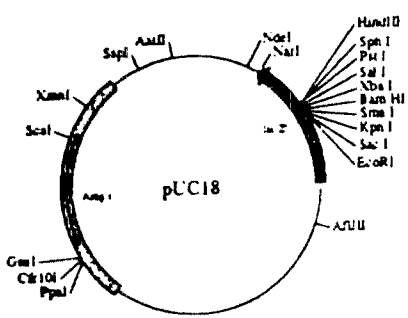
Nsi I

5'~GGCGCGGATGCATTGCGTCTG~3' (21 mer oligonucleotide)

Sst I

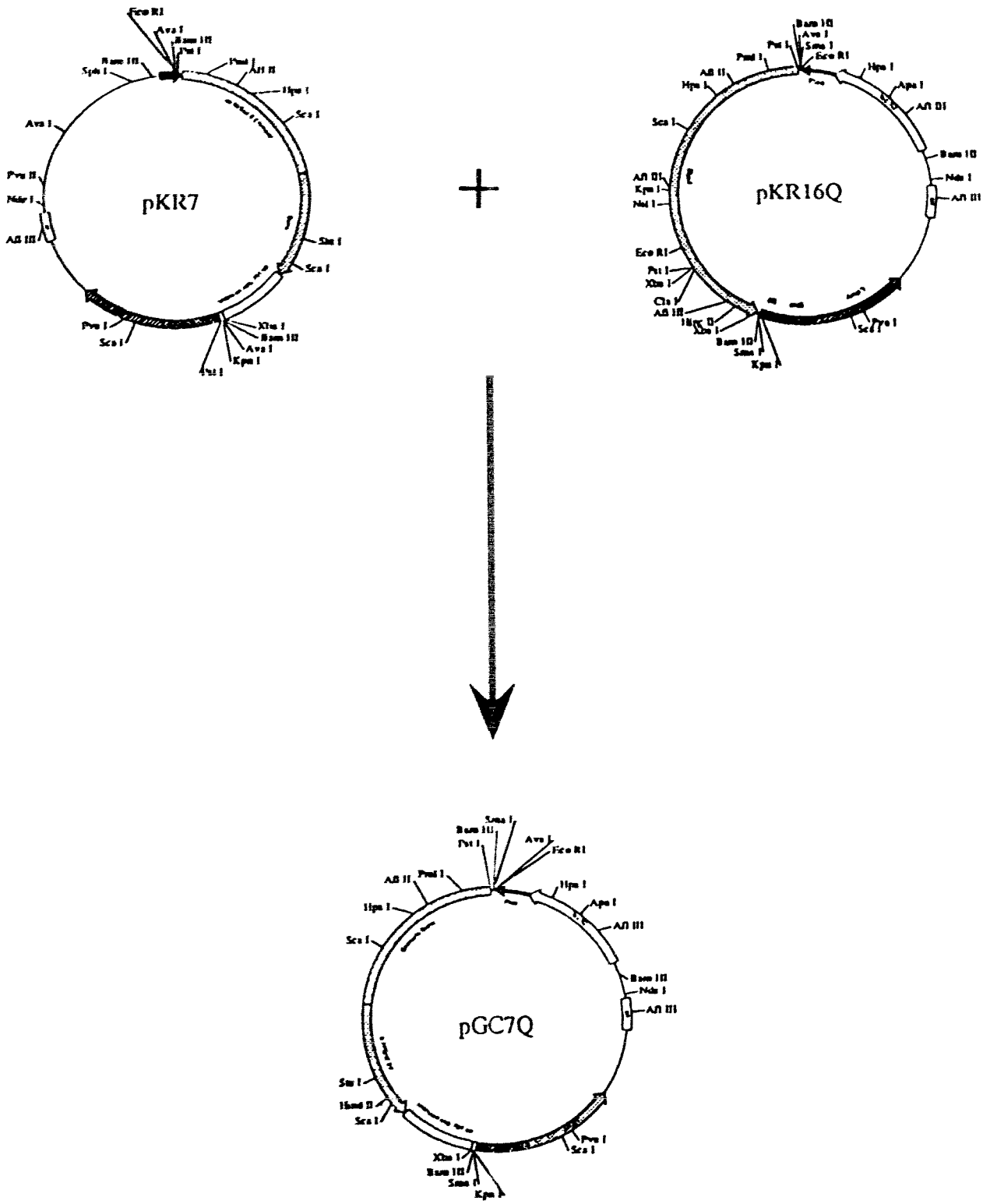
3'~TTTGCATTCAAACGGACTGCTCGAG~5' (24 mer oligonucleotide)





pUC18ΔbIRSB
pUC18ΔbIRSA
pGC6Q
pKR18Q

Figure 7: Cloning Scheme of the Afl III/Sac II Chimeric IleRS Enzyme. The Afl III/Sac II gene was excised from the plasmid, pKR7, and subcloned into the plasmid pKR16Q to improve the expression of the mutant protein. Please see section 2.5.4.3 for a more detailed outline of the cloning steps.


pKR7
pKR16Q
pGC7Q

3.2 PROTEIN EXPRESSION

3.2.1 Predicted Mutant IleRS Protein Constructs

Diagrammatic representations of the predicted primary sequences of the yIRS C-terminal deletion constructs and the chimeric constructs are illustrated in Figures 8 and 9, respectively. Based on sequence alignments with the wild-type bIRS, the predicted secondary structures of the N-terminal domain are also illustrated.

3.2.2 Protein Expression of the C-terminal Deletion IleRS Mutants

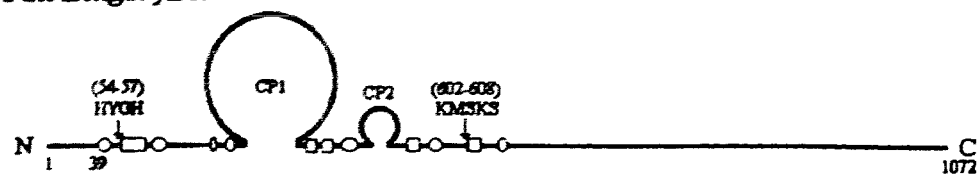
Protein expression in cell lysates of *E. coli* strain DH5α containing the constructs pGC1Q, pGC2Q, pGC3Q, pGC4Q and pGC5Q was determined by SDS-PAGE and western immunoblot analysis. The results showed the overexpression of all five C-terminal deletion mutant proteins with M_r-values corresponding to the predicted molecular weight of each deletion protein (see Figure 10). The expression levels of the C-terminal deletion proteins were comparable to the full length wild-type protein expressed in the same vector.

3.2.3 Protein Expression of the IleRS Chimeras

Constructs pKR5 and pKR7 expressed in *E. coli* strain TG2 and constructs pKR8, pKR9, pGC6Q and pGC7Q expressed in *E. coli* strain DH5α produced recombinant proteins of the predicted molecular weight as shown by SDS-PAGE and western immunoblot analysis (see Figure 11). Protein expression levels using these expression plasmids were deemed as sufficient for subsequent protein purification.

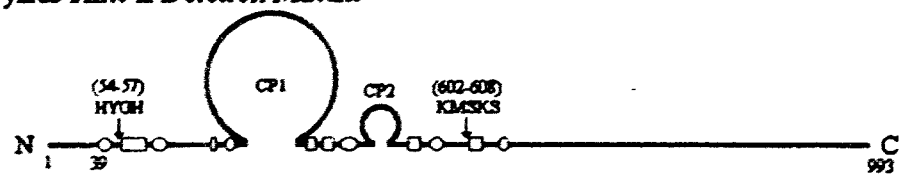
Figure 8: A Diagrammatic Representation of the Yeast Isoleucyl-tRNA Synthetase (yIRS) C-terminal Deletion Constructs. Predicted secondary structural elements, based on primary sequence similarities between *E. coli* methionyl-tRNA synthetase and yIRS, are depicted as rectangles (α-helices) and ovals (β-sheets) along the primary sequence. Connective peptides 1 and 2 (CP1 and CP2) are presented as bulges. The C-terminal deletions were created by utilizing unique restriction sites on the yIRS gene, hence the names of the C-terminal deletion proteins. (Format of the diagram derived from A. Shepard, K. Shiba and P. Schimmel, *PNAS* 89: 9964-9968 (75).

Full Length yIRS



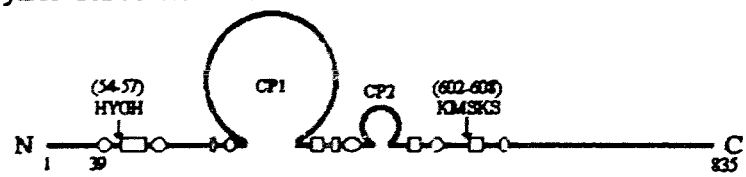
M.W. 123 KDa

yIRS Hinc II Deletion Mutant



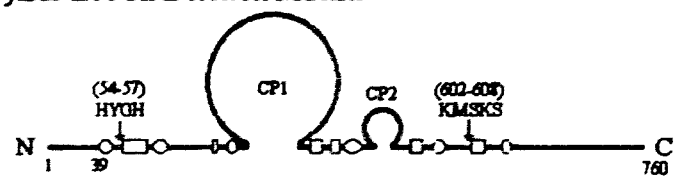
M.W. 114 KDa

yIRS Cla I Deletion Mutant



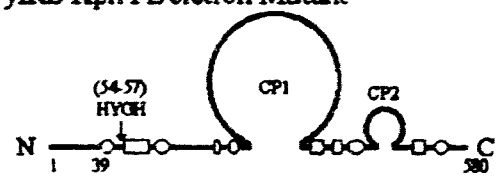
M.W. 96 KDa

yIRS Eco RI Deletion Mutant



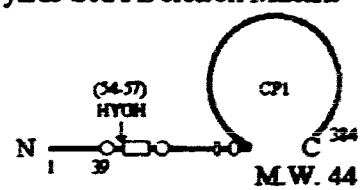
M.W. 88 KDa

yIRS Kpn I Deletion Mutant



M.W. 67 KDa

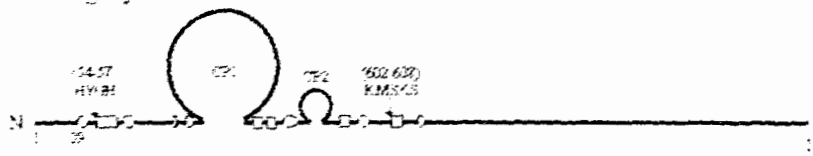
yIRS Sca I Deletion Mutant



M.W. 44 KDa

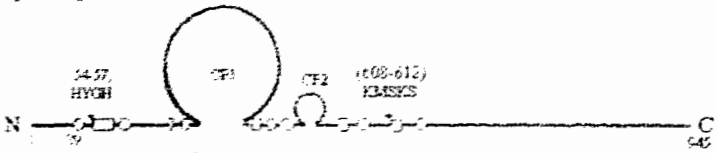
Figure 9: A Diagrammatic Representation of Chimeric Isoleucyl-tRNA Synthetase Constructs. Predicted secondary structural elements, based on primary sequence similarities between *E. coli* methionyl-tRNA synthetase, *E. coli* isoleucyl-tRNA synthetase (bIRS) and yIRS are depicted as rectangles (α-helices) and ovals (β-sheets) along the primary sequence. The blue line and blue shapes represents the yIRS portion of the chimeric enzyme and the red line and red shapes represents the bIRS portion of the chimeric enzyme. (Format of the diagram derived from A. Shepard, K. Shiba and P. Schimmel, *PNAS* 89: 9964-9968 (75)).

Full Length yIRS



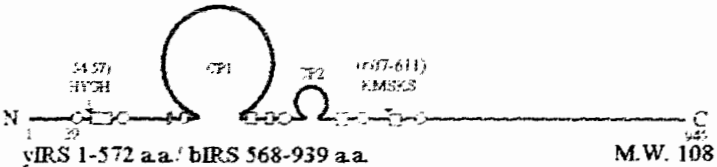
M.W. 123 KDa

Hpa I/Hpa I Chimera



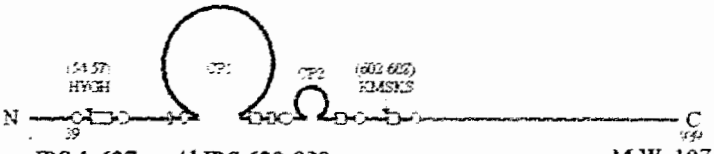
yIRS 1-135 a.a./ bIRS 231-939 a.a. M.W. 107 KDa

Afl III/Sac II Chimera



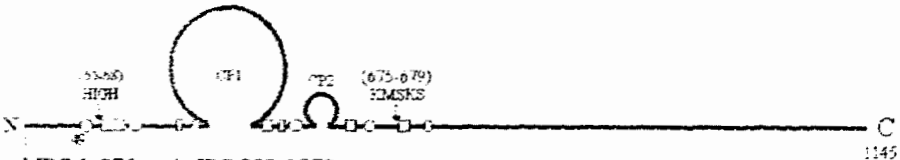
yIRS 1-572 a.a./ bIRS 568-939 a.a. M.W. 108 KDa

Nsi I Chimera



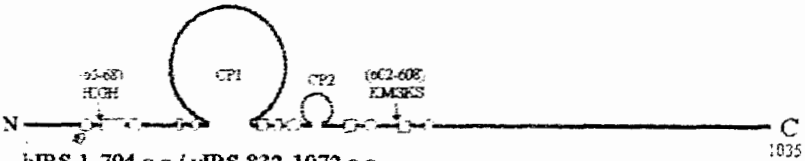
yIRS 1-627 a.a./ bIRS 628-939 a.a. M.W. 107 KDa

Bgl II/Pvu II Chimera



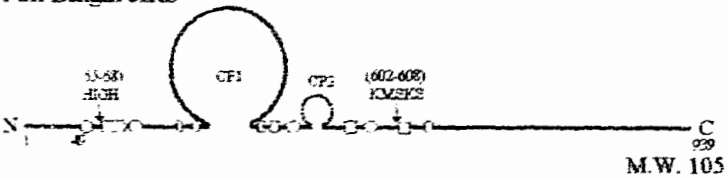
bIRS 1-276 a.a/ yIRS 203-1072 a.a. M.W. 130 KDa

Stu I/Cla I Chimera



bIRS 1-794 a.a./ yIRS 832-1072 a.a. M.W. 116 KDa

Full Length bIRS



M.W. 105 KDa

Figure 10: Western Immunoblot of Crude Cell-Free Extracts of *E. coli* DH5α Strain Containing Plasmids pGC1Q, pGC2Q, pGC3Q, pGC4Q and pGC5Q. Expression of the C-terminal deletion proteins of yIRS was confirmed using polyclonal antibodies developed against the full length yIRS. The lanes are as follows: E.C.L. (Amersham) Molecular Weight Standards (lane 1), purified yIRS (lane 2), pGC1Q (lane 3), pGC2Q (lane 4), pGC3Q (lane 5), pGC4Q (lane 6), pGC5Q (lane 7) and purified bIRS (lane 8). The M_r-values of the molecular weight markers are also labeled.

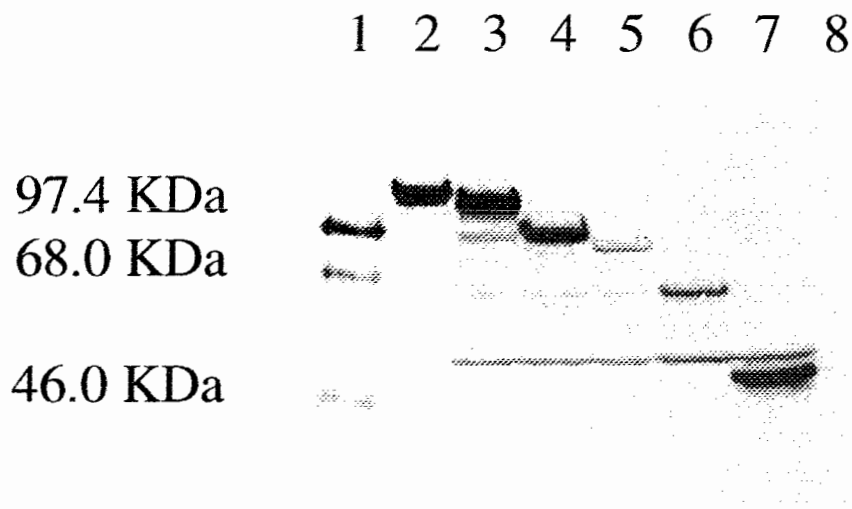
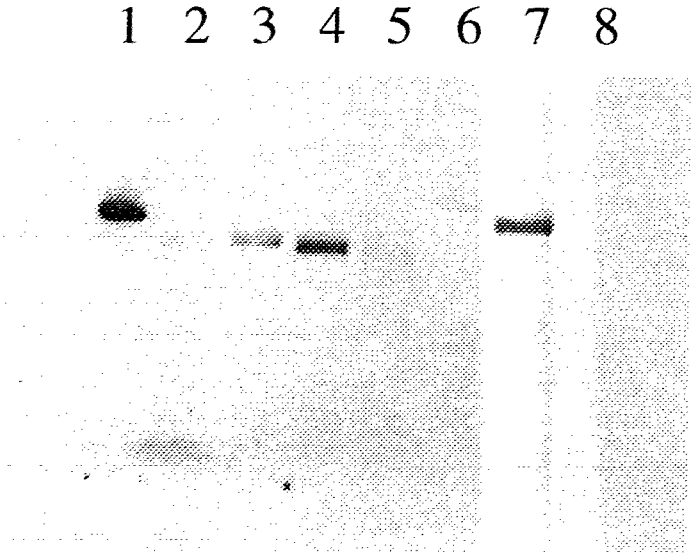
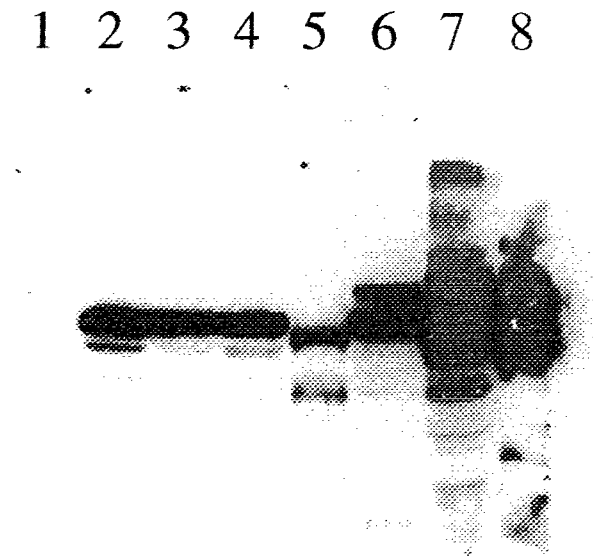

1 2 3 4 5 6 7 8
97.4 KDa
68.0 KDa
46.0 KDa

Figure 11: Western Immunoblot of Crude Cell-Free Lysates of *E. coli* Strain TG2, Containing Plasmids pKR5 and pKR7, and Strain DH5α Harbouring the Plasmids pGC6Q, pKR8 and pKR9. Expression of chimeric IleRS was confirmed using western immunoblotting with **A**: polyclonal antibodies developed against wild-type yIRS and **B**: polyclonal antibodies developed against wild-type bIRS. The lanes are designated as follows: purified yIRS (lane 1), pKR5 (lane 2), pKR7 (lane 3), pGC6Q (lane 4), E.C.L. (Amersham) Molecular Weight Standards (lane 5), pKR8 (lane 6), pKR9 (lane 7) and purified bIRS (lane 8). The M_r-values of the molecular weight standards are labeled.

A



B



3.3 *IN VIVO* AMINOACYLATION ASSAY OF THE MUTANT IleRS PROTEINS

Pseudomonic acid A is a strong competitive inhibitor for the isoleucine binding site of bIRS (37). It was shown by Racher et al. that yIRS has a much higher K_i for pseudomonic acid A (K_i= 1.5 X 10^{-5} M) than bIRS (K_i=2.5 X 10^{-9} M)(61). It has also been shown by Racher et al that *E. coli* cells expressing yeast IleRS ere resistant to pseudomonic acid A whereas *E. coli* cells not expressing yIRS were sensitive to the antibiotic. Thus, yIRS was capable of rescuing the *E. coli* host cells by taking over the function of host's IleRS which was inhibited by the antibiotic. The same *in vivo* assay was performed to determine if any of the mutant IleRS proteins were capable of conferring pseudomonic acid resistance to the *E. coli* host strain.

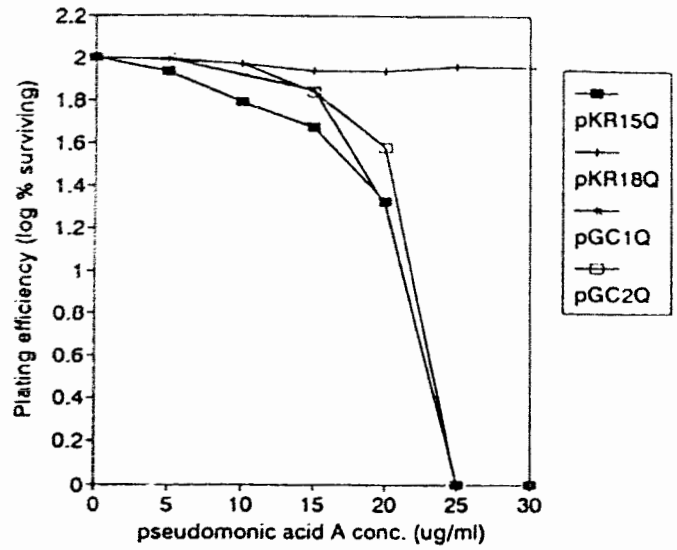
E. coli strain TG2 cells harbouring the mutant IleRS constructs and the appropriate control plasmids were plated on minimal agar supplemented with ampicillin and varying concentrations of pseudomonic acid A. The results are shown in Figure 12. At 25 µg/mL pseudomonic acid A, cells containing the full length wild-type yIRS gene had virtually 100% plating efficiency whereas the cells harbouring the mutant IleRS constructs had almost no colonies of comparable size to the colonies expressing the wild-type yIRS. (Note that "100% plating efficiency" is defined as the number of colonies appearing on a minimal agar plate without pseudomonic acid A. The number of colonies counted on plates supplemented with pseudomonic acid A is measured relative to the number of colonies appearing on the plate without pseudomonic acid). At pseudomonic acid A concentrations higher than 30 µg/mL, no colonies harbouring the mutant IleRS constructs and the control plasmids which lack the yIRS gene were visible. It was noted that the growth of cells expressing some of the mutant proteins were sometimes poorer than cells harbouring the control plasmids which lack the IleRS gene (results not shown). The only anomaly in the results obtained was for pKR9 which had a plating efficiency of 6.5% at concentrations of 25 µg/mL and higher (Figure 12D). This result is misleading because the number of colonies appearing on the control plate (without PA) was 10 fold less than the estimated plated cfu. Therefore, the result should be considered invalid due to poor growth of the colonies on the control plate.

Figure 12: The Relative Plating Efficiencies of *E. coli* Strain TG2 Cells, Containing Various Plasmids, in the Presence of Pseudomonic Acid A. *E. coli* strain TG2 cells, which were infected with the mutant IleRS plasmids, were assayed for resistance against pseudomonic acid A, which is a competitive inhibitor of *E. coli* IleRS (37). Cells were grown at 37°C for 96 hours on minimal agar plates supplemented with various concentrations of pseudomonic acid A. After the growth period, the plates were counted for colonies. The number of colonies present on a plate without pseudomonic acid A was designated as the standard 100% plating efficiency. Note that the assays were performed with the appropriate controls.

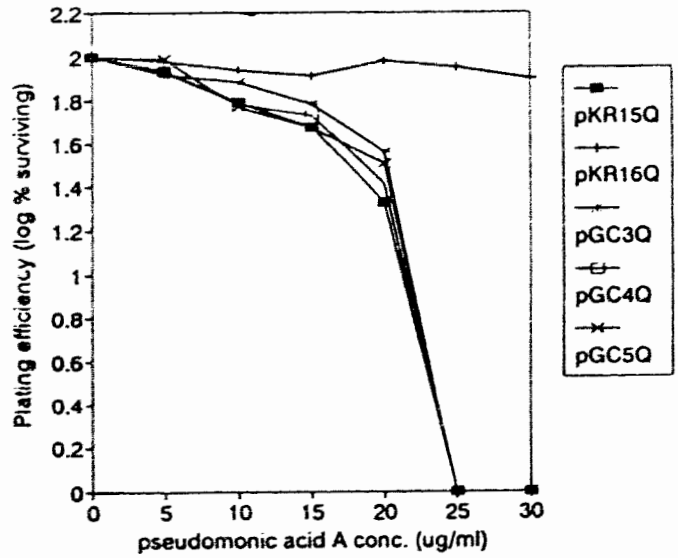
A: *E. coli* strain TG2 cells harbouring plasmids pKR15Q (negative control), pKR18Q (positive control- wild type yIRS), pGC1Q (yIRS Hinc II C-terminal deletion mutant) and pGC2Q (yIRS Cla I C-terminal deletion mutant).

B: *E. coli* Strain TG2 cells containing plasmids pKR15Q (negative control), pKR16Q (positive control- wild type yIRS), pGC3Q (yIRS Eco RI C-terminal deletion mutant), pGC4Q (yIRS Kpn I C-terminal deletion mutant) and pGC5Q (yIRS Sca I C-terminal deletion mutant).

A


Plating efficiency (log % surviving)
2.2
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0
5
10
15
20
25
30
pseudomonic acid A conc. (ug/ml)
pKR15Q
pKR18Q
pGC1Q
pGC2Q

B

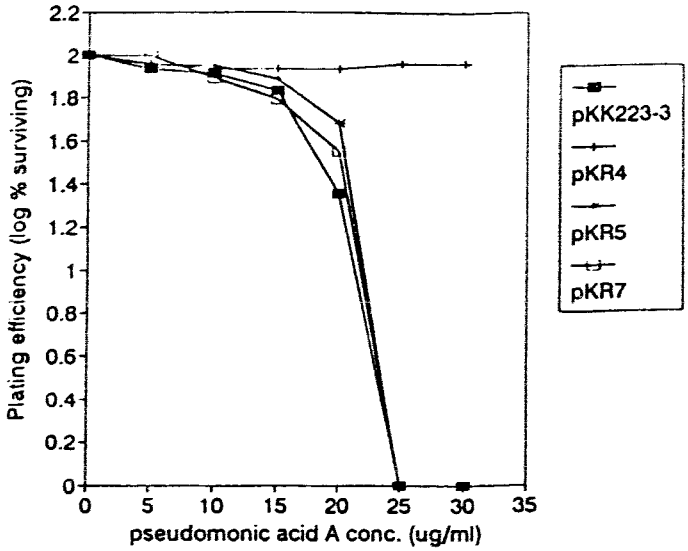

Plating efficiency (log % surviving)
2.2
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0
5
10
15
20
25
30
pseudomonic acid A conc. (ug/ml)
pKR15Q
pKR16Q
pGC3Q
pGC4Q
pGC5Q

C: *E. coli* strain TG2 cells with plasmids pKK223-3 (negative control), pKR4 (positive control- wild type yIRS), pKR5 (Hpa I/Hpa I Chimera) and pKR7 (Afl III/Sac II Chimera).

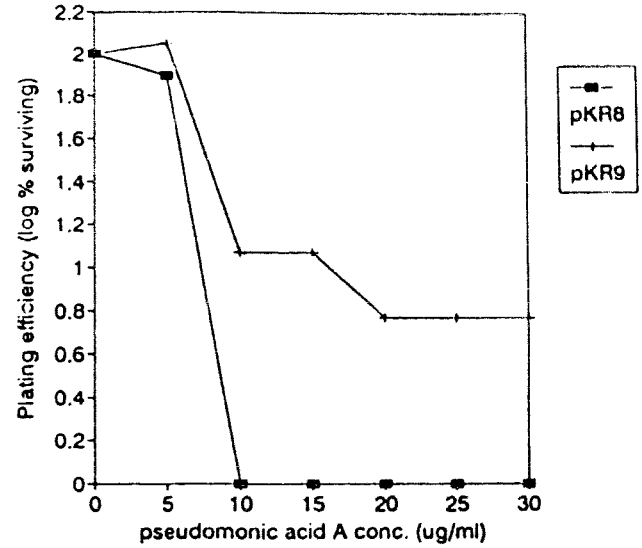
D: *E. coli* strain TG2 cells containing plasmids pKR8 (Bgl II/Pvu II Chimera) and pKR9 (Stu I/ Cla I Chimera).

E: *E. coli* strain TG2 cells containing plasmids pKR15Q (negative control), pKR16Q (control- wild type yIRS), pKR18Q (positive control- wild type yIRS), pGC6Q (Nsi I Chimera) and pGC7Q (Afl III/Sac II Chimera).

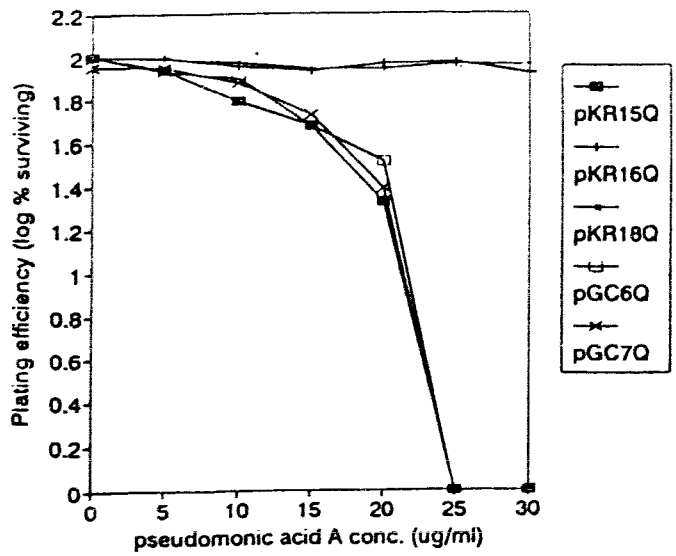
C


Plating efficiency (log % surviving)
pseudomonic acid A conc. (ug/ml)
pKK223-3
pKR4
pKR5
pKR7

D


Plating efficiency (log % surviving)
pseudomonic acid A conc. (ug/ml)
pKR8
pKR9

E


Plating efficiency (log % surviving)
pseudomonic acid A conc. (ug/ml)
pKR15Q
pKR16Q
pKR18Q
pGC6Q
pGC7Q

3.4 *IN VITRO* AMINOACYLATION ASSAY OF MUTANT IleRS PROTEINS

In vitro aminoacylation assays were performed on crude cell lysates of the *E. coli* cells containing the mutant IleRS constructs. Assays were performed using ^{14}C-labeled isoleucine and crude *E. coli* tRNA, in the absence and presence of pseudomonic acid as described in section 2.12.1 and 2.12.3.

Pseudomonic acid A was added to the aminoacylation assay to distinguish the activity of the mutant IleRS proteins and yIRS from the *E. coli* host's IleRS. In a recent publication by Yanasigawa et al. (90), a single amino acid change from phenylalanine to leucine at position #594 was found to be responsible of pseudomonic acid A resistance in the *E. coli* strain PS102. The corresponding amino acid in the yIRS protein at position #594 is an isoleucine. Therefore, based on this evidence, all mutant proteins containing an intact N-terminal domain of yIRS should be resistant to the antibiotic. To determine whether the mutant IleRS proteins were capable of aminoacylating *E. coli* tRNA, assays on crude cell-free lysates of *E. coli* cells expressing these proteins were performed in the absence and presence of pseudomonic acid A. The results are shown in Figure 13. From these results, all the IleRS chimeras and all of the yIRS C-terminal deletion portions, with the exception of the Hinc II C-terminal deletion protein, appeared to be sensitive to pseudomonic acid or have no aminoacylation activity. The yIRS Hinc II C-terminal deletion protein showed partial aminoacylation activity of about 6% relative to the full length yIRS.

Figure 13: Aminoacylation Activity of Various Mutant Isoleucyl-tRNA Synthetase Enzymes in the Absence and Presence of Pseudomonic Acid A. PA denotes Pseudomonic Acid A. Scaling was based on the wild-type yIRS and bIRS enzyme activity in the absence of pseudomonic acid A (as 100%). Aminoacylation assays were performed on crude cell-free lysates of *E. coli* cells expressing mutant protein. Levels of protein expression were estimated by western immunoblot analysis (results not shown). Assays were performed in the absence and presence of 1 μM (final reaction concentration) pseudomonic acid A. It has been found that 1 μM pseudomonic acid A was sufficient to inhibit bIRS at host cell concentrations.

Log % Activity Retained
2.5
2
1.5
1
0.5
0
2.00
2.08
1.82
0.86
1.52
0.02
1.55
0.02
1.33
0.02
1.60
0.02
1.66
0.02
1.73
0.02
1.53
0.02
1.77
0.02
1.93
0.02
2.00
0.02
yIRS
Hinc II Deletion
Cla I Deletion
Eco RI Deletion
Kpan I Deletion
Sca I Deletion
Hpa I/Hpa I Chimera
Afl III/Sac II Chimera
Nsi I Chimera
Bgl II/Pvu II Chimera
Stu I/Cla I Chimera
bIRS
no PA
PA

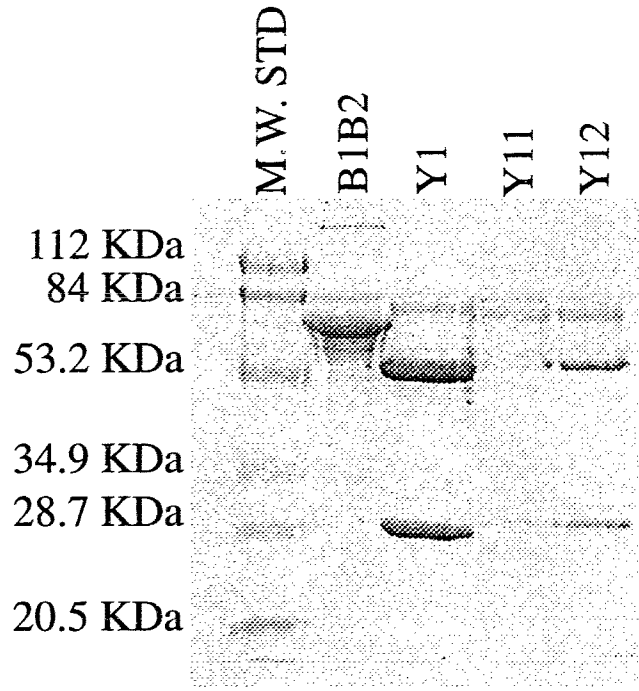
3.5 PURIFICATION OF MONOCLONAL ANTIBODIES FROM ASCITES FLUID

Monoclonal antibodies to the enzymes yIRS and bIRS were purified from ascites fluid and tissue culture respectively as described in section 2.10.4. After precipitation with 50% ammonium sulphate and dialysis of the protein pellet with Buffer A, the protein sample was screened for the presence of antibody on a 12% SDS polyacrylamide gel (results not shown). Most of the protein precipitate was antibody as noted by the characteristic mobility of the light and heavy chains of the monoclonal antibody. It was also noted that the B1B2 monoclonal antibody did not precipitate well in 50% ammonium sulphate. Thus, a sample of the B1B2 monoclonal antibody in DME media was dialyzed to remove the pigment prior to liquid chromatography.

The dialyzed monoclonal antibodies were purified by Fast Protein Liquid Chromatography (FPLC) using an anion-change column (Mono Q). Proteins were eluted from the column with a preprogrammed NaCl gradient and the elution of proteins was monitored by measuring the A_{280}. The elution profiles of each monoclonal antibody are illustrated in Appendix D. Each monoclonal antibody had a different elution profile, which was probably characteristic of the class of the monoclonal antibody. Protein peaks were screened for active antibody by ELISA (results not shown). Typically, the active antibodies was eluted between 80 and 120 mM NaCl except for monoclonal antibody B1B2 which eluted between 180 mM and 220 mM NaCl.

Fractions containing the most active antibodies were then screened for purity by SDS-PAGE (see Figure 14). Monoclonal antibodies Y1, Y11 and Y12 separated into the heavy and light chains as expected for IgG class antibodies. Monoclonal antibody B1B2, on the other hand, did not separate into heavy and light bands but appeared as a single band with the M_r-value at approximately 75 KDa which maybe characteristic of a different class of immunoglobulin (IgA or IgE class) (64).

Figure 14: SDS-PAGE of Concentrated, Purified Monoclonal Antibodies. Aliquots of 10 μL of each of the concentrated, FPLC purified monoclonal antibodies were separated on a 12% polyacrylamide gel. Monoclonal antibodies Y1, Y11 and Y12 separated into the heavy and light chains which are characteristic of IgG class antibodies. The heavy and light chains correspond to approximately 53 KDa and 27 KDa, respectively. The monoclonal antibody B1B2 appeared as only one band with an M_r-value at 75 KDa which is characteristic of a different class of antibody (IgA or IgE (65)). All samples of monoclonal antibody seem to be relatively free of contaminating proteins.

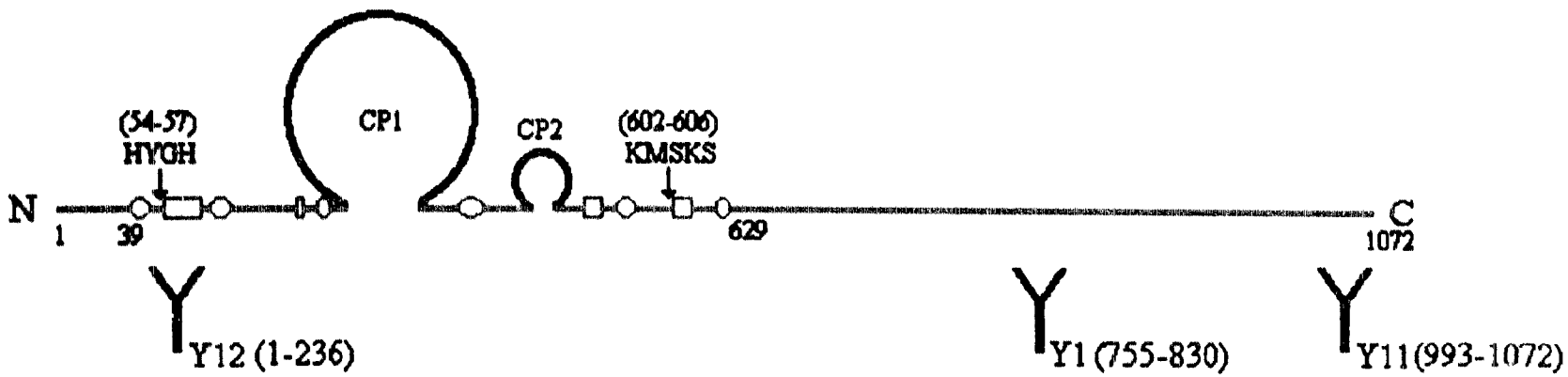

M.W. STD
B1B2
Y1
Y11
Y12
112 KDa
84 KDa
53.2 KDa
34.9 KDa
28.7 KDa
20.5 KDa

3.6 EPITOPE MAPPING OF MONOCLONAL ANTIBODIES

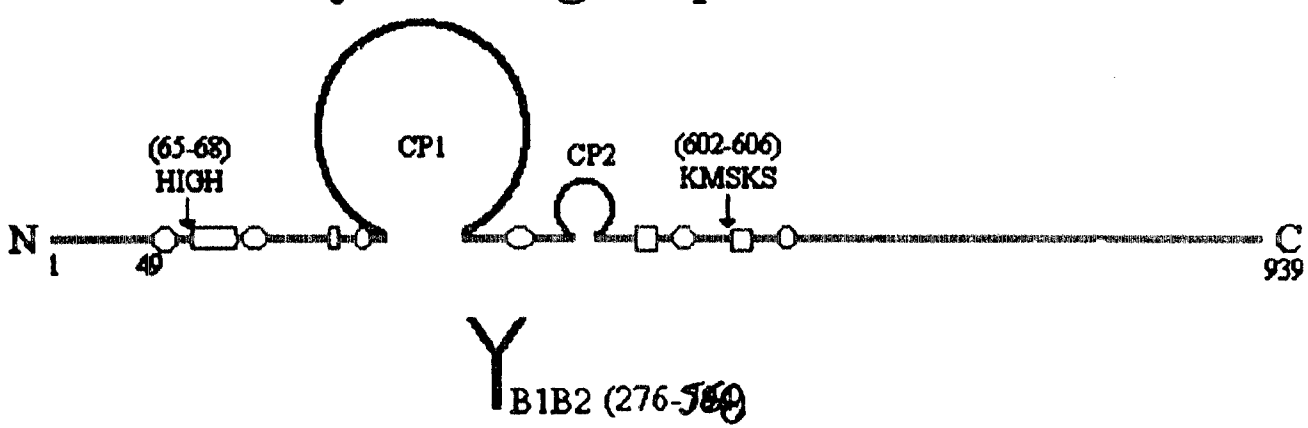
Each monoclonal antibody was tested for their specificity in binding the mutant IleRS proteins by western immunoblotting. The resulting western immunoblots are shown in Appendix E. It was discovered that each monoclonal antibody had a specific region on the IleRS to which it bound. Based on the specificity of each monoclonal for certain mutant IleRS protein and the known amino acid sequence of each mutant IleRS protein, the region to which each monoclonal antibody bound was narrowed down. Figure 15 illustrates the binding sites of the monoclonal antibodies on the yIRS and bIRS proteins. Y12 was discovered to bind to the N-terminal domain of the yIRS protein between amino acid $^{\#}1$-$^{\#}236$. The monoclonal antibodies Y11 and Y1 was found to bind to the C-terminal domain of yIRS between amino acids $^{\#}998$-$^{\#}1072$ and $^{\#}755$-$^{\#}830$, respectively. The monoclonal antibody B1B2 was found to bind between amino acid ($^{\#}279$-$^{\#}568$).

Figure 15: Monoclonal Antibody Binding Maps. From the results of the western immunoblots, we were able to establish the range in which the monoclonal antibodies bind to yIRS and bIRS. Of the three monoclonal antibodies to yIRS, two monoclonal antibodies, Y1 and Y11 were found to bind to the C-terminal domain of yIRS and monoclonal antibody Y12 was found to bind to N-terminal domain. Monoclonal antibody B1B2 to bIRS was found to bind in the middle of bIRS. The span of amino acids in which the monoclonal antibodies bind within is indicated in brackets beside the antibody.

Monoclonal Antibody Binding Map of yIRS



Monoclonal Antibody Binding Map of bIRS



3.7 IMMUNO-AFFINITY CHROMATOGRAPHY

3.7.1 Creation of the Y12 Immuno-Affinity Column

As a tertiary step in the purification of mutant IleRS proteins, an immuno-affinity column was created using the monoclonal antibody Y12. This monoclonal antibody, as mentioned in section 3.6, binds specifically to the N-terminal domain of yIRS. Thus, this monoclonal antibody is ideal in that it can bind to all the yIRS C-terminal deletion proteins as well as three of the five chimeric IleRS enzymes. The immuno-affinity column was created as described in section 2.11 using the Bio-Rad Affi-Gel Hz immuno-affinity kit. The Affi-Gel Hz is a hydrazide-activated support matrix which utilizes the carbohydrate moieties on the Fc region of IgG antibodies. Periodiate oxidation of the vicinal hydroxyls of the carbohydrates on the Fc region of the antibody produces aldehyde functionalities which react with the hydrazide support matrix. This produces an oriented coupling of the antibody, with the antigen binding sites free for binding.

After the coupling the antibody to the matrix, the amount of antibody bound to the matrix was determined by the Bradford Assay. The amount of protein before the coupling process and the protein eluted after the coupling process was determined and the difference is the amount of protein bound to the column. The coupling efficiency of the monoclonal antibody to the column was discovered to be very low with a calculated maximum number of binding sites to be only 2 nmoles.

3.7.2 Testing the Binding Specificity of the Y12 Immuno-Affinity Column

Although it has been shown that the Y12 monoclonal antibody binds specifically to yIRS and not to bIRS (see section 3.2.2.), the interactions between the enzymes and the column matrix may be different from the interactions previously observed between the enzymes and the Y12 antibody. In order to test the binding specificity of Y12 immuno-affinity column, a mixture of

yIRS and bIRS was applied to the column as described in section 2.11.2. Because of the low binding capacity of the column, the amount of protein that would bind to and be eluted from the column would be very small. Thus, the fractions eluted were subjected to two assays to determine if the column specifically bound yIRS.

3.7.2.1 Western-Immunoblot Analysis

Fractions eluted from the immuno-affinity column were subjected to SDS-PAGE and western immunoblotting as described in section (2.7.1 and 2.10.3). The blots were probed separately with monoclonal antibody Y12 and monoclonal antibody B1B2. The resulting blots are shown in Figure 16. From the blots, it appears that both yIRS and bIRS were initially eluted from the column in the low salt buffer wash. In the high salt buffer wash, however, only yIRS was eluted from the column. The elution of yIRS in the initial low salt buffer wash may have been due to an excess of yIRS with respect to the number of antibody binding sites available. The evidence that yIRS can be eluted in the high salt buffer wash confirms that the column can be used to purify proteins with the antigenic site of the Y12 monoclonal antibody.

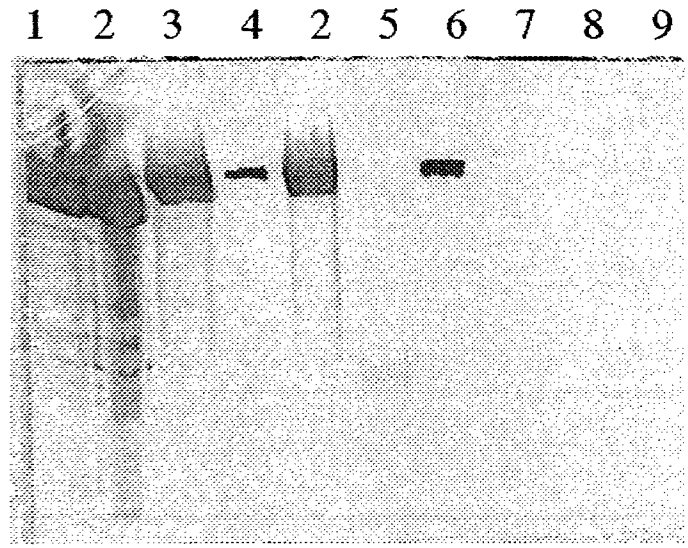
3.7.2.2 Aminoacylation Assays

Aminoacylation assays were performed on some of the protein fractions eluted from the immuno-affinity column. Assays were performed as described in section 2.12.1 using crude yeast tRNA and crude *E. coli* tRNA. The results of the assay are presented in Figure 17. High salt fractions #1 and #3 showed aminoacylation activity with yeast tRNA as well as *E. coli* tRNA. A positive control using pure yIRS showed that yIRS aminoacylated *E. coli* tRNA 2% better than yeast tRNA. High salt fractions #1 and #3 also aminoacylated *E. coli* tRNA more efficiently than yeast tRNA (by roughly 60% and 50% relative to yeast tRNA, respectively), indicating that yIRS was present in the sample. The discrepancy observed between the relative tRNA charging efficiencies of the high salt fractions and the yIRS control (60% and 50% verses 2% greater

efficiency for *E. coli* tRNA relative to yeast tRNA) were most likely due to inconsistencies in the assay. The aminoacylation assays and western immunoblotting analysis (section 3.7.2.1) did show that the high salt fractions were free of bIRS contamination. This assay also showed that the enzyme recovered from the immuno-affinity column retained its activity after binding and eluting from the column.

Figure 16: Western Immunoblot Analysis of Fractions Obtained From the Y12 Immunoaffinity Column. A: probed with monoclonal antibody Y12 as primary antibody and **B:** probed with monoclonal antibody B1B2 as primary antibody. Sample lanes are as follows: A equal molar yIRS/bIRS mixture (lane 1), purified Y12 (lane 2), low salt fraction #1 (lane 3), low salt fraction #5 (lane 4), high molecular weight standard (lane 5), high salt fraction #1 (lane 6), high salt fraction #3 (lane 7), high salt fraction #5 (lane 8) and purified bIRS (lane 9).

A



B

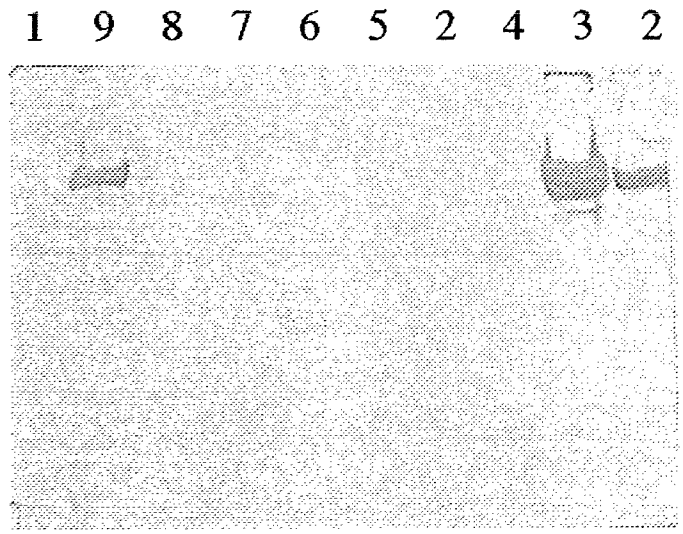
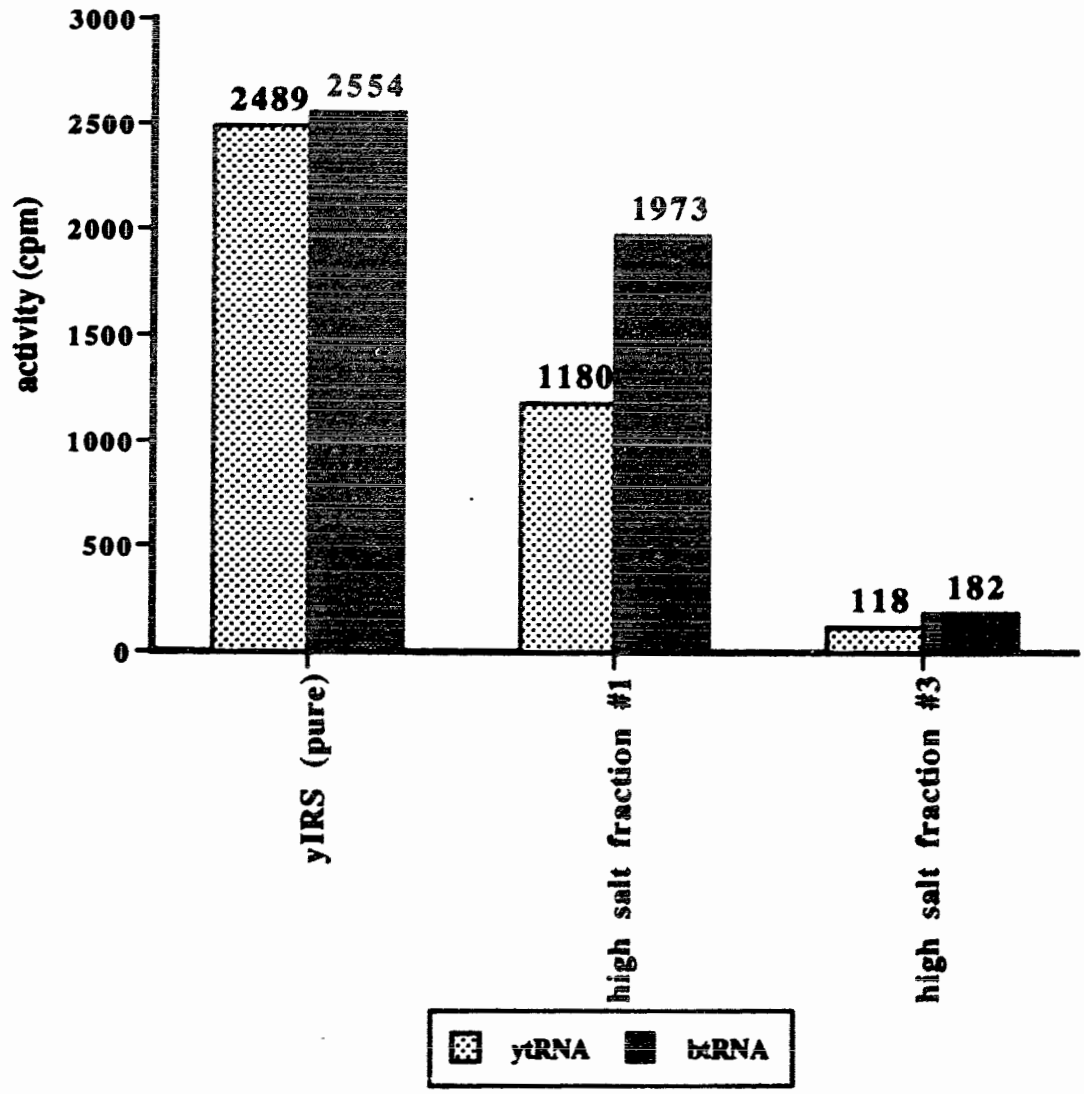


Figure 17: Aminoacylation Assay of Fractions Obtained From the Y12 Immuno-affinity Column. High salt fractions #1 and #3 were assayed for aminoacylation activity with crude yeast tRNA and crude *E. coli* tRNA (Please refer to section 2.12.1 for general assay procedure). Positive controls performed with purified yIRS indicated that yIRS charged the *E. coli* tRNA more efficiently than yeast tRNA (61). High salt fractions #1 and #3 were capable of charging both yeast tRNA and *E. coli* tRNA to roughly the same extent, indicating that yIRS is present in the sample.


3000
2500
2000
1500
1000
500
0
activity (cpm)
2489
2554
1180
1973
118
182
yIRS (pure)
high salt fraction #1
high salt fraction #3
ytRNA
btRNA

3.8 INHIBITION OF IleRS ACTIVITY BY MONOCLONAL ANTIBODIES

Since it was discovered that the monoclonal antibodies bound to specific epitopes on the IleRS's primary sequence, the question arose as to whether or not these antibodies were binding to important sites on the enzyme that would affect the enzymes' activity. Therefore, aminoacylation and aminoadenylation assays were performed to test if any monoclonal antibodies had the ability to inhibit one or both activities in yIRS and bIRS by binding to catalytically important sites on the enzyme.

3.8.1 Aminoacylation Assays With Monoclonal Antibodies

Two sets of assays were performed using the monoclonal antibodies Y1, Y11 and Y12 as possible "inhibitors" to yIRS and the monoclonal antibody B1B2 as a possible "inhibitor" to bIRS.

The first set of assays were conducted using a set molar ratio between enzyme (yIRS or bIRS) and the monoclonal antibody. The enzyme and antibody were pre-incubated for varying periods of time before assaying for aminoacylation activity as described in section 2.12.3.1. The results are shown in Figures 18 and 19. As expected, the higher antibody to enzyme ratio, the greater the inhibition. Of the three monoclonal antibodies to yIRS, only Y12 seems to have a significant affect on aminoacylation activity when compared to the aminoacylation activity of yIRS in the absence of monoclonal antibody. The other two monoclonal antibodies, Y11 and Y1 has little affect on the aminoacylation activity. This is consistent with the evidence that the N-terminal domain of IleRS contains the catalytic core of the enzyme. The monoclonal antibody B1B2 also seems to have little effect on the aminoacylation activity of bIRS despite the fact that it binds in the N-terminal domain of bIRS.

The second set of assays were performed using varying concentrations of monoclonal antibody against a known amount of enzyme. The enzyme was pre-incubated with the monoclonal antibody for 15 minutes prior to assay as described in section 2.12.3.2. The results are shown in Figures 20 and 21. As in the previous assay, the monoclonal antibody Y12 had an inhibitory affect on the aminoacylation activity of yIRS, with greater inhibition at higher concentrations. The monoclonal antibodies, Y1 and Y11, had virtually no inhibitory affect on yIRS at the concentrations tested. The monoclonal antibody B1B2 again showed virtually no affect on the aminoacylation activity of bIRS.

3.8.2 Aminoadenylation Assays with Monoclonal Antibodies

Two sets of aminoadenylation assays were performed to determine if any of the monoclonal antibodies were capable of inhibiting aminoadenylate formation by yIRS and bIRS.

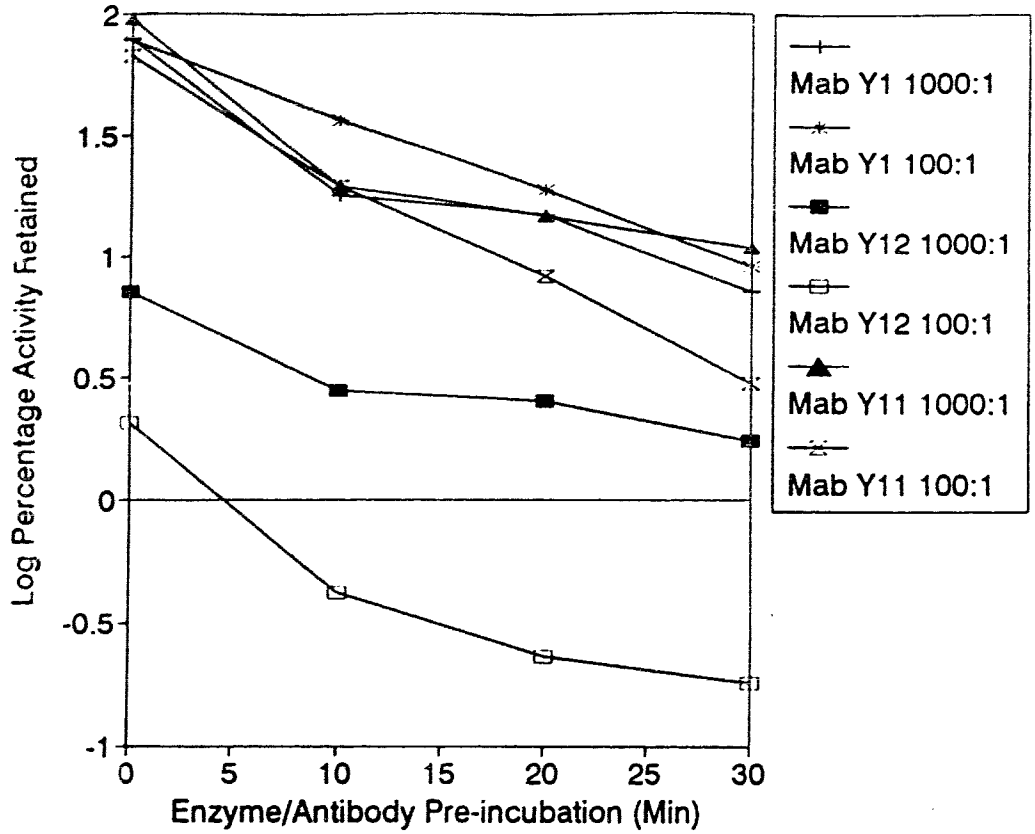
The first set of assays were performed using a set molar ratio of 10:1 between the monoclonal antibody and the enzyme. The enzyme was pre-incubated with the monoclonal antibody for 30 minutes and the mixture was then assayed for aminoadenylate activity (as described in section 2.13.3.1). The results of the assay are illustrated in Figure 22. Of all the antibodies, the monoclonal antibody Y12, which binds to the N-terminal domain of yIRS, was capable of inhibiting aminoadenylate formation. Of the C-terminal binding antibodies, Y1 and Y11, Y1 showed a small degree of inhibition while Y11 showed no inhibition to aminoadenylate formation. The monoclonal antibody B1B2 also showed very little inhibition on aminoadenylation activity of bIRS.

The second set of aminoadenylation assays were performed using varying concentrations of monoclonal antibody as described in section 2.13.3.2. The resulting graphs were very similar to the results from the aminoacylation assays in section 3.8.1. Thus, from these results, it can be

hypothesized that the monoclonal antibody Y12 inhibits aminoadenylate formation and thus in turn inhibits aminoacylation in yIRS. The monoclonal antibodies Y1 and Y11 have very little inhibitory affect on both activities on yIRS and probably doesn't bind in an important substrate recognition or binding site. The monoclonal antibody B1B2 also showed little inhibitory affect to both activities in bIRS and thus, the antibody probably does not bind to an important structural or functional feature on bIRS (see Figures 23 and 24).

Figure 18: Inhibition of Aminoacylation Activity of yIRS by yIRS Monoclonal Antibodies. Scaling was based on the activity of yIRS in the absence of antibody. Monoclonal antibody to enzyme ratios of 1000:1 and 100:1 were used. The enzyme was pre-incubated with the monoclonal antibody on ice for 0 min., 10 min., 20 min. and 30 min. prior to assay. The enzyme-monoclonal antibody mixture was assayed at 30°C for 5 minutes before quenching in 5% cold trichloroacetic acid (TCA). The concentration of enzyme used per 25 μL reaction was 7.8 nM.

Figure 19: Inhibition of Aminoacylation Activity of bIRS by Monoclonal Antibody B1B2. Scaling was based on the activity of bIRS in the absence of antibody. Ratios of 1000:1 and 100:1 of monoclonal antibody B1B2 to bIRS were used. The enzyme was pre-incubated with the antibody prior to assay (Please see section 2.12.3.1 for assay procedure). The concentration of enzyme used per 25 μL reaction was 30 nM.


Log Percentage Activity Retained
2
1.5
1
0.5
0
-0.5
-1
0
5
10
15
20
25
30
Enzyme/Antibody Pre-incubation (Min)
Mab Y1 1000:1
Mab Y1 100:1
Mab Y12 1000:1
Mab Y12 100:1
Mab Y11 1000:1
Mab Y11 100:1

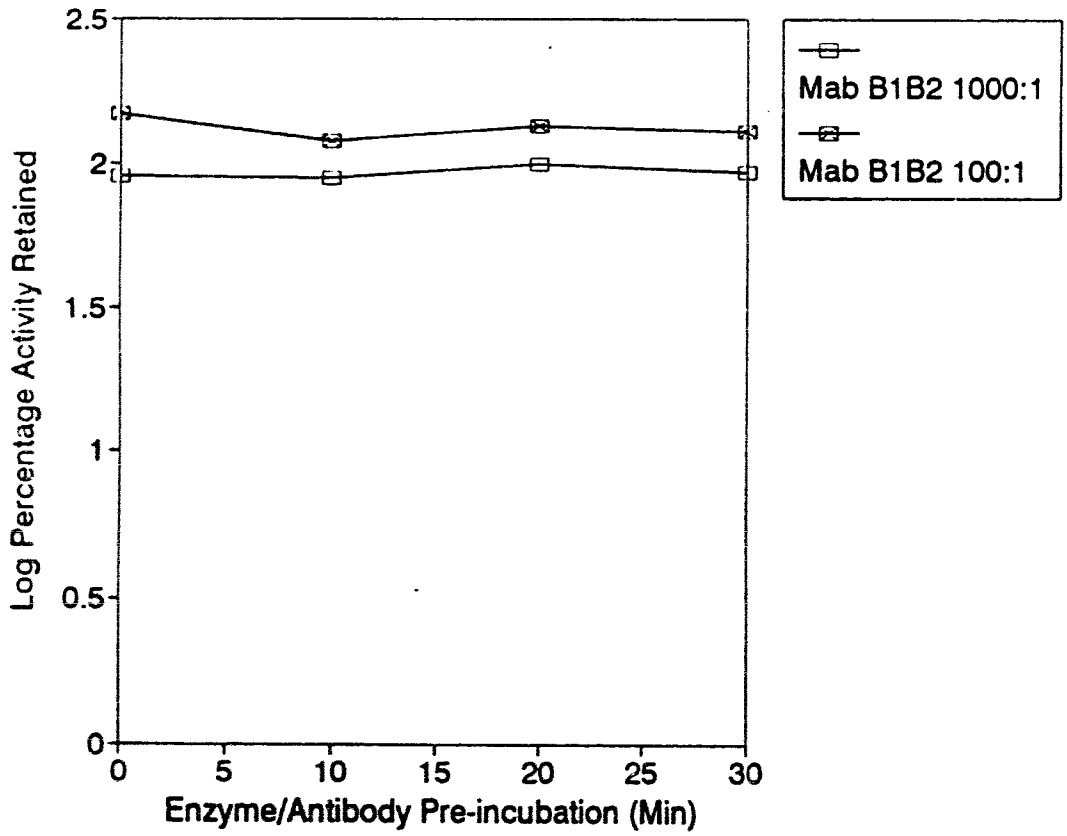
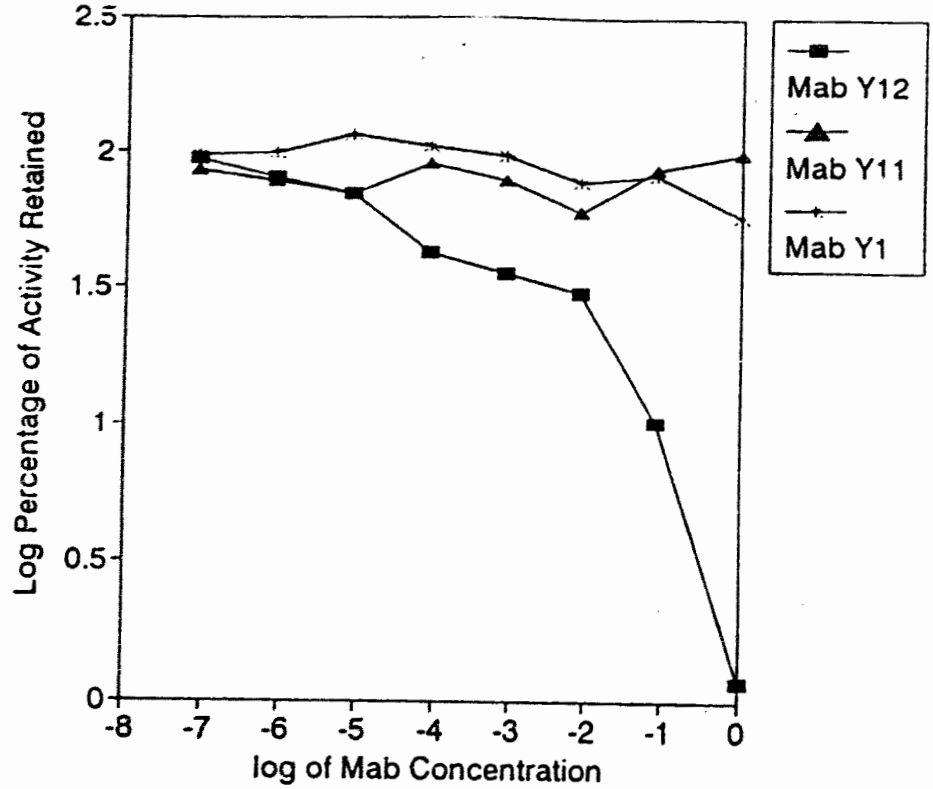

Log Percentage Activity Retained
2.5
2
1.5
1
0.5
0
0
5
10
15
20
25
30
Enzyme/Antibody Pre-incubation (Min)
Mab B1B2 1000:1
Mab B1B2 100:1

Figure 20: Inhibition of Aminoacylation Activity of yIRS at Varying Concentrations of yIRS Monoclonal Antibody. Scaling was based on the activity of yIRS in absence of monoclonal antibody. Serial 10 fold dilutions of monoclonal antibody were made (the original concentration of monoclonal antibody was 1.0 mg/mL). The enzyme was pre-incubated with monoclonal antibody for 15 minutes on ice prior to assay. The assay was performed at 30°C for 5 minutes before quenching in 5% cold TCA. The enzyme concentration used was 10 nM.

Figure 21: Inhibition of Aminoacylation Activity of bIRS at Varying Concentrations of B1B2. Scaling was based on the activity of bIRS in the absence of monoclonal antibody B1B2. Serial 10 fold dilutions of monoclonal antibody were made (the original concentration of monoclonal antibody at 2.0 mg/mL). For the aminoacylation assay, the enzyme-monoclonal antibody mixture was pre-incubated for 15 minutes on ice prior to the assay. The assay conditions were the same as described in figure 19 except crude *E. coli* tRNA was used in the place of crude yeast tRNA. The enzyme concentration used was 30 nM.


2.5
2
1.5
1
0.5
0
Log Percentage of Activity Retained
-8
-7
-6
-5
-4
-3
-2
-1
0
log of Mab Concentration
Mab Y12
Mab Y11
Mab Y1

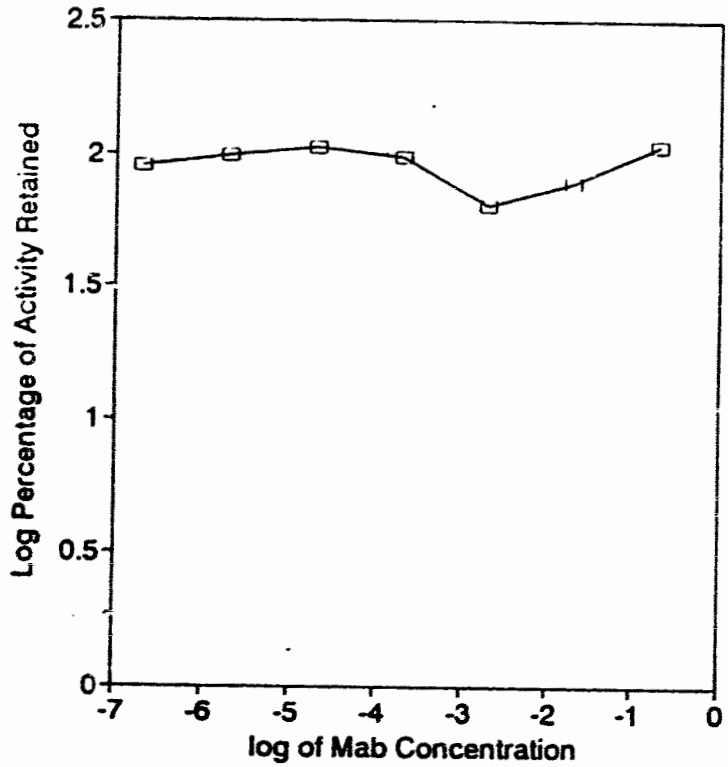

2.5
2
1.5
1
0.5
0
Log Percentage of Activity Retained
-7
-6
-5
-4
-3
-2
-1
0
log of Mab Concentration

Figure 22: Inhibition of IleRS Aminoadenylation Activity by Monoclonal Antibodies. Scaling was based on the aminoadenylation activity of yIRS and bIRS in the absence of monoclonal antibody. An enzyme to monoclonal antibody ratio of 1:10 was used. The enzyme was pre-incubated with the monoclonal antibody for 30 minutes on ice prior to assay. (See section 2.13.3.1 for assay procedure).

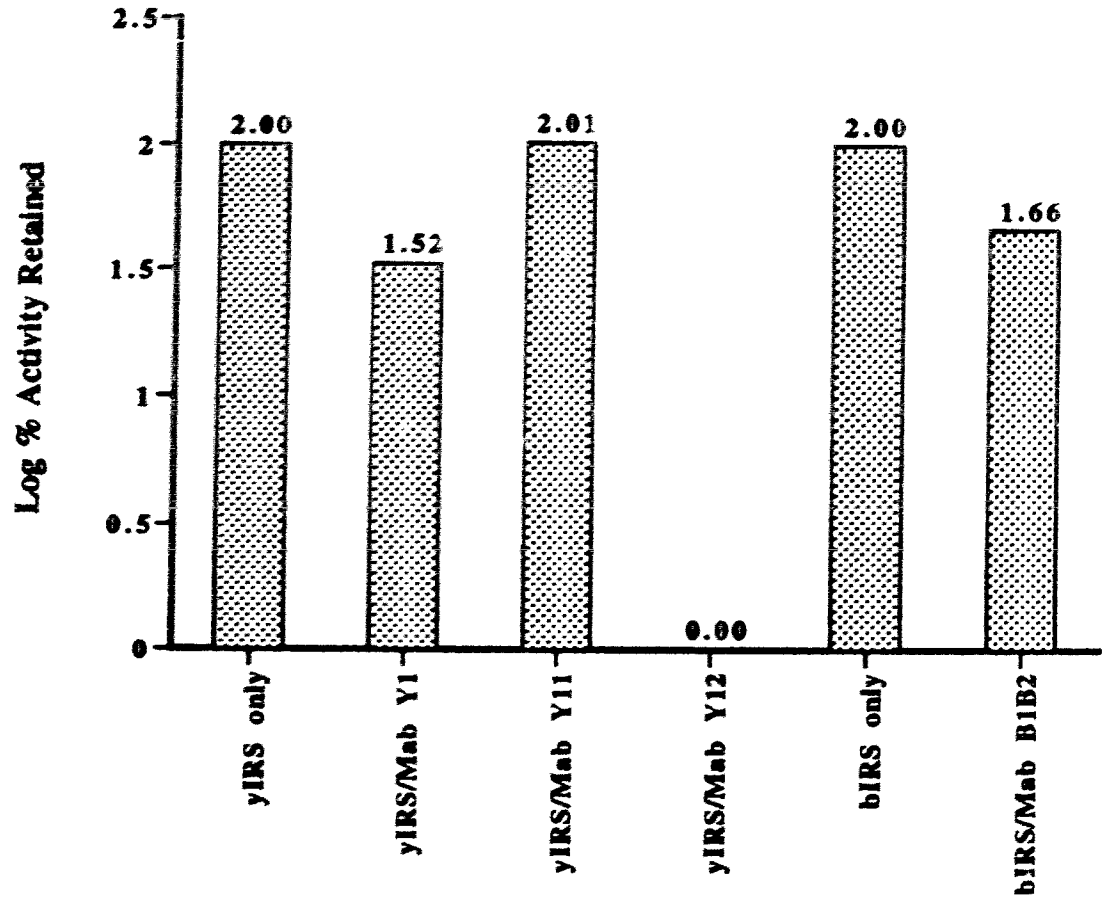
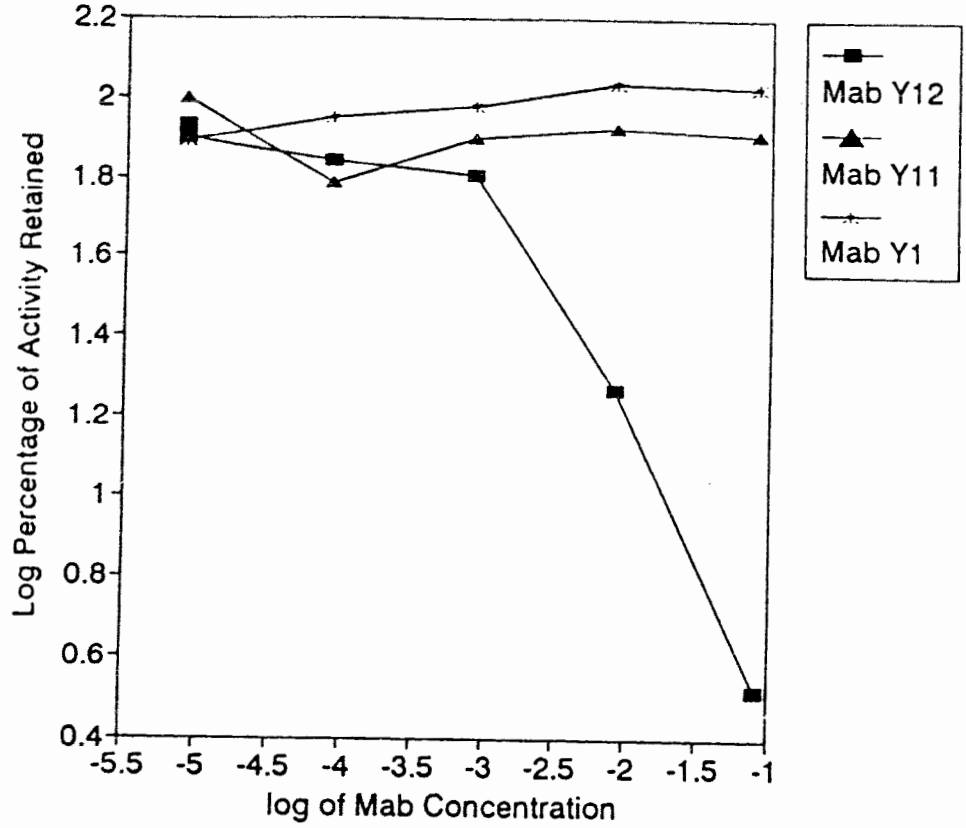
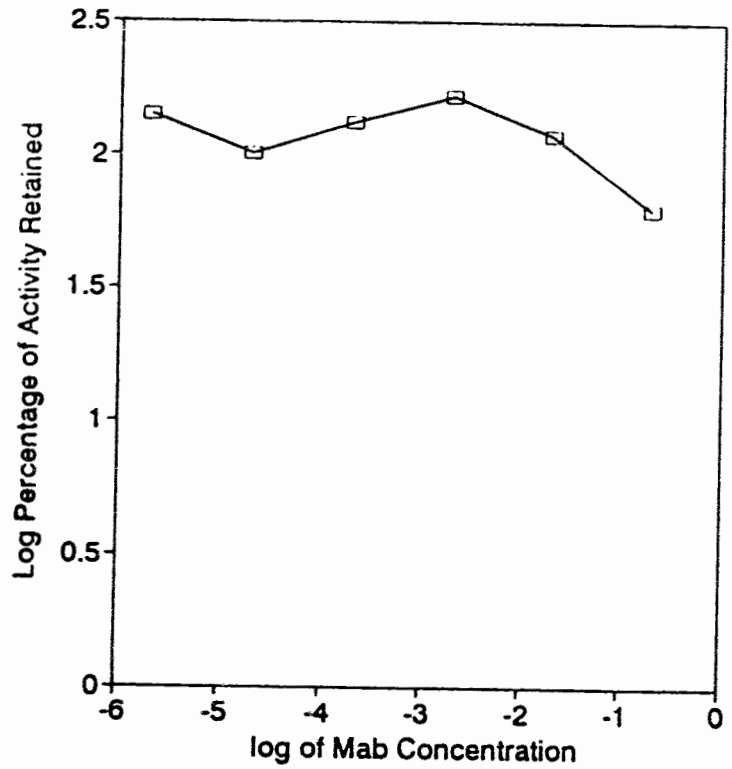

Log % Activity Retained
2.5
2
1.5
1
0.5
0
2.00
1.52
2.01
0.00
2.00
1.66
yIRS only
yIRS/Mab Y1
yIRS/Mab Y11
yIRS/Mab Y12
bIRS only
bIRS/Mab B1B2

Figure 23: Inhibition of Aminoadenylation Activity of yIRS at Varying Concentrations of yIRS Monoclonal Antibodies. Scaling was based on the aminoadenylation activity of yIRS in the absence of monoclonal antibody. As in the aminoacylation assay, serial 10 fold dilutions of monoclonal antibody were made. The enzyme-monoclonal antibody mixture was assayed for aminoadenylation activity at 24°C for 3 minutes. (Please refer to section 2.13.3.2 for general experimental procedure). The concentration of enzyme used was 30 nM.

Figure 24: Inhibition of Aminoadenylation Activity of bIRS at Varying Concentrations of B1B2. Again, the scaling was based on the activity of the enzyme in the absence of monoclonal antibody. For the aminoadenylation assay, the enzyme-monoclonal antibody mixture was also pre-incubated for 15 minutes on ice prior to the assay. Please refer to section 2.13.3.2 for assay conditions. The enzyme concentration used for the aminoadenylation assay was 60 nM.


Log Percentage of Activity Retained
2.2
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
-5.5 -5 -4.5 -4 -3.5 -3 -2.5 -2 -1.5 -1
log of Mab Concentration
Mab Y12
Mab Y11
Mab Y1


Log Percentage of Activity Retained
2.5
2
1.5
1
0.5
0
-6 -5 -4 -3 -2 -1 0
log of Mab Concentration

3.9 ISOLATION AND PURIFICATION OF CHIMERIC IleRS ENZYMES

3.9.1 Purification of the Afl III/Sac II Chimera

A large scale (7.5 L) preparation of *E. coli* strain DH5α harbouring the plasmid pGC7Q was grown in a fermentor as described in section 2.8.4.1. The overexpressed Afl III/Sac II chimeric protein was purified initially by anion-exchange chromatography (see section 2.11.2). The elution profile is shown in Figure 25.

SDS-PAGE and western immunoblot analysis of the peak fractions indicated that the Afl III/Sac II chimera eluted from the Q Sepharose column between 4 and 5.5 hours after the start of the gradient (approximately between 300-500 mM KCl). In addition, western immunoblot analysis showed that the Afl III/Sac II chimera eluted from the column after the *E. coli* host IleRS (see Figures 26 and 27).

Fractions containing the Afl III/Sac II chimera were then pooled and purified on a Hydroxylapatite column (Macro-Prep HA from Bio-Rad) as described in section 2.11.3. Bound protein was eluted from the column with a linear gradient from 10 mM-400 mM potassium phosphate. The elution profile is illustrated in Figure 28. No significant protein peaks were observed during the course of the gradient. A 400 mM phosphate wash of the column after the end of the gradient showed two minor peaks. SDS-PAGE and western immunoblot analysis of various samples taken over the course of the gradient elution and the 400 mM phosphate wash showed that most of the protein eluted near the end of the gradient and at the 400 mM phosphate wash. Western immunoblot analysis also showed that some of the Afl III/Sac II chimera eluted during the initial 10 mM phosphate wash (see Figure 29). This difference in the binding (or lack of binding) of the Afl III/Sac II chimera may be due to a number of reasons. One reason may be that this chimeric enzyme may exist in two different forms, one that does not bind to the column and one that binds well to the column.

Figure 25: Purification of Afl III/Sac II Chimera by Anion-Exchange Chromatography. A crude cell lysate from a 7.5 L culture of *E. coli* DH5α cells expressing the chimera was purified on a Q Sepharose column. Peak fractions were assayed for the presence of the chimera by SDS-PAGE and western immunoblotting (see figures 26 and 27). The Afl III/Sac II Chimera was eluted from the column between 4 - 5.8 hours after the start of the gradient, at a salt concentration of approximately 430-570 mM KCl. Shaded peaks denoted fractions collected.

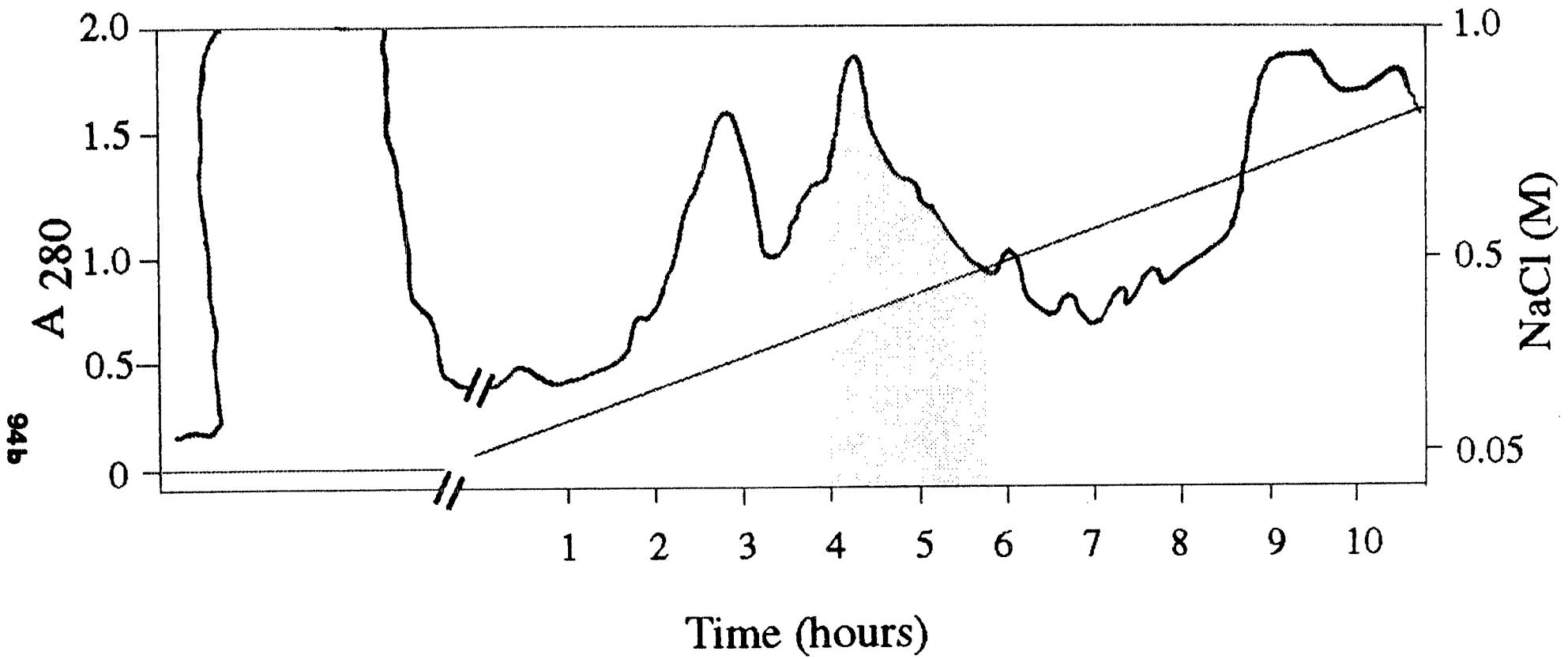
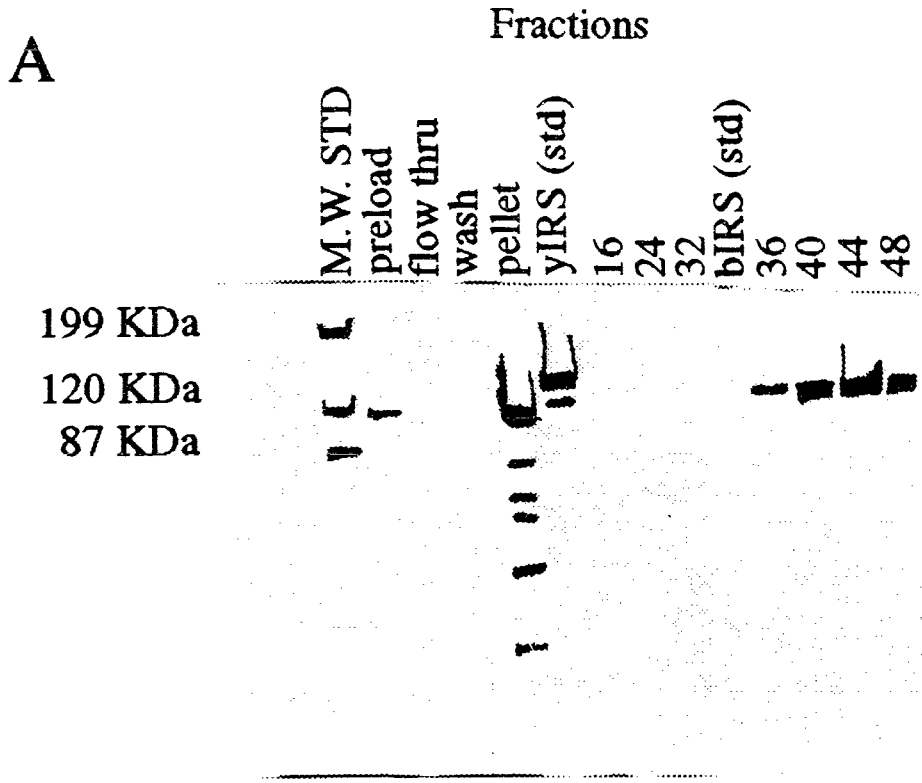

2.0
1.5
1.0
0.5
0
A 280
1.0
0.5
0.05
NaCl (M)
1
2
3
4
5
6
7
8
9
10
Time (hours)

Figure 26: Western Immunoblot Analysis of Peak Fractions Obtained From the Purification of Afl III/Sac II Chimera on the Q Sepharose Column Using Monoclonal Antibody Y12. Immunoblots were probed with monoclonal antibody Y12 as primary antibody. The Afl III/Sac II Chimera was found to elute from the column within the second major peak after the start of the gradient (see figure 25). Fractions containing the chimera were pooled.

A


Fractions
M.W. STD
preload
flow thru
wash
pellet
yIRS (std)
16
24
32
bIRS (std)
36
40
44
48
199 KDa
120 KDa
87 KDa

B

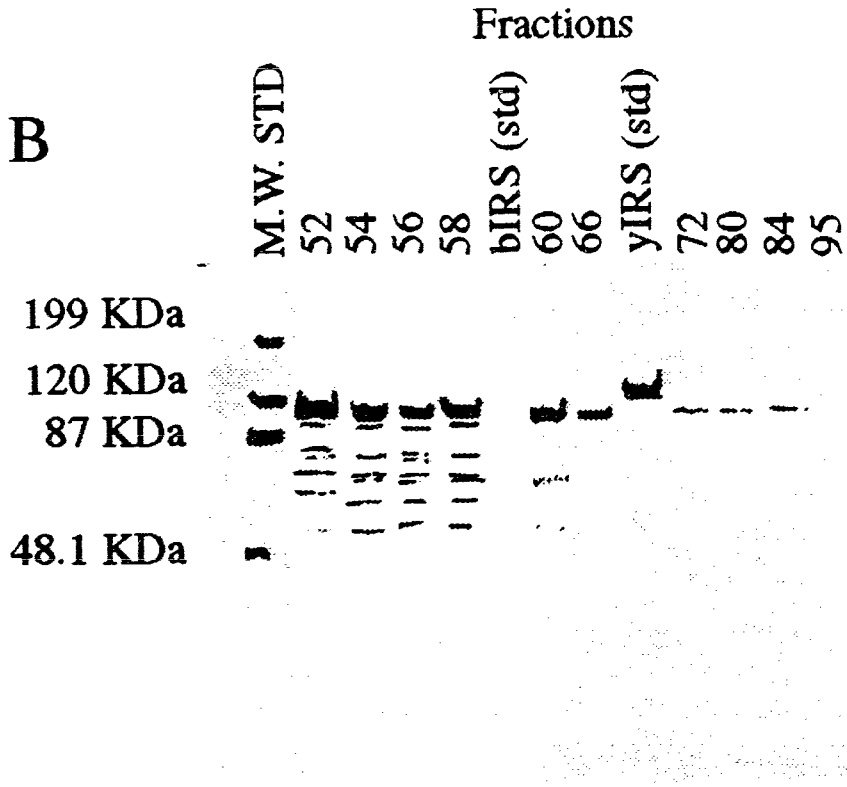
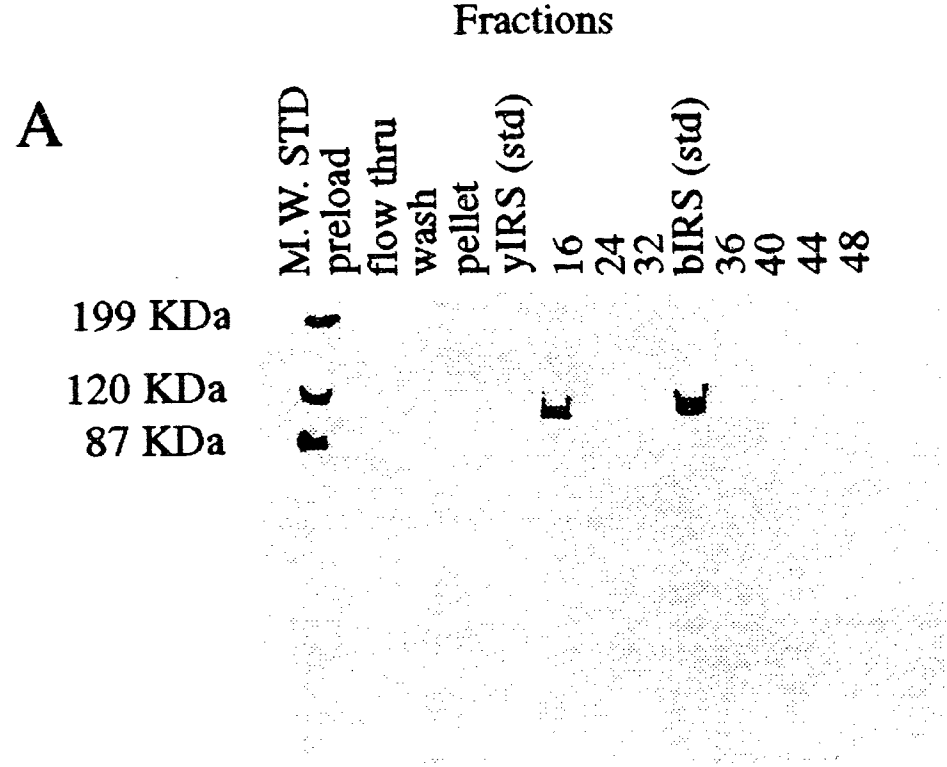

Fractions
M.W. STD
52
54
56
58
bIRS (std)
60
66
yIRS (std)
72
80
84
95
199 KDa
120 KDa
87 KDa
48.1 KDa

Figure 27: Western Immunoblot Analysis of Peak Fractions Obtained From the Purification of Afl III/Sac II Chimera on the Q Sepharose Column Using Monoclonal Antibody B1B2. Immunoblots (figure 26) were stripped and re-probed with monoclonal antibody B1B2 to check for the point of elution of *E. coli* host IleRS. It was discovered that the host IleRS eluted from the column earlier than the Afl II/Sac II Chimera, with approximately 180 mM KCl. Note that in the preload and the pellet samples, the host IleRS does not appear on the western immunoblots. The host IleRS appears on the blot at fraction #16 because it was concentrated in the column. From the blots, there appears to be no significant contamination of the Afl III/Sac II Chimera with host IleRS.


Fractions
A
M.W. STD
preload
flow thru
wash
pellet
yIRS (std)
16
24
32
bIRS (std)
36
40
44
48
199 KDa
120 KDa
87 KDa

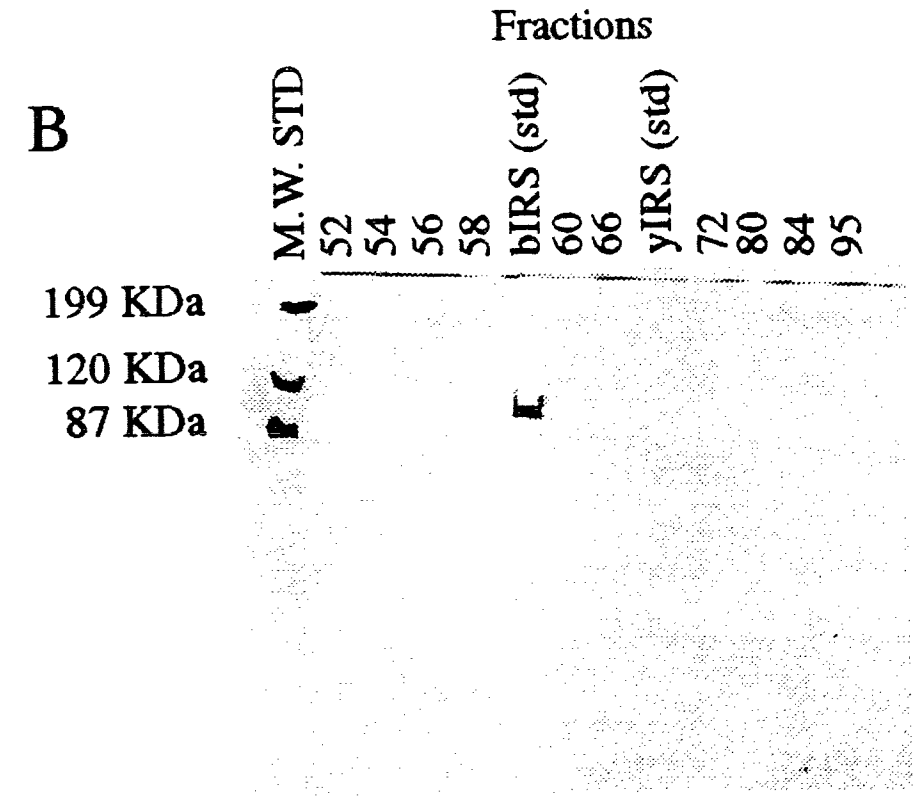

Fractions
B
M.W. STD
52
54
56
58
bIRS (std)
60
66
yIRS (std)
72
80
84
95
199 KDa
120 KDa
87 KDa

Figure 28: Secondary Purification of Afl III/Sac II Chimera by Hydroxylapatite Affinity Chromatography. Fractions from the Q Sepharose column containing the Afl III/Sac II Chimera were pooled and diluted 5 times prior to loading onto a hydroxylapatite column. No major peaks other than the flow through, low phosphate buffer wash and the high phosphate buffer wash were noted. Western immunoblot analysis of these fractions (figure 29), showed that a small amount of the chimera eluted with 400 mM phosphate. Analysis of the flow through and initial low phosphate washed showed that the some of the chimera did not bind to the column. The shaded peaks denote the fractions containing the chimera that bound to the column.

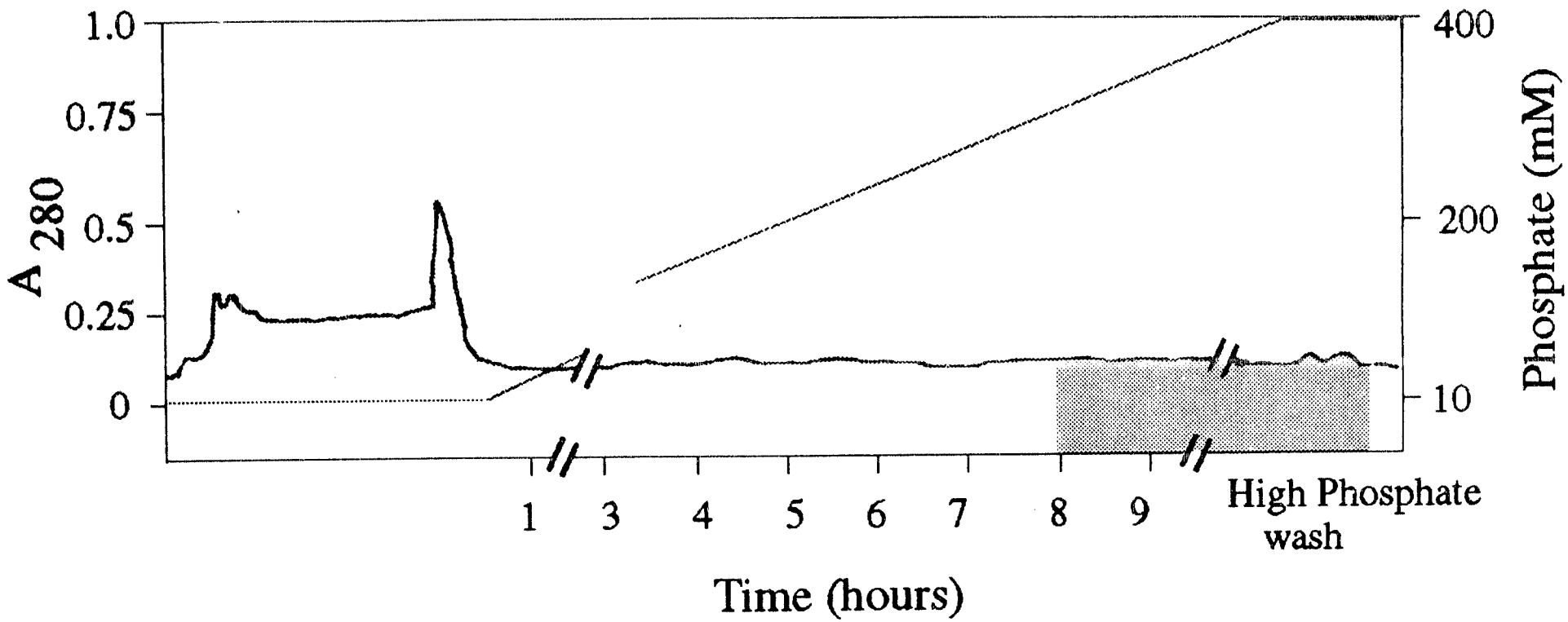
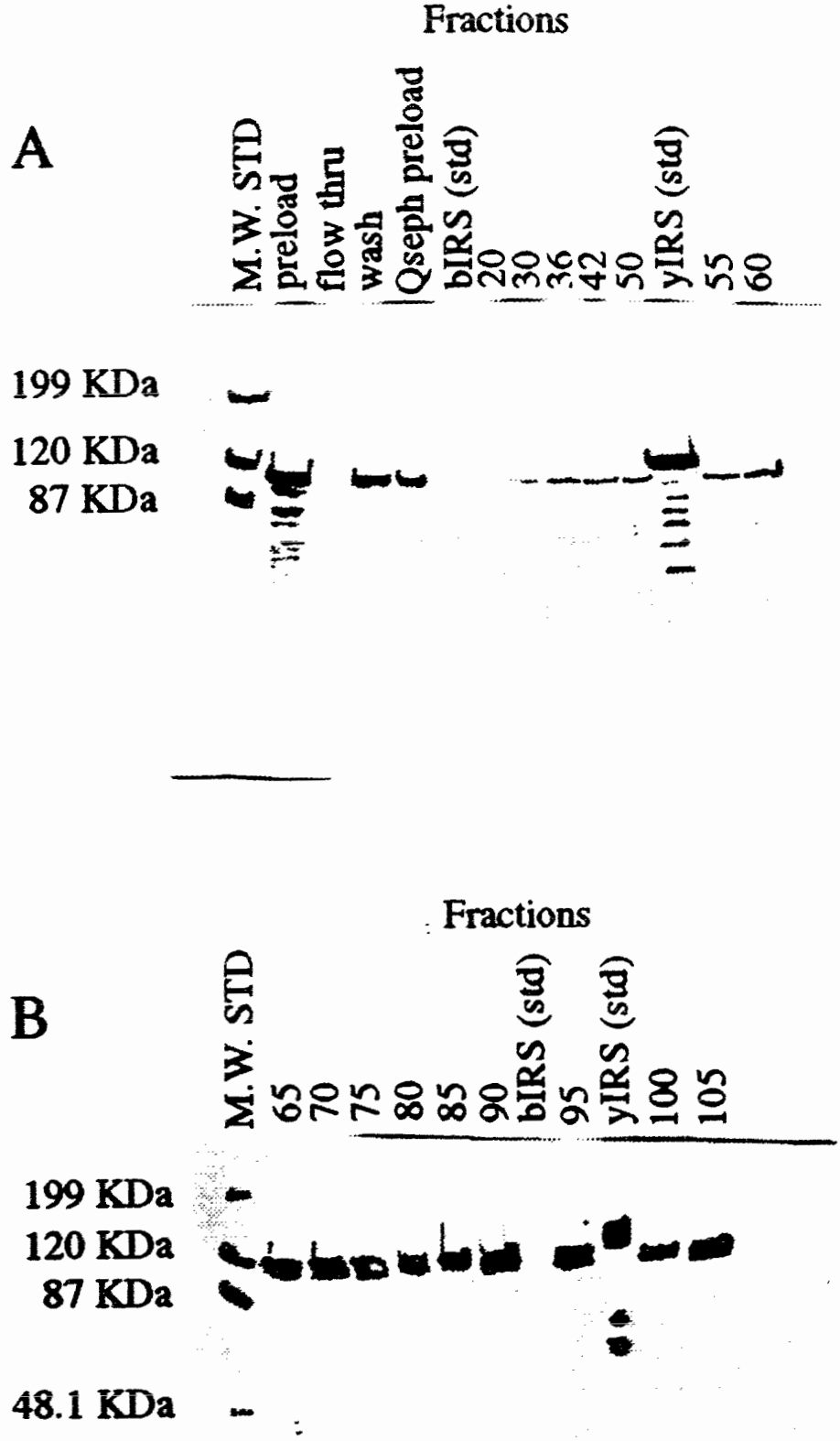

1.0
0.75
0.5
0.25
0
A 280
400
200
10
Phosphate (mM)
1
3
4
5
6
7
8
9
High Phosphate wash
Time (hours)

Figure 29: Western Immunoblot Analysis of Various Fractions From the Hydroxylapatite Purification of the Afl III/Sac II Chimera. Fractions were assayed with the monoclonal antibody Y12. The blots showed that the chimera was eluted from the column over a very broad range, with most of the chimera eluting with 400 mM phosphate.


Fractions
A
M.W. STD
preload
flow thru
wash
Qseph preload
bIRS (std)
20
30
36
42
50
yIRS (std)
55
60
199 KDa
120 KDa
87 KDa
Fractions
B
M.W. STD
65
70
75
80
85
90
bIRS (std)
95
yIRS (std)
100
105
199 KDa
120 KDa
87 KDa
48.1 KDa

3.9.2 Purification of the Nsi I Chimera

A 7.5 L growth of E. coli strain DH5α containing the plasmid pGC6Q was carried out and the overexpressed Nsi I chimera was purified, firstly, by anion-exchange chromatography (see section 2.11.2). The elution profile of the Nsi I chimera is illustrated in Figure 30. SDS-PAGE and western immunoblot analysis showed that the Nsi I chimera eluted from the Q Sepharose column over a wider range than the Afl III/Sac II chimera, between 3.5 and 5.5 hours after the start of the gradient (approximately between 200 mM and 500 mM KCl). The Nsi I Chimera eluted earlier from the column, relative to the Afl III/Sac II chimera, yet later that the E. coli host's IleRS as shown by the western immunoblots (see Figures 30 and 31). The double bands observed in the western immunoblots may be due to aggregation of the chimera with itself or other bands as the lower band observed corresponds to the estimated M_r of the monomeric Nsi I chimera.

The fractions containing the Nsi I chimera were pooled and loaded onto a hydroxylapatite column for the secondary purification step. The elution profile is illustrated in Figure 33. Unlike the purification of the Afl III/Sac II Chimera on the hydroxylapatite column, a number of protein peaks were eluted over the course of the 10 mM-400 mM potassium phosphate gradient. After the end of the gradient, a 400 mM potassium phosphate wash of the column was performed and a single protein peak was eluted. Analysis of various fractions taken along the gradient and the 400 mM phosphate wash by SDS-PAGE and western immunoblotting (Figures 34) showed that the Nsi I chimera was eluted from the column over a very wide range, with most of the chimera eluted in the last peak, during the 400 mM phosphate wash. Unlike the Afl III/Sac II chimera, little or no Nsi I chimera was eluted during the 10 mM phosphate wash.

Figure 30: Purification of Nsi I Chimera by Anion-Exchange Chromatography. A crude cell-free lysate from a 7.5 L culture of *E. coli* strain DH5α expressing the Nsi I Chimera was purified using a Q Sepharose column. The point of elution of the Nsi I Chimera from the column was identified by a combination of SDS-PAGE and Western Immunoblot analyses (figures 31 & 32). The Nsi I chimera eluted over a wider range than the Afl III/Sac II Chimera, at a salt concentration of 350-650 mM KCl. The shaded peaks denote the fractions collected.

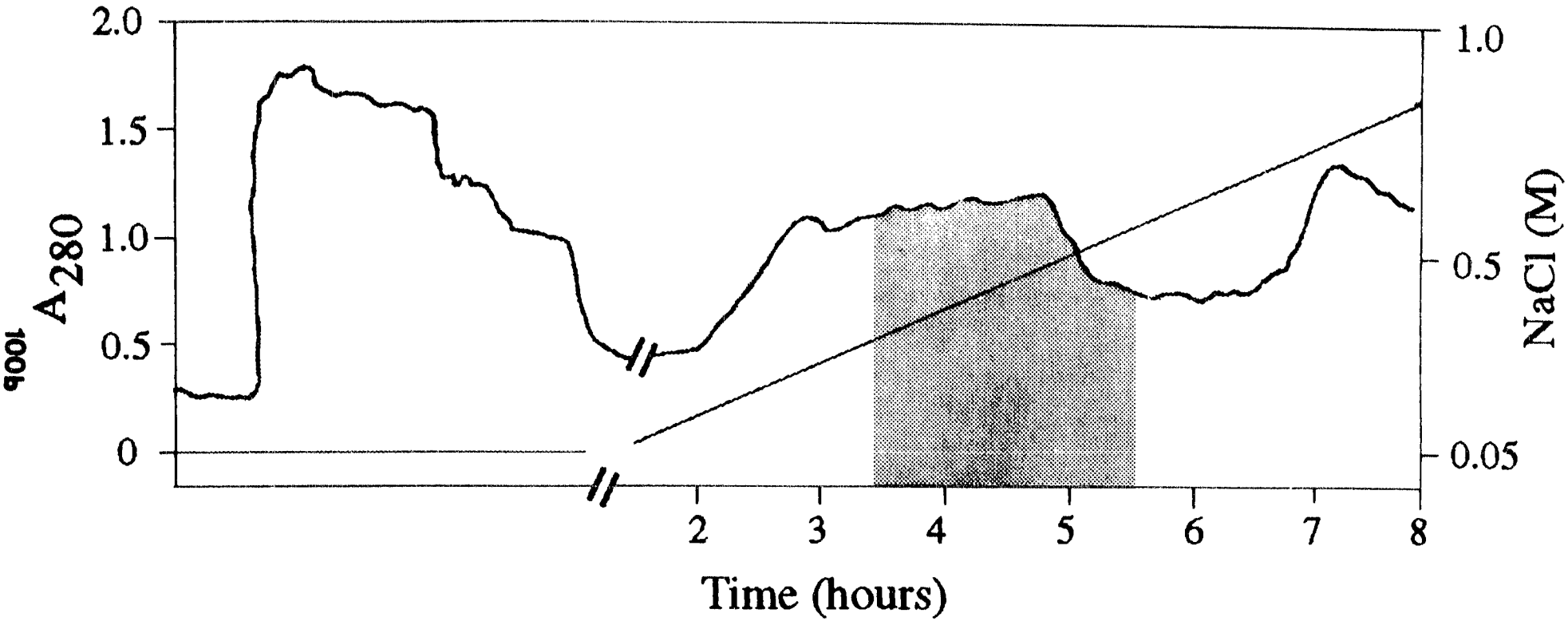
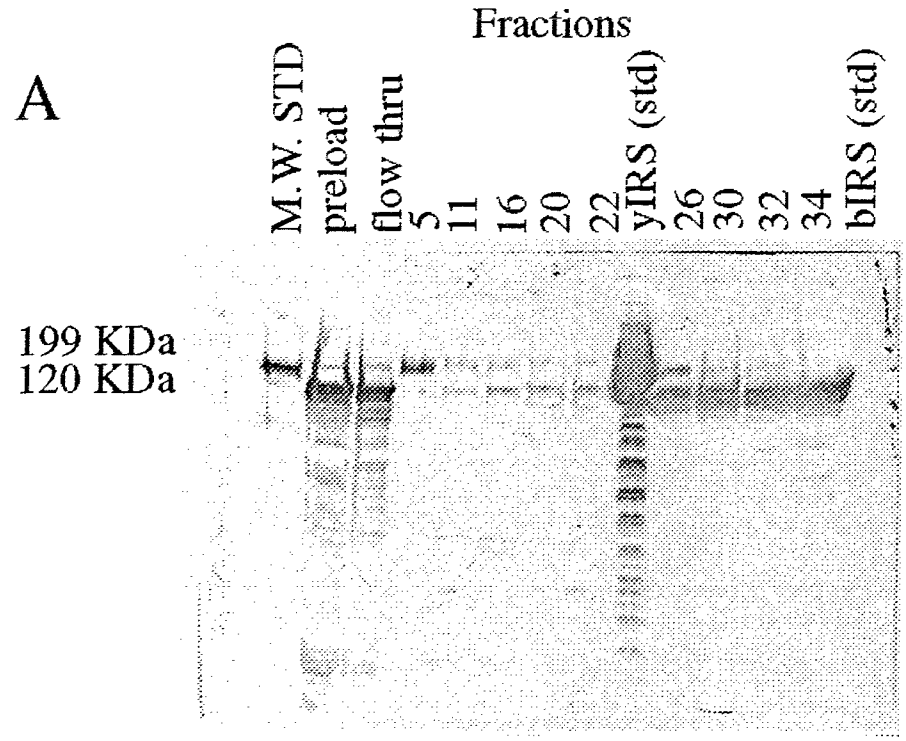

2.0
1.5
1.0
0.5
0
A280
1.0
0.5
0.05
NaCl (M)
2
3
4
5
6
7
8
Time (hours)

Figure 31: Western Immunoblot Analysis of Peak Fractions From the Purification of Nsi I Chimera by Anion-Exchange Chromatography Using Monoclonal Antibody Y12. Peak fractions were probed with monoclonal antibody Y12. The Nsi I Chimera was found to elute over a very wide range from the Q Sepharose column. Most of the chimera was eluted at about 4 hours after the start of the gradient. The unusual double banding pattern observed in the later fractions may to due to aggregation of the chimera. The lower band major band (at approximately 110 KDa) corresponds to the M_r-value of the Nsi I chimera.

A


Fractions
M.W. STD
preload
flow thru
5
11
16
20
22
yIRS (std)
26
30
32
34
bIRS (std)
199 KDa
120 KDa

B

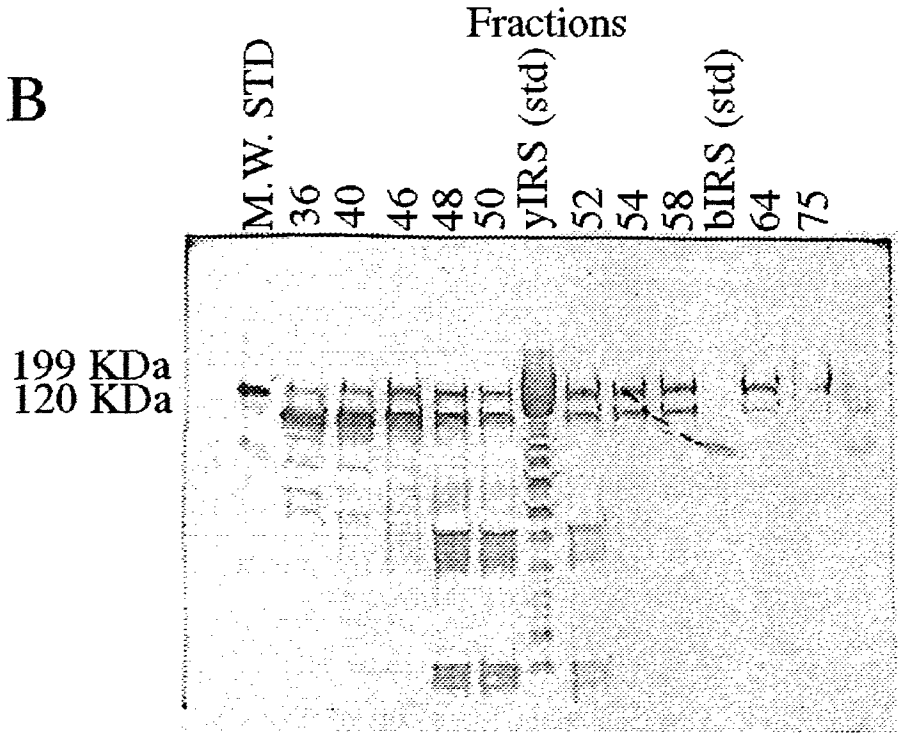
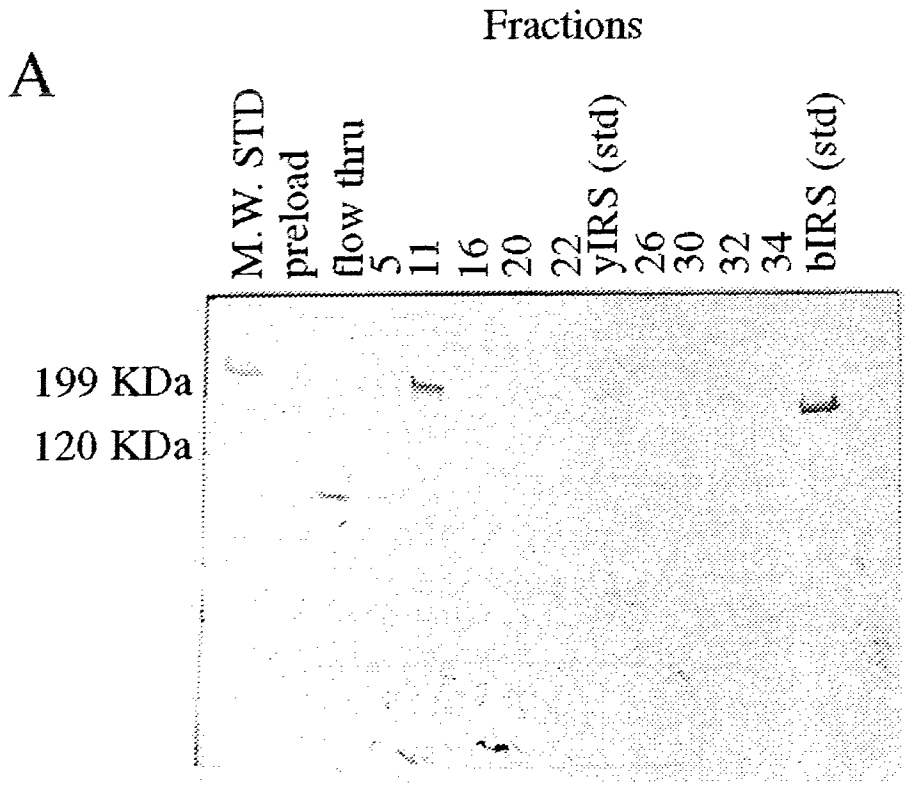

Fractions
M.W. STD
36
40
46
48
50
yIRS (std)
52
54
58
bIRS (std)
64
75
199 KDa
120 KDa

Figure 32: Western Immunoblot Analysis of Peak Fractions From the Purification of Nsi I Chimera by Anion-Exchange Chromatography Using the Monoclonal Antibody B1B2. The blots (figure 31) were stripped and re-probed with monoclonal antibody B1B2 to determine the point of elution of the host IleRS. As with the Afl III/Sac II Chimera purification, the host IleRS eluted earlier in the gradient relative the Nsi I Chimera. The host IleRS eluted from the column at a salt concentration of approximately 150 mM KCl and was not found in any of the later fractions that contained the Nsi I Chimera.

A


Fractions
M.W. STD
preload
flow thru
5
11
16
20
22
yIRS (std)
26
30
32
34
bIRS (std)
199 KDa
120 KDa

B

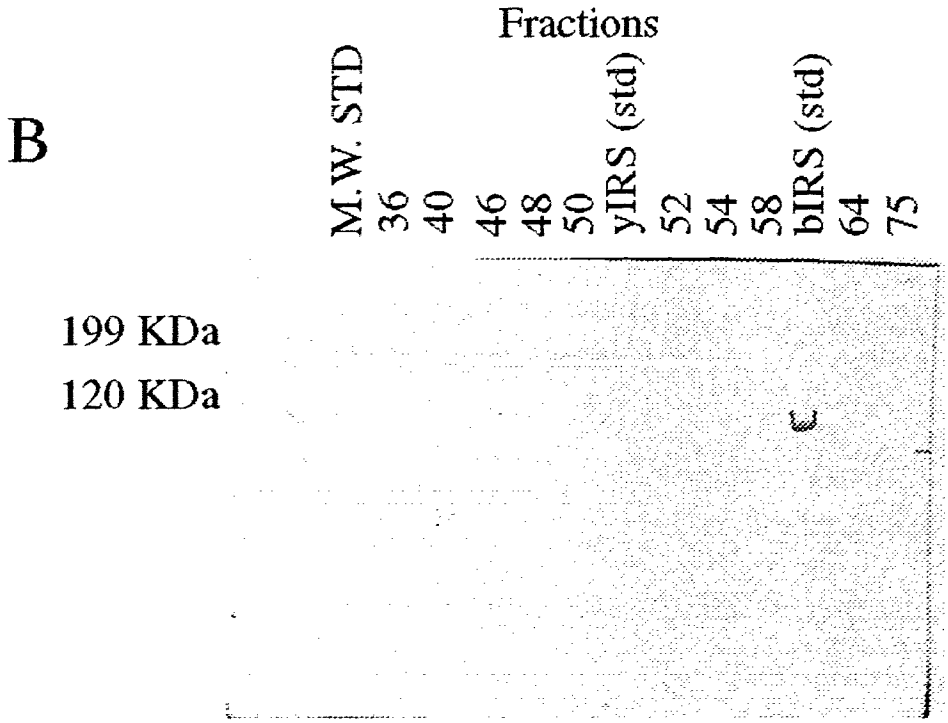

Fractions
M.W. STD
36
40
46
48
50
yIRS (std)
52
54
58
bIRS (std)
64
75
199 KDa
120 KDa

Figure 33: Secondary Purification of Nsi I Chimera By Hydroxylapatite Affinity Chromatography. Fractions from the Q Sepharose column containing Nsi I Chimera were pooled and diluted 5 fold prior to loading onto a hydroxylapatite column. Peak fractions were assayed for the presence of chimera by SDS-PAGE and western immunoblotting (figure 34). This chimera was found to stick very well to the column, eluting only with 400 mM phosphate. The shaded peaks indicate the point of elution of the Nsi I Chimera.

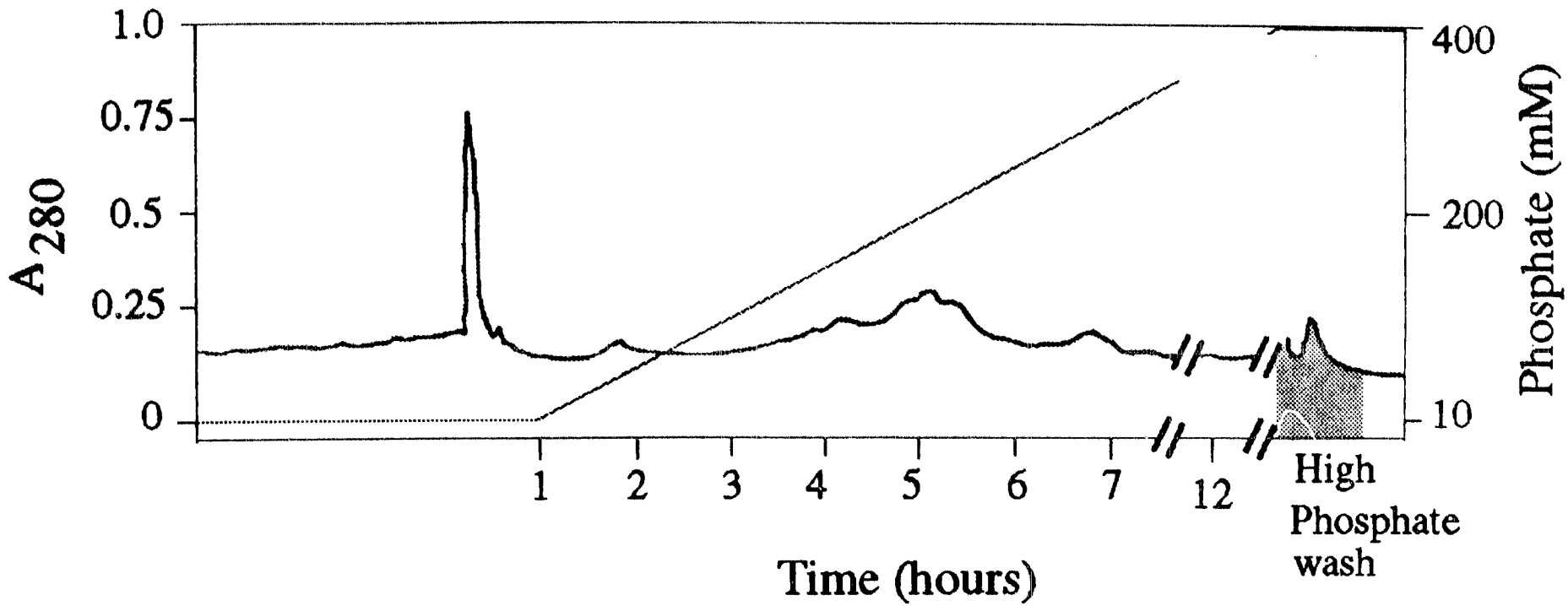
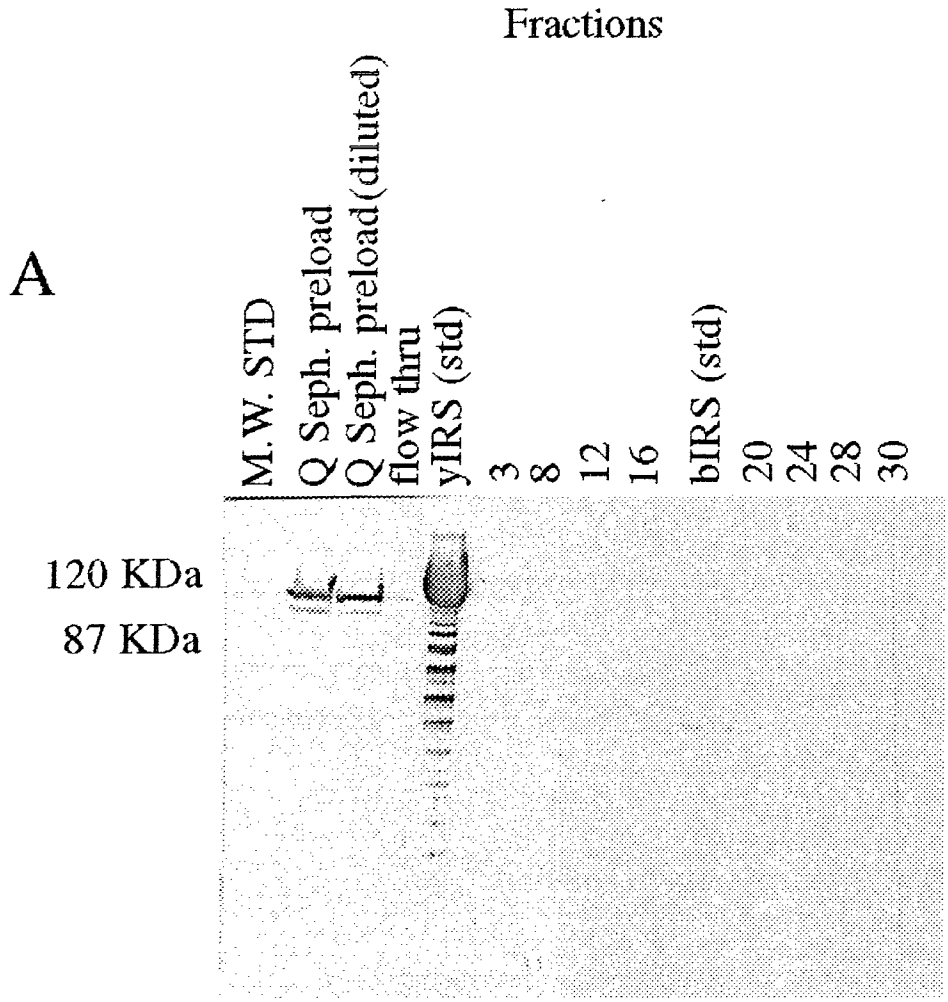
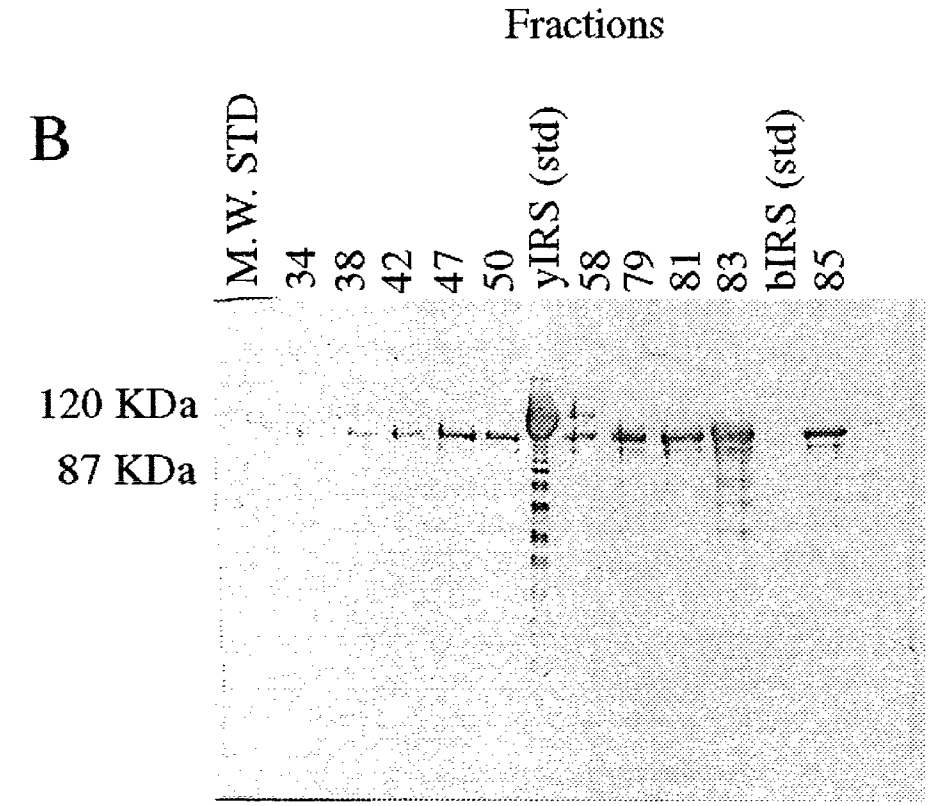

1.0
0.75
0.5
0.25
0
A_{280}
400
200
10
Phosphate (mM)
1
2
3
4
5
6
7
12
High
Phosphate
wash
Time (hours)

Figure 34: Western Immunoblot Analysis of the Peak Fractions From the Hydroxylapatite Column. Peak fractions were probed with the monoclonal antibody Y12. Blot B confirms the presence of Nsi I Chimera in the 400 mM phosphate wash fractions.


Fractions
A
M.W. STD
Q Seph. preload
Q Seph. preload(diluted)
flow thru
yIRS (std)
3
8
12
16
bIRS (std)
20
24
28
30
120 KDa
87 KDa


Fractions
B
M.W. STD
34
38
42
47
50
yIRS (std)
58
79
81
83
bIRS (std)
85
120 KDa
87 KDa

3.10 AMINOADENYLATION ASSAYS OF PURIFIED CHIMERAS

Previous aminoacylation assays of the chimeric enzymes have shown that none of the chimeras seem to the have the ability to aminoacylate tRNA. Purified samples of the Nsi I and Afl III/Sac II chimeras (Figure 35) were assayed for aminoadenylation activity in the absence and presence of pseudomonic acid A as described in section 2.13.1. In theory, these two chimeric enzymes should be resistant to the inhibitory affect of pseudomonic acid A as these two chimeric enzymes contain the N-terminal domain of yIRS. Thus, this assay is able to distinguish the activity observed from any bIRS contamination that might have occurred during the course of purification. The results of the assay is shown in Figure 36. In the absence and presence of pseudomonic acid A, the Nsi I chimera appears to have aminoadenylation activity whereas the Afl III/Sac II does not have any activity in the absence or presence of pseudomonic acid A. Successive assays gave similar results. The similar magnitude of activity observed in the Nsi I chimera in the absence and presence of pseudomonic acid A also indicates that the enzyme is free from bIRS contamination. The lack of activity observed for the Afl III/Sac II in the absence of pseudomonic acid A indicates that this particular chimera is not only inactive but also free from contaminating bIRS.

Figure 35: SDS-PAGE Analysis of Hydroxylapatite Purified Chimeras. Pooled and concentrated samples of hydroxylapatite purified chimeras were separated on a 12% polyacrylamide gel. The samples obtained were relatively pure and appear free from contaminating proteins. Bands correspond to the predicted molecular weights of the chimeras.

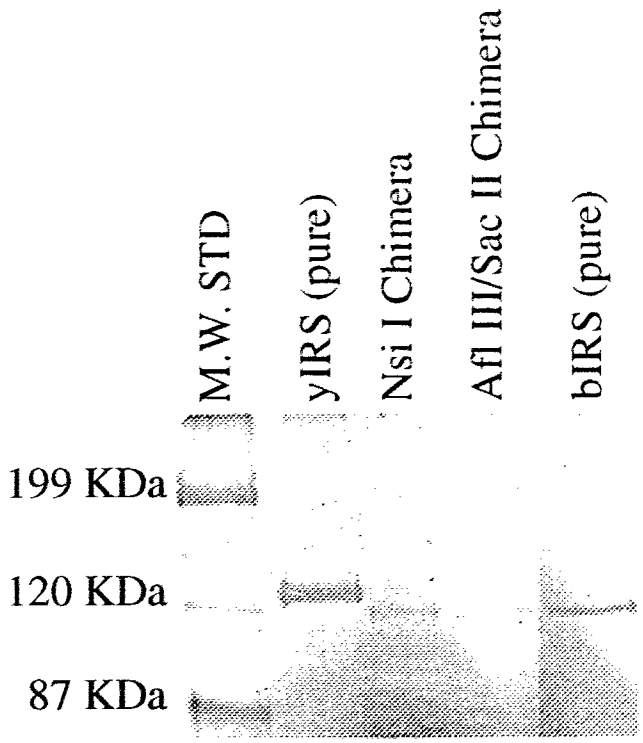
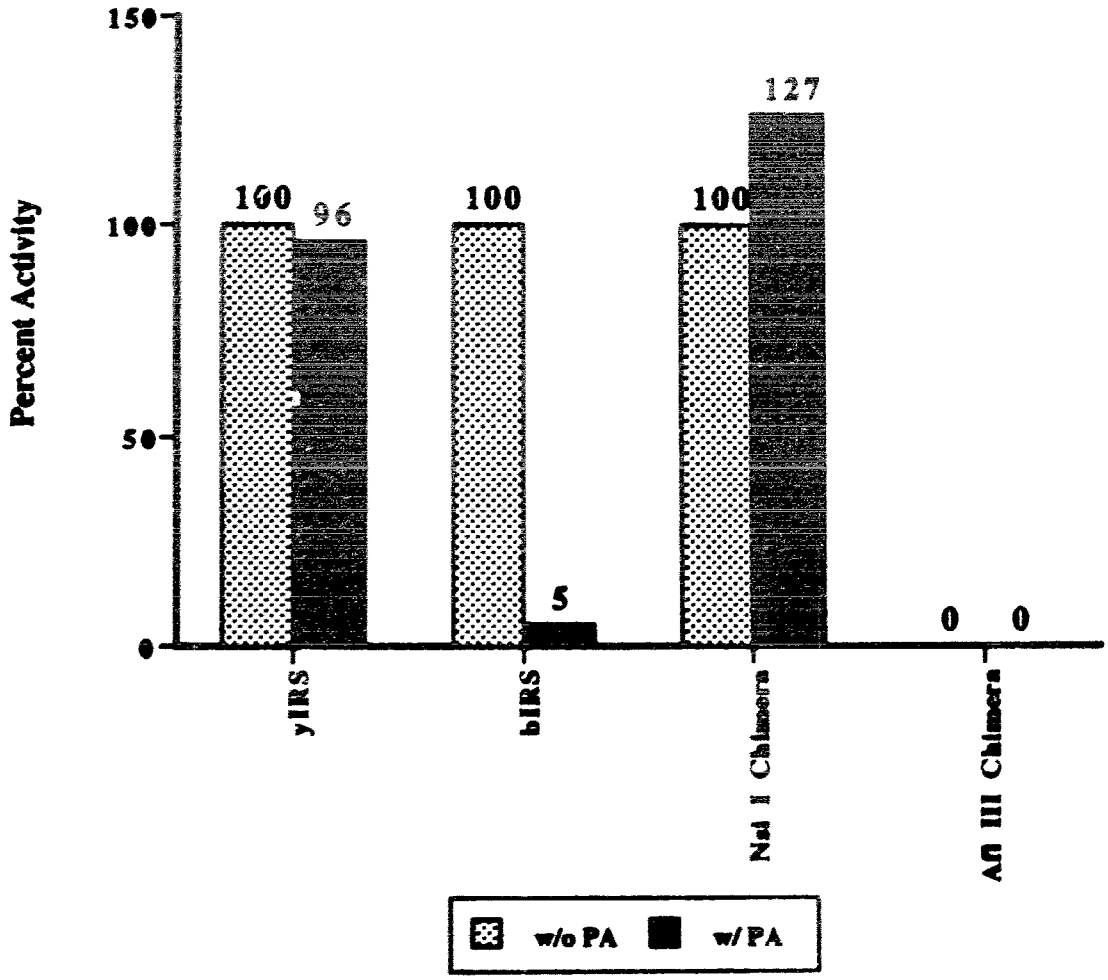

M.W. STD
yIRS (pure)
Nsi I Chimera
Afl III/Sac II Chimera
bIRS (pure)
199 KDa
120 KDa
87 KDa

Figure 36: Aminoadenylation Activity of Purified Chimeric Enzyme in the Absence and Presence of Pseudomonic Acid A. PA denotes Pseudomonic Acid A. Purified chimeric enzymes Afl III/Sac II and Nsi I were assayed for aminoadenylation activity in the absence and presence of pseudomonic acid A along with yIRS and bIRS as controls. Scaling was based on comparing the activity of the enzyme in the absence of pseudomonic acid A to the activity of the enzyme in the presence of 1 μM (final reaction concentration) pseudomonic acid A. The activity of the enzyme in the absence of pseudomonic acid A designated as 100% activity.


150
100
50
0
Percent Activity
100
96
100
5
100
127
0
0
yIRS
bIRS
Nsi I Chimera
Afl III Chimera
w/o PA
w/ PA

4. DISCUSSION:

The aminoacyl-tRNA synthetases are enzymes which are responsible for the ligation of amino acids to their cognate tRNAs. There are 20 aminoacyl-tRNA synthetases, one for each of the 20 natural amino acids. Some amino acids, such as serine, can have as many as 6 cognate tRNAs. Therefore, each aminoacyl-tRNA synthetase must be able to distinguish its cognate tRNAs from other tRNAs in addition to recognising its own amino acid substrate.

The isoleucyl-tRNA synthetase was selected as the enzyme of study because of this synthetase's ability to accept various substrates and because of its editing function (37, 41, 78, 79). *In vivo*, IleRS has been shown to bind valine at its amino acid binding site because of valine's structural similarity to isoleucine (30). The frequency which valine was found to be mischarged to $tRNA^{ile}$ in rabbit reticulocyte globin was 1/3000 (44). The natural editing function of IleRS hydrolyses either the incorrectly aminoadenylated amino acid (step 1 of the reaction) or the incorrectly aminoacylated tRNA (step 2 of the reaction) before it is released from the enzyme. In the absence of this editing function, the misincorporation of valine for isoleucine was theoretically calculated to be 1/16 (55). A study of the discrimination factors involved in the acceptance/rejection of amino acids in the amino acid binding site of IleRS was conducted by Cramer *et al.* (18). The relation between these discrimination factors, which are involved in the editing function of IleRS, to hydrophobic interaction between the amino acid and the amino acid binding site of IleRS was calculated and it was found that the difference in the ΔG value between the substrate amino acid, isoleucine and the non-substrate amino acid, valine was 3 KJ for the first discrimination factor and 11 KJ for the second discrimination factor (see reference 18 for more details). Cramer *et al.* also calculated an overall discrimination factor (D) for all the amino acids by IleRS and it was calculated that valine had the highest D value. According to Cramer *et al.*, this high D value cannot be accounted for by hydrophobic interaction between the amino acid and IleRS alone, thereby suggesting that other unknown factors besides hydrophobicity also play a role in the discrimination of amino acids by IleRS (18).

Our laboratory is also studying the editing and misincorporation as a means to introduce novel amino acids into proteins (60) and we are interested to know where the editing site is located. By disabling the editing function of IleRS we could promote the binding of novel amino acids to this mutant IleRS and hopefully, catalyse the aminoacylation of a novel amino acid to tRNA. To develop this 21st aminoacyl-tRNA synthetase, which would catalyse the aminoacylation of novel amino acids to tRNA, a thorough understanding of IleRS's editing site(s), binding sites and catalytic site is required.

Previous studies have shown that the N-terminal nucleotide-binding domain of Class I aaRSs contains the catalytic site (21, 28, 43, 53). C-terminal deletions of MetRS and TyrRS of 200 amino acids and 100 amino acids, respectively, do not affect the aminoadenylation activity of these two class I synthetases, suggesting that the N-terminal nucleotide-binding domain is functionally independent of the C-terminal domain (5, 26, 87). Presently, the three-dimensional structure of IleRS is not known. Thus, comparative sequence analysis of bIRS and yIRS with MetRS, whose three-dimensional structure has been solved, was used to as a guide in the design of IleRS mutants. Although yIRS and bIRS catalyse the same reaction and are similar in structure, their specificities towards tRNA and various IleRS inhibitors are different. By creating chimeras composed of portions of the yIRS and bIRS enzymes, it was hoped that we could identify the catalytically important segments responsible for aminoacylation and aminoadenylation (see Figure 9). C-terminal deletion proteins of the yIRS enzyme were created to find the minimal protein sequences required for aminoacylation and aminoadenylation activity (see Figure 8).

In order to characterise the structural elements involved in isoleucyl-tRNA synthetase substrate recognition, binding and editing, the C-terminal deletion mutants of yIRS and the chimeric IleRS variants were first generated in sufficient quantities for large scale purification (see Figure 10). Subsequent *in vivo* and *in vitro* assays were performed to determine whether the variants were capable of aminoacylation and/or aminoadenylation activity. Lastly, a tertiary

purification technique for the purification of IleRS variants was developed using monoclonal antibodies specific for wild-type yIRS. These monoclonal antibodies were characterised and epitope binding sites on the primary sequence of IleRS were mapped (see Figure 15). In addition, the inhibitory effect of the monoclonal antibodies on IleRS activity was studied.

4.1 EXPRESSION OF THE C-TERMINAL DELETIONS AND CHIMERAS OF ISOLEUCYL-tRNA SYNTHETASE

The heterologous expression of yeast IleRS in *E. coli* was described previously (61). The same system was used to express the IleRS variant genes in an *E. coli* host strain using three different *E. coli* expression vectors. Unfortunately, the expression and purification of the recombinant IleRS proteins could not be followed by conventional aminoacylation assays because the variant enzymes potentially have altered substrate specificity or they may be completely inactive. Thus, the criterion for the overexpression of the recombinant variants was the appearance of a protein of the anticipated molecular weight on SDS-PAGE gels and western immunoblots (see Figures 10 and 11).

Three different *E. coli* expression systems were used to produce the various recombinant IleRS mutants. The N-terminal yIRS/C-terminal bIRS chimeras (Hpa I/Hpa I and Afl III/Sac II Chimeras) were expressed in constructs pKR5 and pKR7 (60), respectively, using the same expression system as the pKR4 yIRS expression vector (61). The N-terminal bIRS/C-terminal yIRS chimeras (Bgl II/Pvu II and Stu I/Cla I Chimeras) were expressed as plasmids pKR8 and pKR9 (60), respectively, using the regulatory sequences of the bIRS expression vector, pMT521 (88). The five yIRS C-terminal deletion genes, the Nsi I chimeric gene and the Afl III/Sac II chimeric gene were expressed using the pKR15Q expression plasmid (60) (constructs pGC1Q, pGC2Q, pGC3Q, pGC4Q, pGC5Q, pGC6Q and pGC7Q, respectively). Western immunoblot analysis showed that all the variant IleRS gene constructs produced recombinant protein of the

predicted molecular weight. The blots also showed that the pKR15Q expression system produced higher levels of recombinant protein in comparison to the other two expression vectors. The difference in the level of protein expression can be attributed to a number of factors. The expression vector, pKR15Q, is a high copy number plasmid that contains a lac I^q gene which controls the "ptac" promoter (see material and methods section 2.4.1.7). Therefore, this plasmid does not require a special host strain to regulate the expression of the recombinant proteins. The plasmids, pKR4, pKR5 and pKR7, on the other hand, are lower copy number plasmids which require a specialised *E. coli* host strain with a lac I^q gene to regulate the "tac" promoter. The expression of the Bgl II/Pvu II and Stu I/Cla I chimeras in constructs pKR8 and pKR9, respectively, is regulated by the *E. coli* promoter sequence of the bIRS gene (88). As such, these two chimeric constructs do not require a special *E. coli* host strain but the number of plasmids per cell is lower when compared to the pKR15Q expression vector. In summary, it appeared that the pKR15Q expression system yielded the highest level of recombinant protein expression due to the high copy number of this plasmid.

A comparison of the level of protein expression among the IRS constructs under the control of the same expression vector was made. Among the C-terminal deletion mutants, the construct pGC3Q, expressing the yIRS Eco RI deletion mutant, consistently showed a lower level of recombinant protein expression when compared with the other C-terminal deletion constructs (see Figure 10). The N-terminal yIRS/C-terminal bIRS chimeric constructs also showed a different level of recombinant protein expression between pKR8 and pKR9, where the Bgl II/Pvu II chimera (pKR8) was expressed at a consistently lower level than the Stu I/Cla I chimera (see Figure 11). The variance in the level of protein expression within a given expression system may be due to some of the recombinant proteins being improperly folded *in vivo*. Therefore, they are more rapidly turned over.

4.2 AMINOACYLATION ACTIVITY OF THE IleRS VARIANTS

In vivo and *in vitro* assays were performed to determine whether any of the mutant IleRS variants have the ability to aminoacylate tRNA. Both *in vivo* (Figure 12) and *in vitro* (Figure 13) assays were conducted in the presence of pseudomonic acid A, a competitive inhibitor of *E. coli* IleRS. The inhibitor was added to the assays to reduce the activity contributed by endogenous *E. coli* IleRS. Pseudomonic acid A is believed to be an analogue of isoleucyladenylate (90) and is believed to inhibit bIRS by blocking the amino acid binding site (37). Previous studies showed that pseudomonic acid A has a very weak inhibitory effect towards crude aaRSs preparations from rat liver (37). A more recent study by Racher *et al.* showed that the inhibitory effect of pseudomonic acid A on bIRS (K_i=2.5X10^{-9}) is four orders of magnitude greater than that of yIRS (K_i=1.5X10^{-5}) (61). Since it is believed that the N-terminal domain of IleRS contains the catalytic and isoleucine binding sites (7, 64, 68), we hypothesised that some of the chimeric IleRS variants, containing the N-terminal portion of bIRS, may be sensitive to this antibiotic. These chimeras include the Stu I/Cla I Chimera, the Bgl II/Pvu II Chimera and the Hpa I/Hpa I Chimera. Assuming that the N-terminal nucleotide binding fold remains intact in these chimeric enzymes, the Stu I/Cla I Chimera, which contains the entire N-terminal domain of bIRS, is predicted to be sensitive to pseudomonic acid A (see Figure 9). The Bgl II/Pvu II and Hpa I/ Hpa I Chimeras (see Figure 9) each have a predicted nucleotide-binding fold comprised of both yIRS and bIRS enzymes. The residues in each chimera which interact with pseudomonic acid A are unknown, therefore, it is difficult to speculate whether or not these two chimeras are sensitive to pseudomonic acid as the junction between the yIRS and the bIRS portions of the chimeric enzymes may have disrupted the folding of the isoleucine binding site. However, the other two chimeric enzymes (Afl III/Sac II and Nsi Chimeras), both contain the majority of the N-terminal domain of yIRS and therefore, may be resistant to pseudomonic acid A. The yIRS C-terminal deletion proteins, if they contain an intact nucleotide-binding fold should contained the amino acid binding site and should also be resistant to pseudomonic acid.

In vivo aminoacylation assays were performed using increasing concentrations of pseudomonic acid A to determine if any of the mutant IleRS variants were able to confer pseudomonic acid A resistance to the *E. coli* host strain. Previously, this assay showed that *E. coli* cells expressing the wild-type yIRS were able resist the effect of pseudomonic acid A at concentrations that would otherwise kill the *E. coli* cells not expressing the yIRS enzyme (61). Hence, yIRS is resistant to pseudomonic acid A and is capable of taking over the function of the *E. coli* host IleRS which was inhibited by the antibiotic (61). The results obtained from the *in vivo* aminoacylation assays showed that none of the IleRS variants was capable of "rescuing" the host cell from the effects of pseudomonic acid A. In addition, it was noted that some colonies expressing IleRS variants grew more poorly at a given pseudomonic acid A concentration relative to the control colonies which harboured only the control vector. From *in vivo* assays, we can conclude that none of the IleRS variants were capable of aminoacylating *E. coli* $tRNA^{ile}$.

In vitro aminoacylation studies were conducted on crude cell-free lysates of *E. coli* cells expressing the yIRS C-terminal deletion mutants. These assays were performed using crude *E.coli* tRNA as yIRS has been found to aminoacylate *E. coli* $tRNA^{ile}$ as efficiently as yeast $tRNA^{ile}$ under assay conditions (Racher, 1991). All of the C-terminal deletion mutants, except for the yIRS C-terminal Hinc II, were unable to aminoacylate crude *E. coli* tRNA in the presence of pseudomonic acid A (see Figure 13). The Hinc II deletion protein was shown to have partial aminoacylation activity on the order of about 6% relative to the full length yIRS. This supports the hypothesis that the C-terminal domain is required for tRNA binding and/or recognition in Class I aaRSs. As shown in previous studies, C-terminal deletions of MetRS and TyrRS of 200 amino acids and 100 amino acids, respectively, eliminated aminoacylation activity in both of these Class I synthetases (5, 87). From the results we obtained, deletions of the C-terminal domain of IleRS of 79 amino acids or more, effectively reduces aminoacylation activity to zero.

Although MetRS and IleRS belong to the same subclass, there is little sequence homology between the two synthetases other than in the N-terminal nucleotide-binding domain. The *E. coli* MetRS is an α_2 dimeric synthetase comprised of two subunits of 676 amino acids each and a relative molecular weight of 76,000 daltons for each subunit (23). The *E. coli* IleRS is a monomeric synthetase of 939 amino acids in length and has a relative molecular weight of 105,000 daltons (88). (The yeast IleRS is 1076 amino acids in length and has a relative molecular weight of approximately 123,000 daltons (49)). Previous studies have shown that mild proteolysis of the native MRS dimer, with trypsin, removed 130 amino acids from the C-terminal end of the native subunit to yield a fully active monomeric synthetase, which was 556 amino acids in length (15). An equivalent deletion of 130 amino acids in IleRS would have resulted in total loss of aminoacylation activity. Thus, unlike methionyl-tRNA synthetase (MetRS), which belongs in the same subclass as IleRS, IleRS appears to be unable to tolerate C-terminal deletions. Crystallographic studies on the 556 amino acid tryptic fragment of the monomeric *E. coli* MetRS has shown that residues $^{\#}$527-$^{\#}$551 of the C-terminal end of the synthetase loop back and interact with the N-terminal domain, thereby forming a bridge between the N-terminal and C-terminal domains (12). Thus, the last 130 amino acids of the C-terminal end of the native *E. coli* MetRS 676 amino acid subunit may be responsible for the formation of a dimeric interface between the two subunits (75). This is consistent with the research on yeast MetRS which was found to be monomeric and has a smaller C-terminal domain compared to the *E. coli* MetRS (86). An additional deletion of 70 amino acids from the C-terminal end of the truncated, monomeric *E. coli* MetRS abolishes aminoacylation activity in the synthetase but not aminoadenylation activity (5). Therefore, the importance of the C-terminal end of IleRS may be its interaction with the N-terminal domain. The C-terminal domain may function to stabilise the N-terminal domain and/or facilitate the binding of tRNA for aminoacylation.

Aminoacylation assays on the chimeric variants using crude *E. coli* tRNA in the presence of pseudomonic acid and, crude yeast tRNA in the absence of pseudomonic acid A, showed that

the chimeric IleRS variants were not able to aminoacylate tRNA from either species (see Figure 13. Note: results from the aminoacylation assays performed with yeast tRNA is not shown). One previous study on chimeras of aminoacyl-tRNA synthetase has been documented (81). Of the five intra-domain chimeras designed by Starzyk *et al.*, which were comprised of an N-terminal portion of *E. coli* MetRS and a C-terminal portion of *E. coli* IleRS, only three were expressed as proteins and none of the three were active (81). Thus, it appears that tRNA specificity in IleRS cannot be switched by simply swapping the domains of synthetases.

The *in vivo* and *in vitro* aminoacylation assays have established that none of the IleRS variants were capable of aminoacylation under assay conditions. However, it should be noted that the results from the aminoacylation assay do not rule out the possibility that some of the IleRS variants may have aminoadenylation activity. The differences observed in the sizes of the colonies between the control plates and some of the plates containing colonies expressing IleRS mutants, in the *in vivo* aminoacylation assay, may be due to the sequestering of isoleucine, by the IleRS variants with aminoadenylation activity.

4.3 AMINOADENYLATION ACTIVITY OF THE Afl III/Sac II AND Nsi I CHIMERAS

In order to ascertain whether any of the IleRS variants have aminoadenylation activity, the variants must first be purified from the *E. coli* host IleRS. Two of the ten variants were chosen for purification, the Afl III/Sac II Chimera and the Nsi I Chimera. Both these enzymes are comprised of the majority of the N-terminal domain of yIRS and the C-terminal domain of bIRS. We believed that the Afl III/Sac II Chimera and the Nsi I Chimera were most likely to have an intact nucleotide-binding fold and resistance to pseudomonic acid A. The only difference in the between the two chimeras lies in the position in which the N-terminal yIRS portion was joined to the C-terminal portion of bIRS. The Afl III/Sac II Chimera is composed of amino acids #1-#572 of yIRS and #568-#939 of bIRS, giving a protein with a predicted molecular weight of 107866. Based on primary sequence alignment with bIRS, the junction between the yIRS and bIRS halves

of the Afl III/Sac II Chimera lies within the α_d helix of the nucleotide-binding fold (see Figure 37). The Nsi I chimera contains more of the nucleotide-binding fold of yIRS from amino acid #1-#628. The C-terminal domain of the Nsi I Chimera is composed of amino acids #628-939 of bIRS, thereby giving a protein with a predicted molecular weight of 107254. This puts the junction between the two halves of the chimera at the beginning of the β_f β-sheet at the end of the N-terminal domain (see Figure 37). Thus, the Nsi I Chimera can be considered as an inter-domain chimera and the Afl III/Sac II Chimera as an intra-domain chimera (see Figure 9). It was hoped that these two chimeras would help identify catalytically important segments in IleRS.

The aminoacylation assays have shown that neither of the chimeric enzymes was capable of aminoacylating *E. coli* tRNA in the presence of pseudomonic acid or aminoacylating yeast tRNA in the absence of pseudomonic acid. As mentioned, it is possible that the chimeric IleRS enzymes might have the ability to form the isoleucyladenylate. Using the protocol established for the purification of yIRS from *E. coli* host IleRS (61), an attempt was made to purify the two chimeric variants from the host IleRS. Analysis of the protein sequences of the two chimeric variants by the program, CHARGEPRO (16), predicted that both these chimeras have an isoelectric point at 5.48, compared to bIRS and yIRS which have predicted isoelectric points at 5.9 and 5.62, respectively. Thus, we hypothesised that the two chimeras can be purified from the *E. coli* host IleRS using anion-exchange and hydroxylapatite chromatography.

Aminoadenylation assays, otherwise known as active-site titration assays (27), are commonly used to determine the number of amino acid binding sites (active sites) within a given protein sample. Assays were conducted on the hydroxylapatite column-purified chimera samples to establish if the two chimeras were able to bind isoleucine and catalyse the formation of the isoleucyladenylate. The assays were performed in the absence and presence of pseudomonic acid to see firstly, if the sample of chimeric IleRS was free of bacterial IleRS contamination and secondly, to determine if pseudomonic acid A resistance has been conferred. Of the two chimeras, only the Nsi I Chimera showed aminoadenylation activity (see Figure 36). In addition,

the yIRS portion of the Nsi I Chimera made this chimera pseudomonic acid A resistant. The Afl III/Sac II chimera, on the other hand, showed no activity in the absence or presence of pseudomonic acid A and was, essentially, a dead enzyme.

Despite the similarity in protein sequence between the Afl III/Sac II Chimera and the Nsi I Chimera, one chimera was able to retain the ability to form the aminoadenylate and one was not. We have hypothesised that the catalytic site of the Afl III/Sac II Chimera was disrupted by the junction made between the yIRS and bIRS portions of the enzyme. The α_d helix of the Rossmann Fold, where the junction was made, may have been disrupted and in turn, may have caused misalignment of the KMSKS sequence which is known to be associated with ATP in the catalytic site (51, 90). Recently, it has been proposed that movement of the KMSKS loop, during the transition state of amino acid activation, brings the basic residues closer to the phosphates of the ATP molecule (74). Disrupting the position of the KMSKS loop would result in an inactive enzyme. In the Nsi I Chimera, the junction between the yIRS and bIRS portions of the enzyme is located at the fringe of the N-terminal domain and thus, we hypothesise that the isoleucine binding site and the catalytic site remained intact. In addition, the KMSKS sequence in the Nsi I Chimera is part of the yIRS portion of the enzyme and, as such, probably remained in the correct orientation for catalytic activity. We also hypothesise that the ability of the Nsi I Chimera to resist the effects of pseudomonic acid A was conferred by the yIRS N-terminal domain in the chimera. Thus, the determinant(s) for pseudomonic acid resistance is probably located within the N-terminal domain of the enzyme. This supports the findings by Yanasigawa *et al.* which showed that a single amino acid change from phenylalanine to leucine at position #594 in bIRS was responsible for pseudomonic acid A resistance in *E. coli* strain PS102 (90) (see Figure 37). In the Afl III/Sac II Chimera, the yIRS portion of the chimera does not include the equivalent of the #594 site bIRS. The C-terminal domain of the Afl III/Sac II chimera, which contains amino acids #568-939 of bIRS, does contain the phenylalanine but the alignment of the phenylalanine was shifted by four amino acids due to the junction made between the two halves of the Afl III/Sac II chimera. It

was difficult to speculate if pseudomonic acid A resistance was conferred to the Afl III/Sac II Chimera based on this misalignment. The Nsi I Chimera, on the other hand, was found to contain an isoleucine at the equivalent site of the phenylalanine found in bIRS. Since the alignment of the Nsi I Chimera was not as drastically disrupted as in the Afl III/Sac II Chimera, this isoleucine at #594 of the Nsi I sequence may be responsible for pseudomonic acid A resistance in the Nsi I Chimera.

From the results obtained, formation of the aminoadenylate in the N-terminal domain of IleRS appears to be independent of the C-terminal domain. We hypothesise that disruption of the nucleotide-binding fold in the N-terminal domain led to the inactivation of the catalytic site in the Afl III/Sac II Chimera. The results also showed that tRNA specificity cannot be interchanged by swapping domains between synthetases. Therefore, we hypothesise that the C-terminal domain and the N-terminal domain are both required for the recognition and binding of the cognate tRNA in IleRS.

4.4 CHARACTERISATION OF MONOCLONAL ANTIBODIES TO yIRS AND bIRS

The high specificity of antibodies to their respective antigens is the basis behind all immunochemical techniques used today. Antibodies are produced by B cells as part of the immune response by the host towards a foreign substance. Each B cell produces antibody towards one antigenic site. Monoclonal antibodies are created by a fusion of a single B cell with a sarcoma cell to form what is called a hybridoma. The term monoclonal applies to the fact that one hybridoma cell produces an antibody with one specific antigen binding site whereas polysera contains a variety of antibodies produced by a number of B cells, each of which producing an antibody with a different antigen binding specificity. Thus, monoclonal antibodies have higher specificities towards one antigen while polysera has a larger repertoire of antigen binding sites.

Western immunoblotting analysis of the IleRS variants, using the four monoclonal antibodies developed against the *E. coli* and yeast IleRS enzymes, allowed us to characterise the antigen binding specificities of the four monoclonal antibodies (see Figure 15). These monoclonal antibodies were invaluable in monitoring of variant IleRS purification.

Immuno-affinity chromatography is a powerful technique which utilises the specificity of antibodies to purify a desired antigen. This technique has been widely used in the purification of protein (47, 77). Preliminary work on developing an immuno-affinity technique for the purification of mutant IleRS enzymes yielded poor results. The Y12 immuno-affinity column was found to have a very low antigen binding capacity and thus, it was difficult to determine if the column bound specifically to yIRS. Western immunoblotting analysis of the high salt fractions, showed that yIRS did bind to the column in very small amounts (nmolar amounts) and that sample was free from bIRS contamination (see Figure 16). Aminoacylation assays (Figure 17) were conducted on fractions eluted with high salt to assay whether the enzyme which was eluted retained the ability to aminoacylate crude *E. coli* tRNA and/or crude yeast tRNA. Previously, it has been shown that yIRS is capable of aminoacylating *E. coli* tRNA as efficiently as yeast tRNA whereas bIRS was capable of only aminoacylating *E. coli* tRNA under the same assay conditions (61). The high salt fraction containing yIRS was found to charge both *E. coli* and yeast tRNA. The differences in the charging activities observed with yeast tRNA and *E. coli* tRNA may have been due to the relative concentration of $tRNA^{ile}$ in each of the crude tRNA preparations. In addition, the concentration of the tRNA between the yeast and *E. coli* tRNA stocks may have been slightly different. Thus, the results obtained from the aminoacylation assays were not as reliable as the results obtained from the western immunoblots. Thus, we hypothesised that the IleRS variants can be purified by immuno-affinity chromatography providing that a higher antigen-binding capacity column could be produced.

Since it was found that the monoclonal antibodies bound to specific epitopes on the primary sequence of IleRS, it would be interesting to see whether the monoclonal antibodies were

capable of inhibiting aminoacylation and/or aminoadenylation activity. A previous study conducted on the inhibiting ability of an N-terminal binding monoclonal antibody against yeast glutaminyl-tRNA synthetase (GlnRS), which belongs to the same class as IleRS, has been documented (46). This monoclonal antibody, which binds within the first 205 amino acids of the N-terminal domain, was not capable of inhibiting aminoacylation or aminoadenylation activity in yeast GlnRS. This result contributed to the conclusion that the first 205 amino acids of the N-terminal domain of GlnRS are dispensable (46). The results from the inhibitory studies on yIRS and bIRS, using the monoclonal antibodies against these two synthetases, showed that only monoclonal antibody, Y12, which binds within the first 236 amino acids of the N-terminal domain of yIRS, was capable of inhibiting both aminoacylation and aminoadenylation in yIRS. Thus, unlike yeast GlnRS, yIRS does not have a dispensable N-terminal domain. The results obtained from the studies using monoclonal antibody B1B2 on the bIRS enzymes showed that this antibody was not capable of inhibiting either aminoacylation or aminoadenylation, despite the fact that it binds to the N-terminal domain of bIRS. We hypothesise that this monoclonal antibody may be a weak binding antibody and as such was not capable of blocking substrate binding. Inhibitory studies on the C-terminal binding antibodies, Y1 and Y11 showed that neither antibody was capable of inhibiting aminoadenylation. The monoclonal antibody Y1 was shown to inhibit aminoacylation to a minor extent whereas monoclonal antibody Y11 was not capable of inhibiting aminoacylation. Therefore, Y11 was not binding to an important site that would inhibit aminoacylation and Y1 may be binding weakly to a site that might be a recognition site for the binding of tRNA.

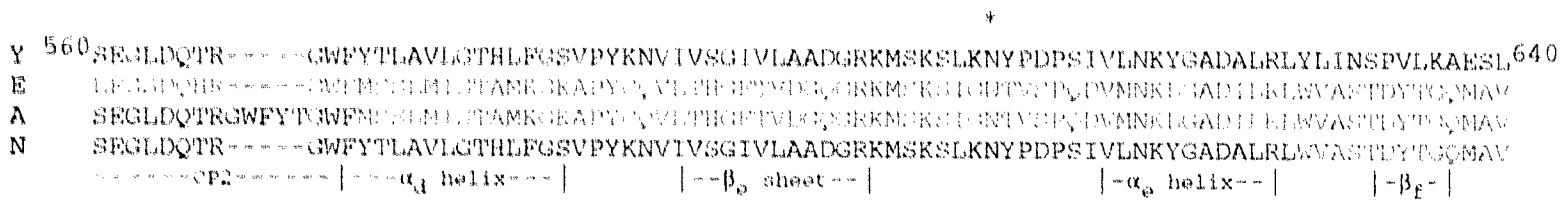


Figure 37 : Primary Sequence Alignments of the Nsi I and Afl III/Sac II Chimeras with yIRS and bIRS. The junctions made between the yIRS and bIRS portions of the chimeras are indicated by the colour change in the sequence. Note that blue represents the yIRS portion of the chimera and red represents the bIRS portion of the chimera. Gaps in the sequence alignments are denoted by (-) and the (*) denotes the postulated pseudomonic acid determinant at position #594 of the bIRS enzyme. Y denotes the wild-type yIRS sequence, E denotes the wild-type bIRS sequence, A denotes the Alf III/Sac II Chimera sequence and N denotes the Nsi I Chimera sequence. The predicted secondary structural features are based on the alignment of bIRS with MetRS (Shiba & Schimmel PNAS 89:1880-1884 (75)).

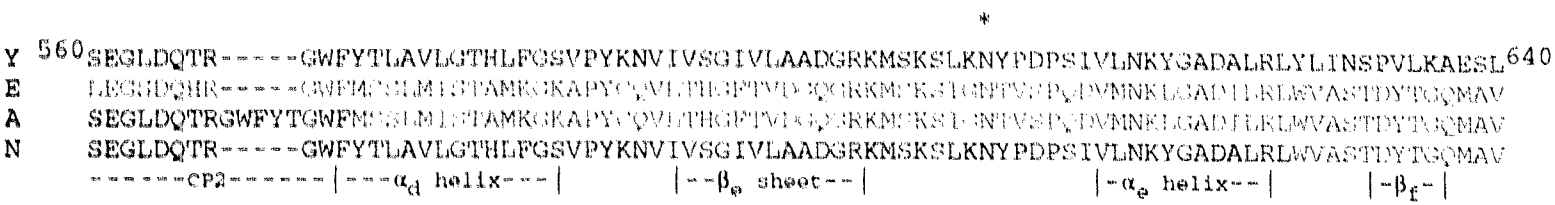


Figure 37 : Primary Sequence Alignments of the Nsi I and Afl III/Sac II Chimeras with yIRS and bIRS. The junctions made between the yIRS and bIRS portions of the chimeras are indicated by the colour change in the sequence. Note that blue represents the yIRS portion of the chimera and red represents the bIRS portion of the chimera. Gaps in the sequence alignments are denoted by (-) and the (*) denotes the postulated pseudomonic acid determinant at position #594 of the bIRS enzyme. Y denotes the wild-type yIRS sequence, E denotes the wild-type bIRS sequence, A denotes the Alf III/Sac II Chimera sequence and N denotes the Nsi I Chimera sequence. The predicted secondary structural features are based on the alignment of bIRS with MetRS (Shiba & Schimmel PNAS 89:1880-1884 (75)).

4.5 CONCLUSION

In order to obtain a better understanding of the editing site(s), substrate binding sites and the catalytic site of isoleucyl-tRNA synthetase, C-terminal deletion mutants and chimeric IleRS enzymes were engineered and successfully expressed in *E. coli* using recombinant techniques. Preliminary studies of the C-terminal deletion mutants and the chimeric IleRS enzymes have shown that none of the IleRS variants were capable of aminoacylating tRNA. This suggests that the C-terminal domain of IleRS is not dispensable and tRNA specificity cannot be switched by simply swapping C-terminal domains between the *E. coli* and yeast IleRS enzymes. Two purified chimeric IleRS enzymes were assayed for aminoadenylation activity. The results from the aminoadenylation assays suggests that the N-terminal nucleotide-binding domain is catalytically independent of the C-terminal domain. Disruption of the N-terminal nucleotide-binding in the Afl III/Sac II Chimera probably resulted in the inactivation of this chimera. The Nsi I chimera was hypothesised to contain an intact nucleotide-binding fold because of its ability to form the aminoadenylate. In addition, the Nsi I chimera was able to retain pseudomonic acid A resistance from the N-terminal yIRS portion of the chimera. This suggests that the determinant(s) for pseudomonic acid resistance is located in the N-terminal domain of yIRS. Based on the assay results, we hypothesise that the binding of the cognate $tRNA^{ile}$ by IleRS requires recognition and/or binding sites in both the N-terminal and C-terminal domains of the protein.

Monoclonal antibodies developed against yIRS and bIRS were characterised and their antigenic sites on the IleRS primary sequence were mapped. One monoclonal antibody, Y12, was found to inhibit both aminoacylation and aminoadenylation in yIRS, thereby suggesting that monoclonal antibody Y12 binds at the catalytic site of yIRS. Preliminary work on developing an immuno-affinity column has shown that yIRS can be purified from endogenous IleRS by using the specificity of monoclonal antibodies. Thus, this is a feasible method for the purification of IleRS variants.

From the results of these studies, we have not be able to determine the minimal protein sequence required for aminoadenylation activity in yIRS. Purification of the yIRS C-terminal deletion proteins from the endogenous host IleRS is necessary before aminoadenylation assays can be performed. Immuno-affinity chromatography can be used to purify the C-terminal deletion proteins, providing that a column with a higher antigen-binding capacity could be achieved. We suggests the use of the cyanogen bromide method (19) to couple the antibody to the column matrix as this method has a high coupling efficiency and does not require extensive buffer exchange.

Further studies on the chimeric variants of IleRS may lead to the design of a fully functional chimeric enzyme with altered substrate specificity. We have shown that the Nsi I Chimera was capable of aminoadenylation activity. Using the Nsi I Chimera as a model, chimeras with random junctions (between the yIRS and bIRS fragments) around the C-terminal end of the nucleotide-binding fold near the Nsi I restriction site in yIRS, can be generated using Bal 31 exonuclease (72) which has 3' and 5' exonuclease activity. Once the plasmid containing either the yIRS gene or the bIRS genes has be linearlised using a unique restriction site around the end of the nucleotide-binding fold, Bal 31 can be used to "chew" back the 5' and 3' ends of the gene to generate random fragments. These fragments can then be ligated to create yIRS/bIRS chimeras with random points of junction. Another method for generating chimeric enzymes was described by Ogawa *et al.* (57). This method depends on the natural recombination process that occurs between two genes that express homologous proteins. Recombination events would occur at higher frequencies at regions of high homology between the two proteins. Thus, proteins with high homology would generate more chimeras. The homologous genetic recombination method for generating chimeric enzymes had been shown to yield chimeras whose parental proteins have as low as 50% homology (52). However, its is not know if proteins of lower homology, like *E. coli* IleRS and yeast IleRS (~27% homology (17)),

would yield chimeras by this method. These two methods for generating chimeras may help in the characterisation of the structural elements responsible for editing, substrate recognition and binding and catalytic activity in IleRS.

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APPENDICES:

APPENDIX A: PLASMID MAPS

Figure A1: Regulatory Sequences and Partial Restriction Map of the Expression Vector, pKK223-3. Note that "rrnB" denotes the termination sequences T_1 and T_2 and "5S" denotes the 5S RNA gene. The pKK223-3 vector carries the gene for ampicillin resistance.

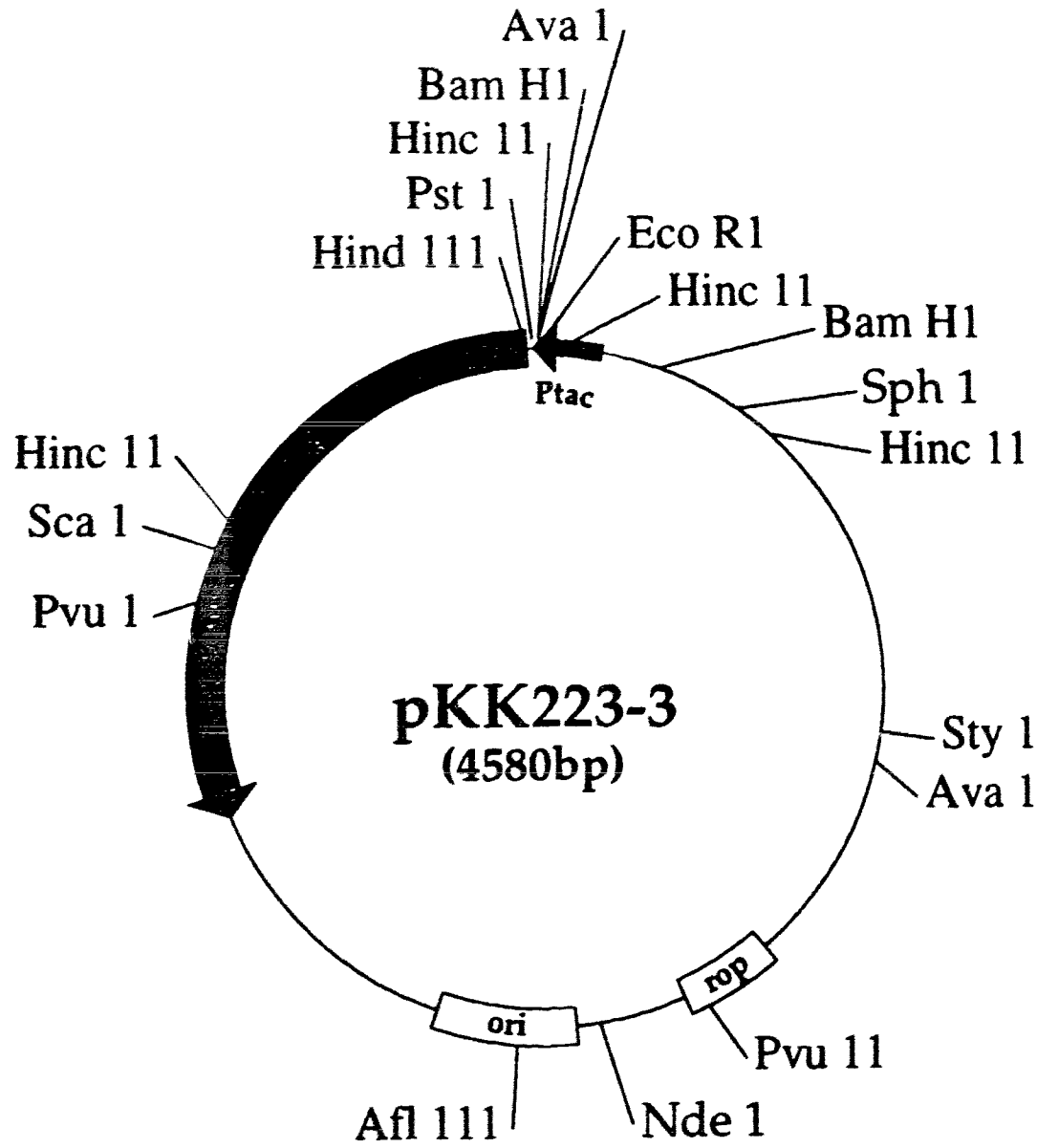

Ava 1
Bam H1
Hinc 11
Pst 1
Hind 111
Eco R1
Hinc 11
Bam H1
Sph 1
Hinc 11
Sty 1
Ava 1
Pvu 11
Nde 1
Afl 111
Hinc 11
Sca 1
Pvu 1
Ptac
ori
rop
pKK223-3
(4580bp)

Figure A2: Regulatory Sequences and Partial Restriction Map of the pKR3 Construct. Plasmid pKR3 is a derivative of pUC18 and was used as an intermediate in the reconstruction of the yIRS gene. Please refer to section 2.4.2.3 for cloning procedure.

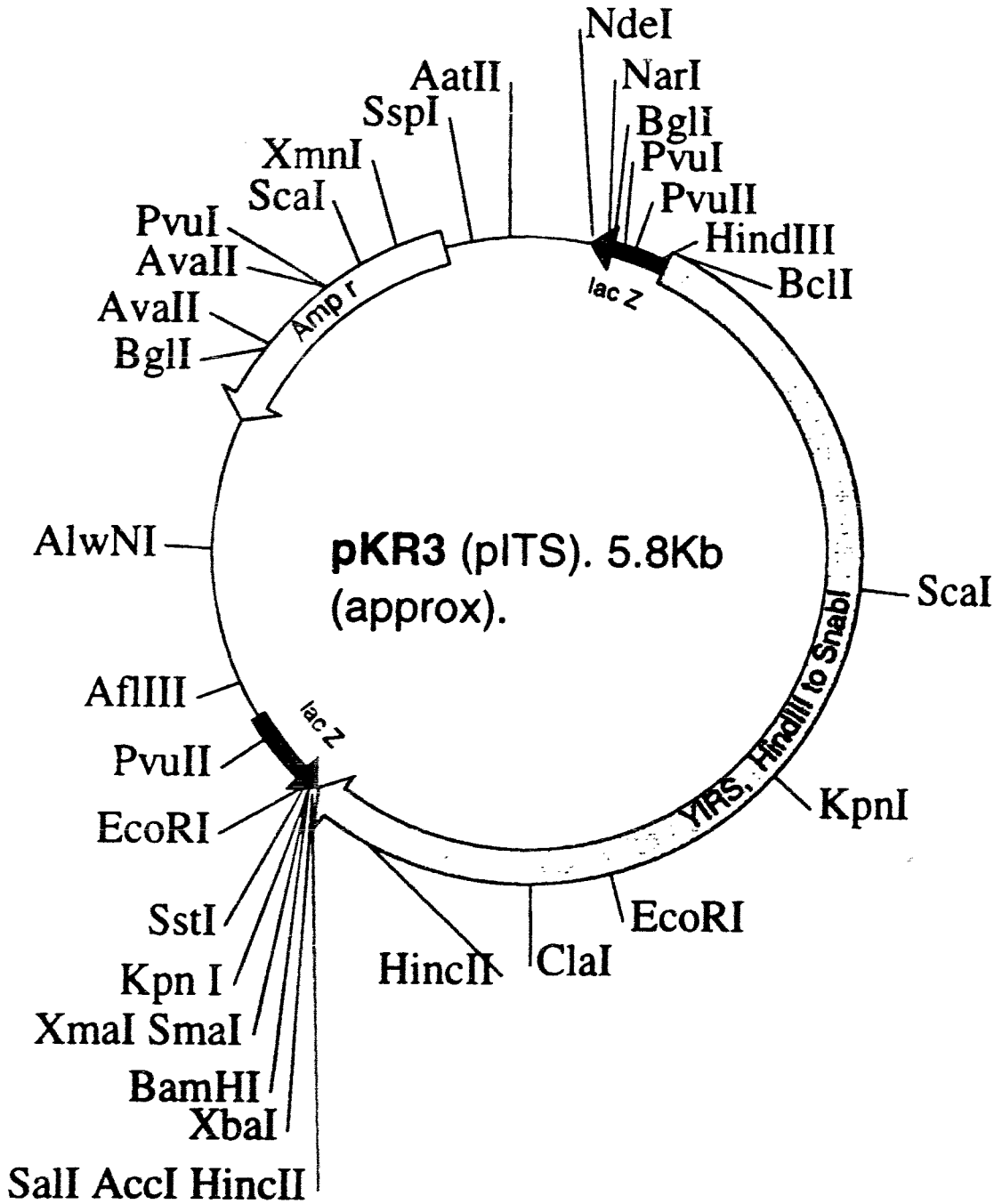
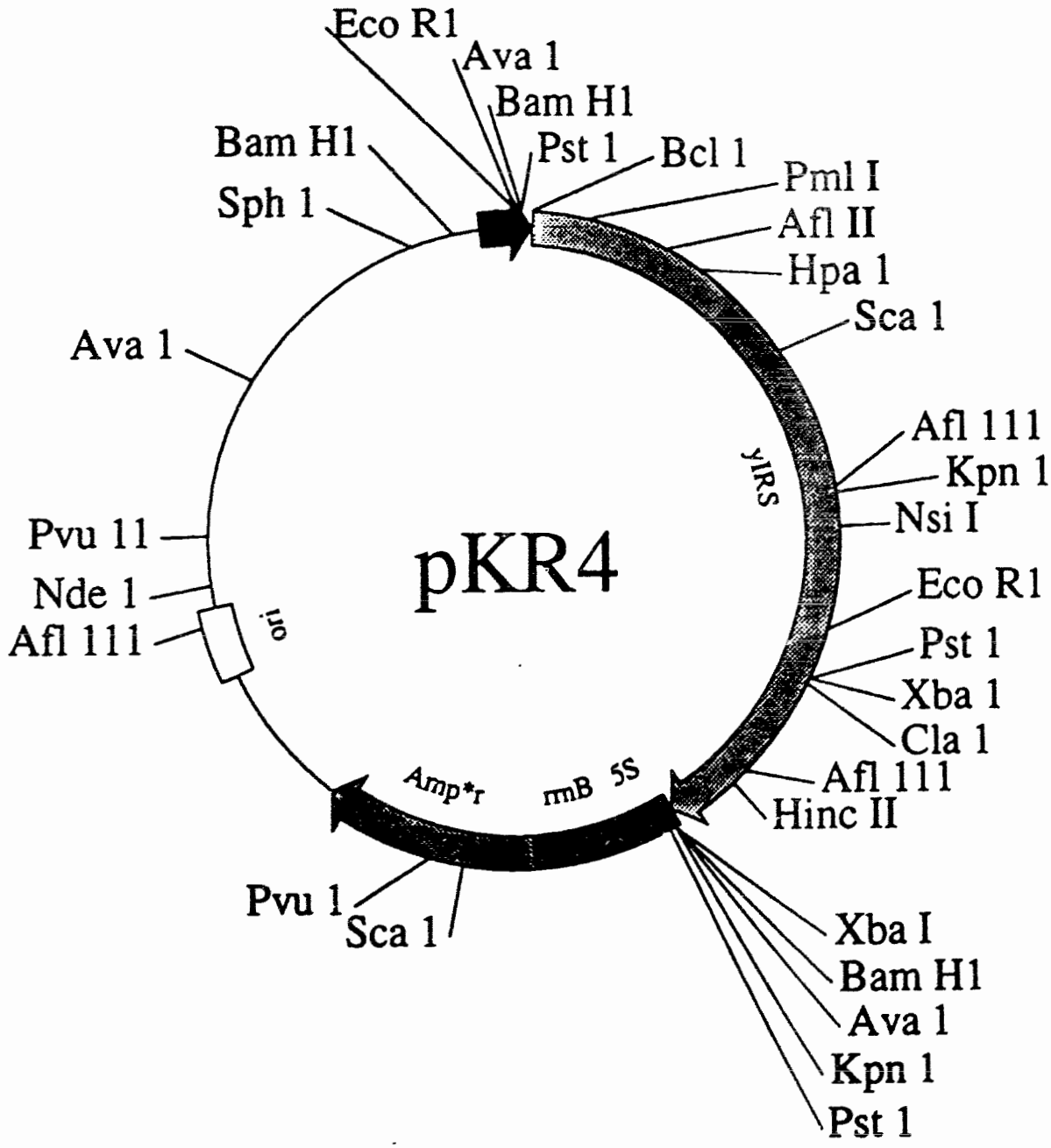
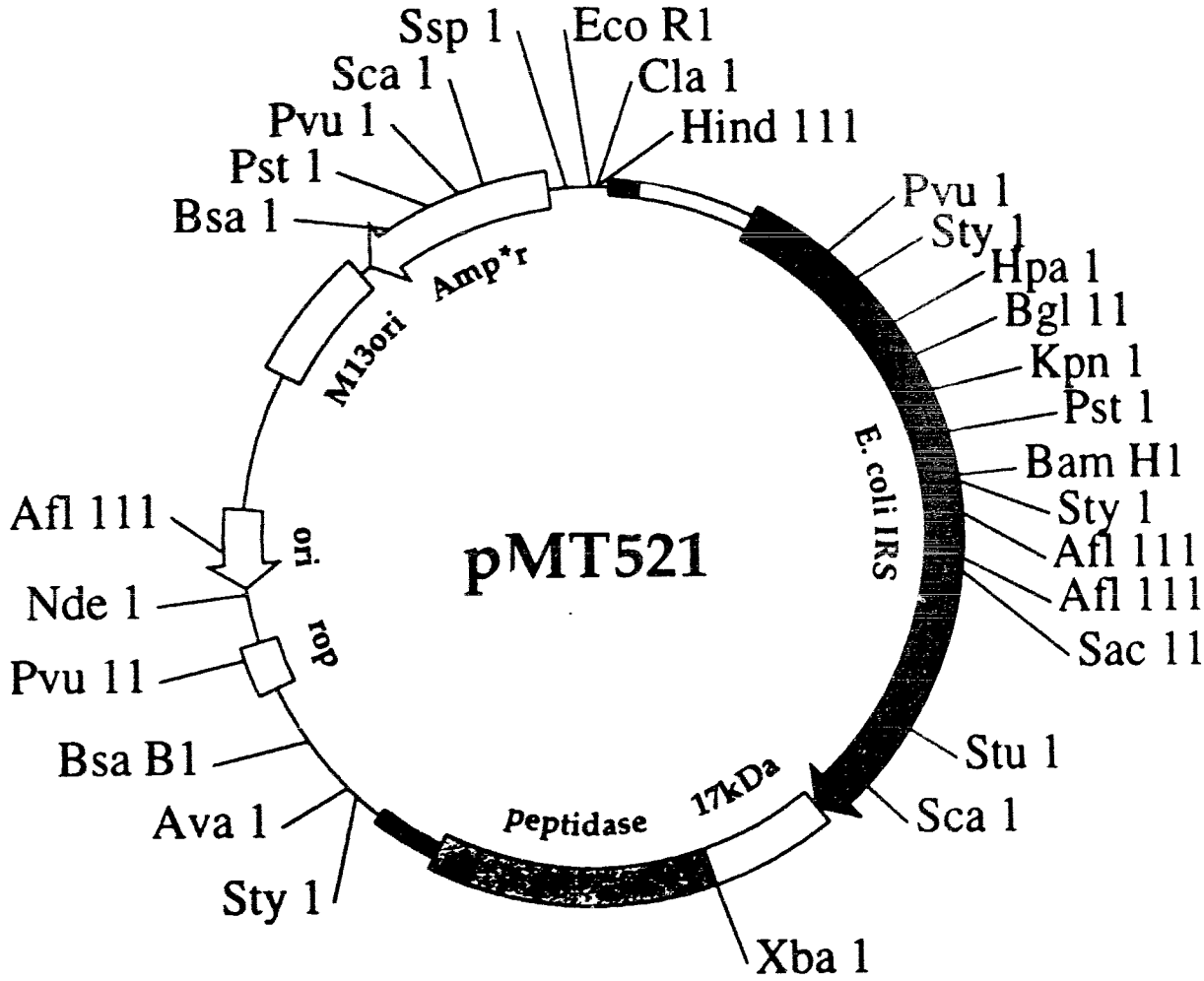

NdeI
NarI
BglI
PvuI
PvuII
HindIII
BclI
ScaI
KpnI
EcoRI
ClaI
HincII
SstI
Kpn I
XmaI SmaI
BamHI
XbaI
SalI AccI HincII
AflIII
PvuII
EcoRI
AlwNI
AvaII
BglI
PvuI
AvaII
XmnI
ScaI
SspI
AatII
lac Z
Amp r
lac Z
pKR3 (pITS). 5.8Kb
(approx).
VIRS, HindIII to SnaBI

Figure A3: Regulatory Sequences and Partial Restriction Map of the Expression Vector, pKR4. The plasmid, pKR4, was derived from the plasmid pKK223-3 and contains the entire coding sequence of the yIRS gene along with the "improved ribosome-binding site".



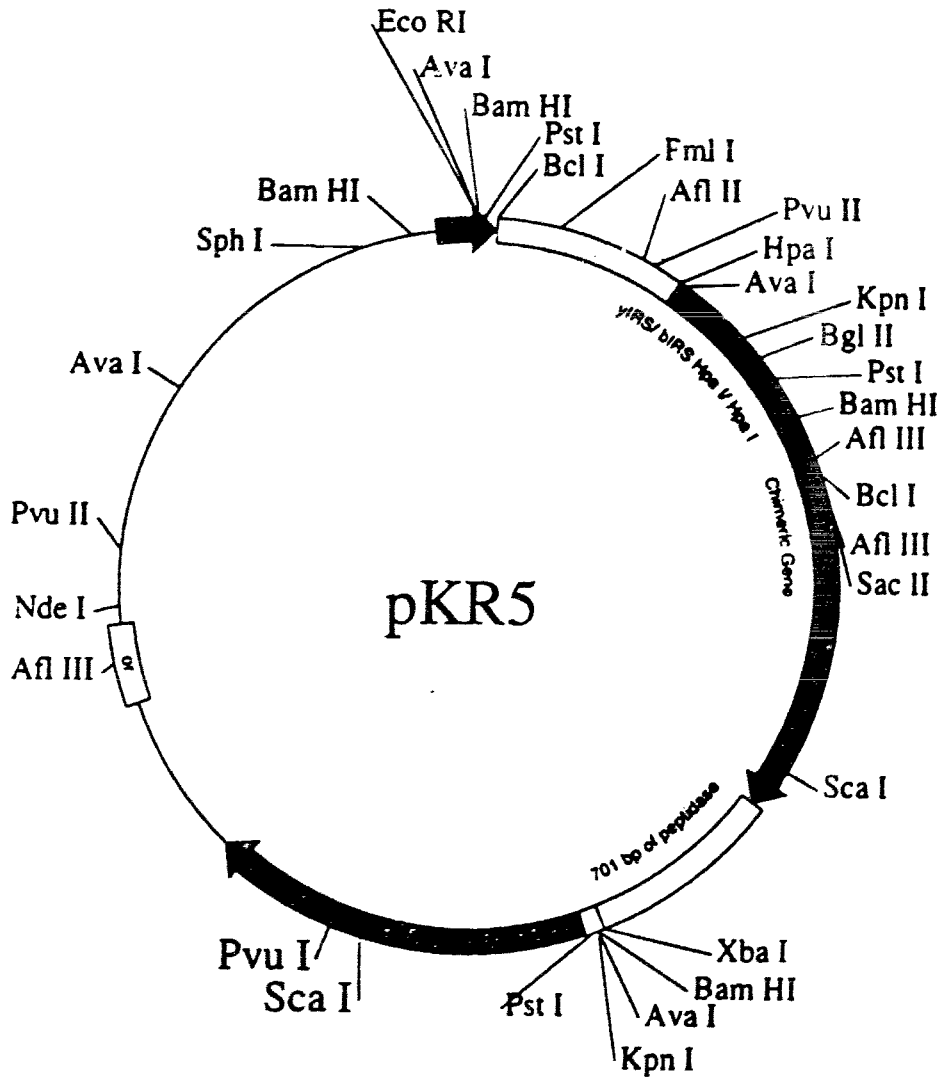
M_r value for yIRS: 122983 Da

Figure A4: Regulatory Sequences and Partial Restriction Map of the Expression Vector, pMT521. This plasmid contains the gene of the *E. coli* Isoleucyl-tRNA Synthetase under the regulation of its own promoter. This plasmid contains the "ori" and "rop" sequences from bacteriophage λ and the M13 ori sequence.



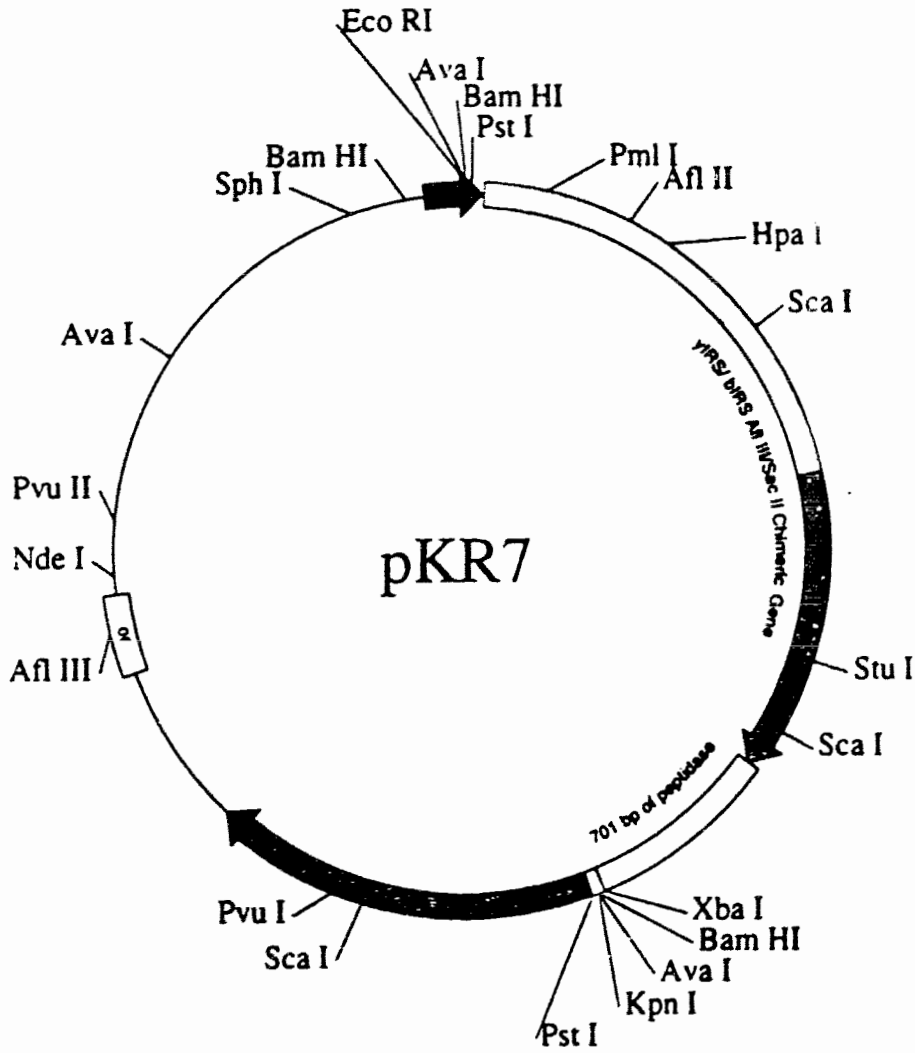
M_r value for bIRS: 104988 Da

Figure A5: Regulatory Sequences and Partial Restriction Map of the Expression Vector, pKR5. pKR5 is a derivative of pKR4 and contains the Hpa I/Hpa I yIRS/bIRS chimeric gene under the regulation of the *ptac* promoter. The light gray represents the yIRS portion of the chimeric gene and the dark gray represents the bIRS portion of the gene.



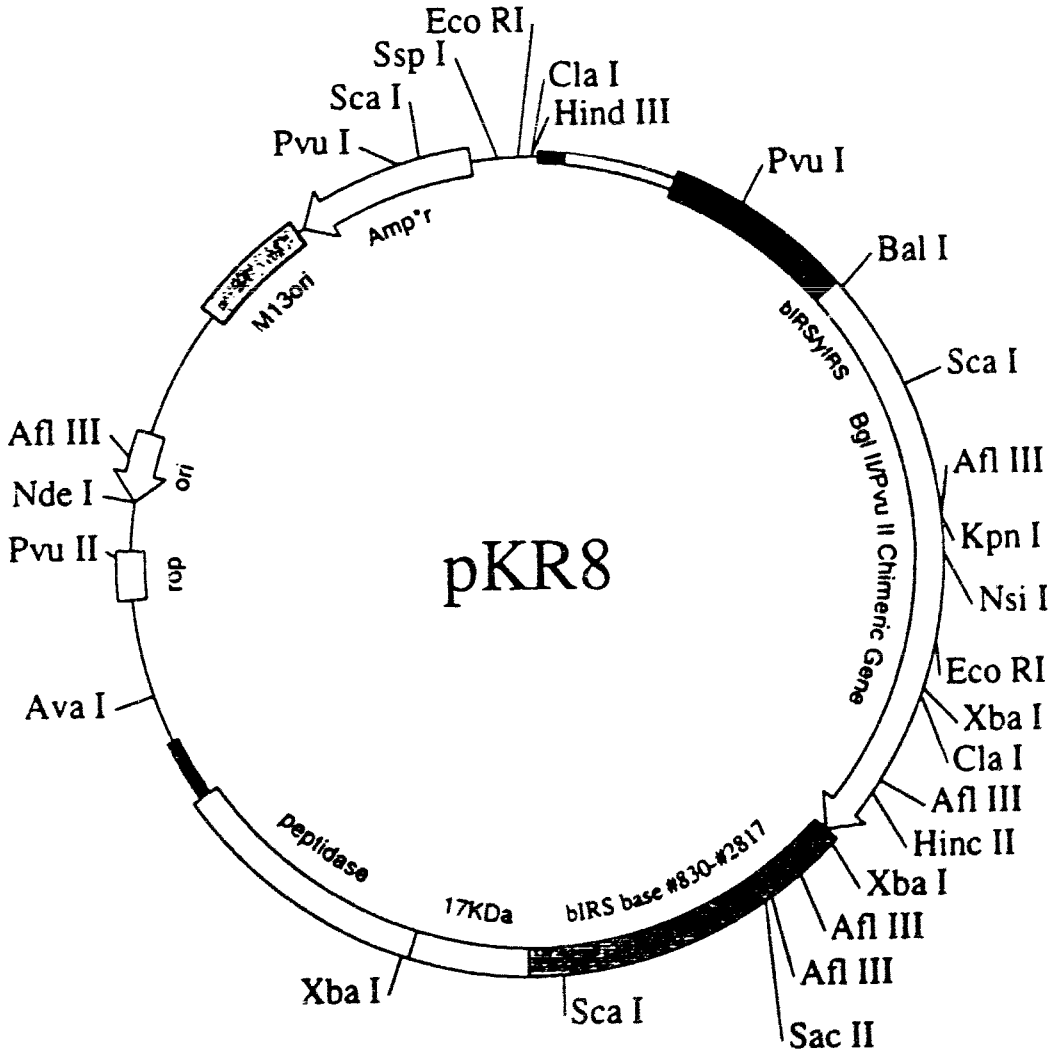
M_r value for Hpa I/Hpa I Chimera: 106654 Da

Figure A6: Regulatory Sequences and Partial Restriction Map of the Expression Vector, pKR7. pKR7, like pKR5, was derived from the plasmid pKR4. pKR7 contains the Afl III/Sac II chimeric gene under the regulation of the *ptac* promoter. The light gray represents the yIRS portion of the chimeric gene and the dark gray represents the bIRS portion of the gene.



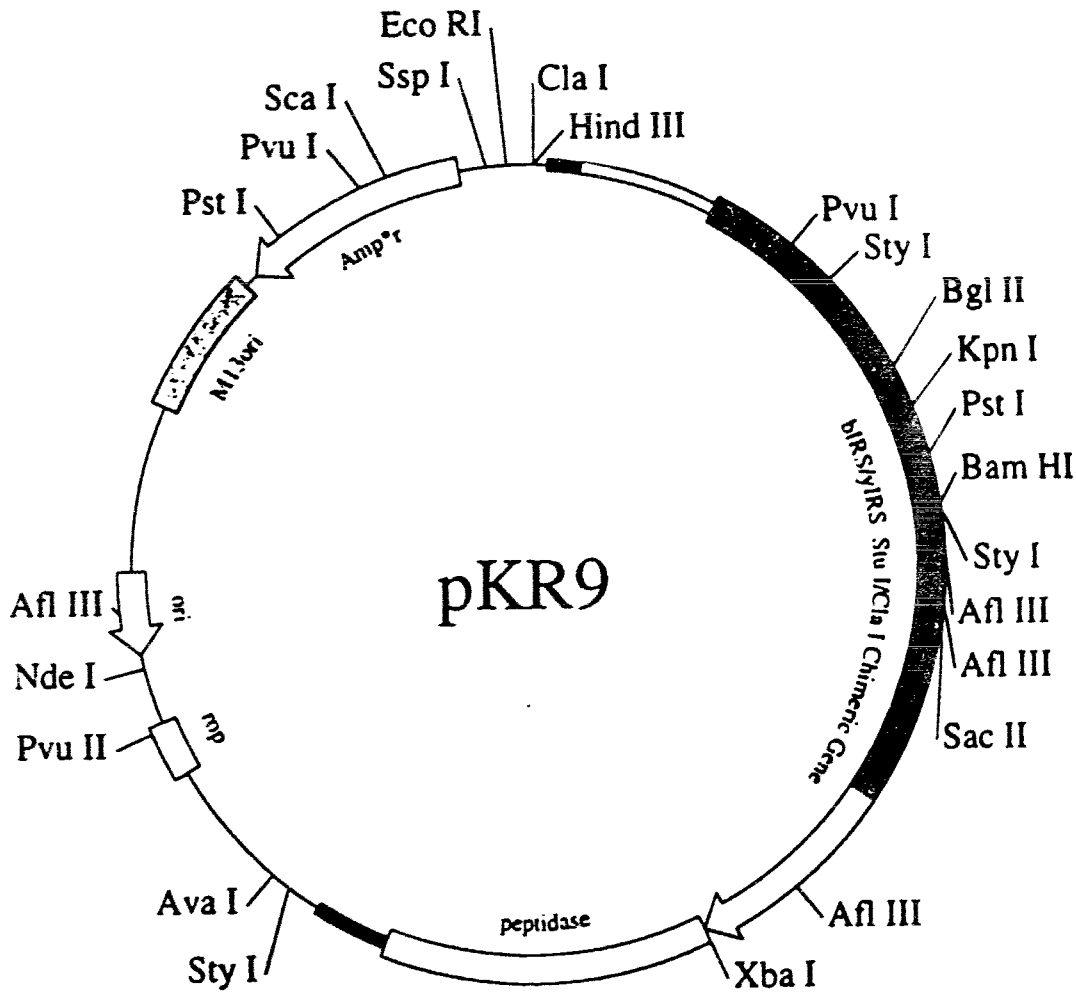
M_r value for Afl III/Sac II Chimera: 107886 Da

Figure A7: Regulatory Sequences and Partial Restriction Map of the Expression Plasmid, pKR8. A 2.6 kb fragment of the C-terminal region of the yIRS gene (including the stop codon) was inserted into the plasmid pMT521 to create the construct, pKR8, and the chimeric gene Bgl II/Pvu II. The dark gray represents the bIRS portion of the chimeric gene and the light gray represents the yIRS portion of the chimeric gene. pKR8 also contains the C-terminal end of the bIRS gene from base #830-#2817 located 3' to the Bgl II/Pvu II chimeric gene. This C-terminal bIRS sequence is not transcribed.



M_r value for Bgl II/Pvu II Chimera: 130084 Da

Figure A8: Regulatory Sequences and Partial Restriction Map of the Expression Plasmid, pKR9. The plasmid construct, pKR9, is a derivative of the plasmid pMT521 and contains the chimeric gene Stu I/Cla I under the regulation of the bIRS promoter. The dark gray represents the bIRS portion of the chimeric gene and the light gray represents the yIRS portion of the gene.



M_r value for Stu I/Cla I Chimera: 116346 Da

Figure A9: Regulatory Sequences and Partial Restriction Map of the Vector, pEND. The plasmid, pEND, is a modification of pUC19 with termination codons in all three reading frames. This plasmid was used to create the C-terminal deletion genes of yIRS.

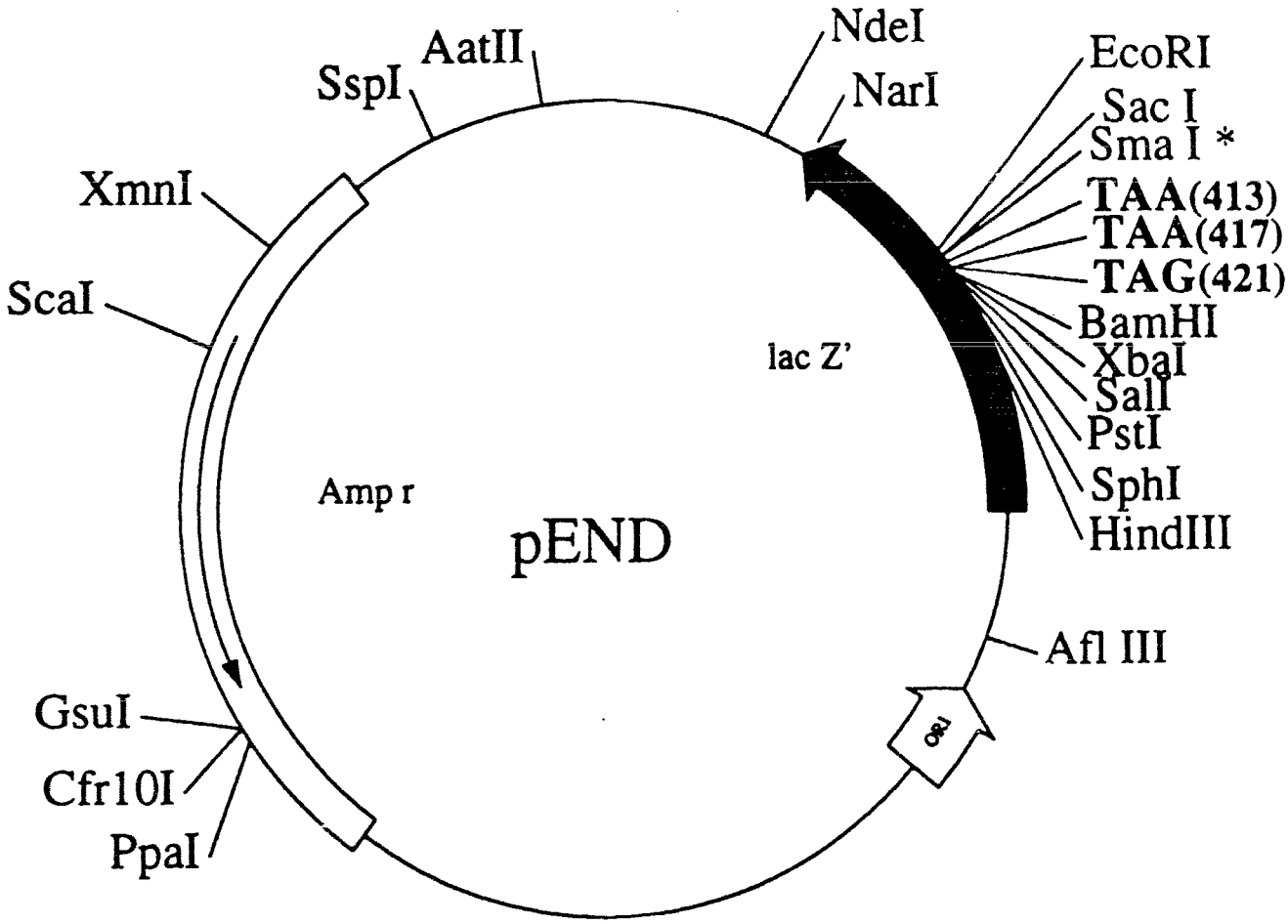

NdeI
AatII
SspI
NarI
EcoRI
Sac I
Sma I *
XmnI
TAA(413)
TAA(417)
TAG(421)
ScaI
BamHI
lac Z'
XbaI
SalI
PstI
Amp r
pEND
SphI
HindIII
Afl III
ori
GsuI
Cfr10I
PpaI

Figure A10: Regulatory Sequences and Partial Restriction Map of the Expression Vector, pLmp10. The terms "lambda OL P_L" refer to the leftward promoter of bacteriophage λ, "nutL" and "nutR" refer to the anti-termination for transcription which is initiated at "P_L".

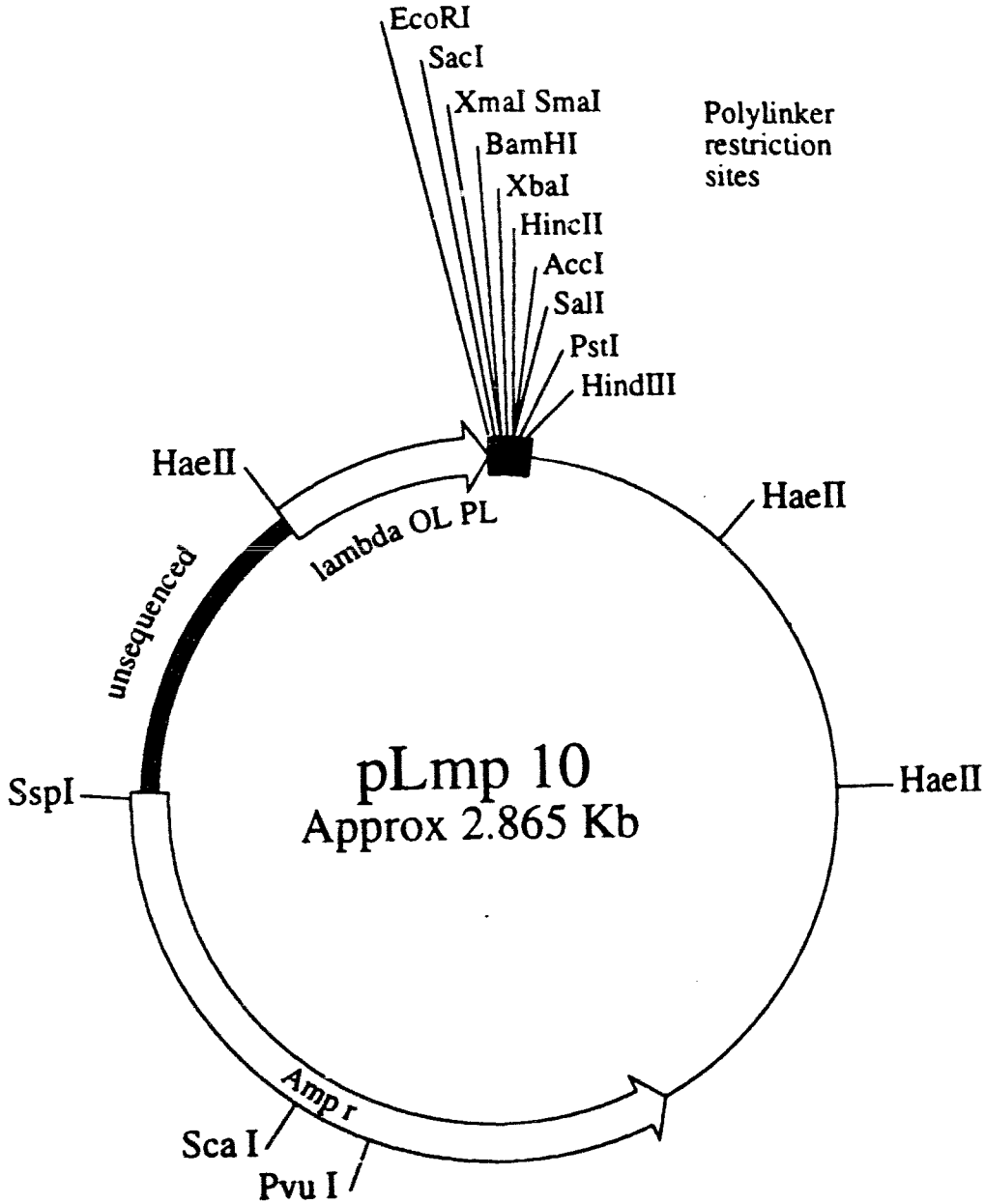

EcoRI
SacI
XmaI SmaI
BamHI
XbaI
HincII
AccI
SalI
PstI
HindIII
Polylinker
restriction
sites
HaeII
HaeII
HaeII
lambda OL PL
unsequenced
pLmp 10
Approx 2.865 Kb
SspI
Amp r
Sca I
Pvu I

Figure A11: Regulatory Sequences and Partial Restriction Map of the plasmid pEND-ITS. This plasmid was derived from the plasmid pEND and contains the yIRS gene. This plasmid was used as an intermediate to create the yIRS Hinc II deletion gene.

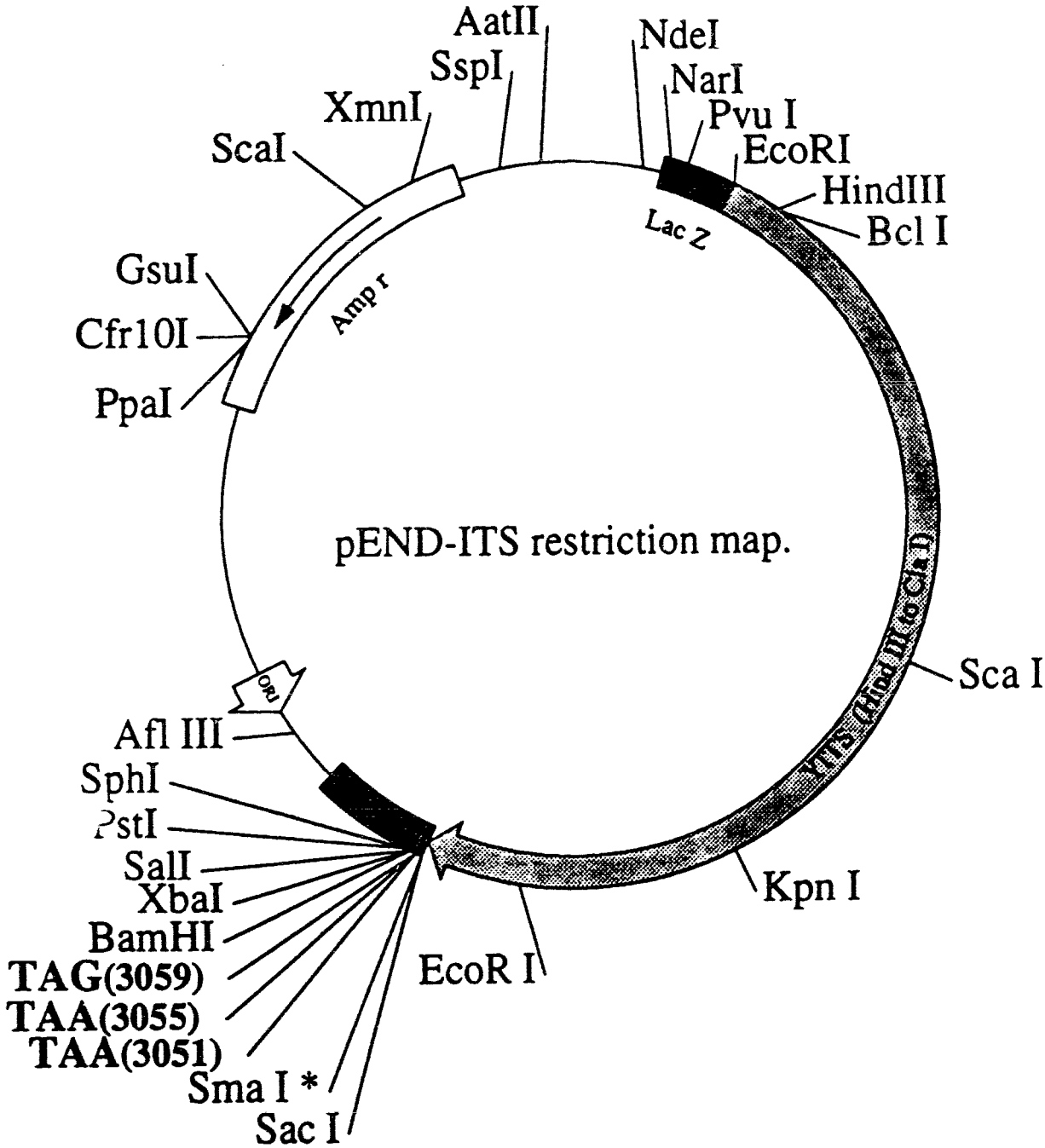
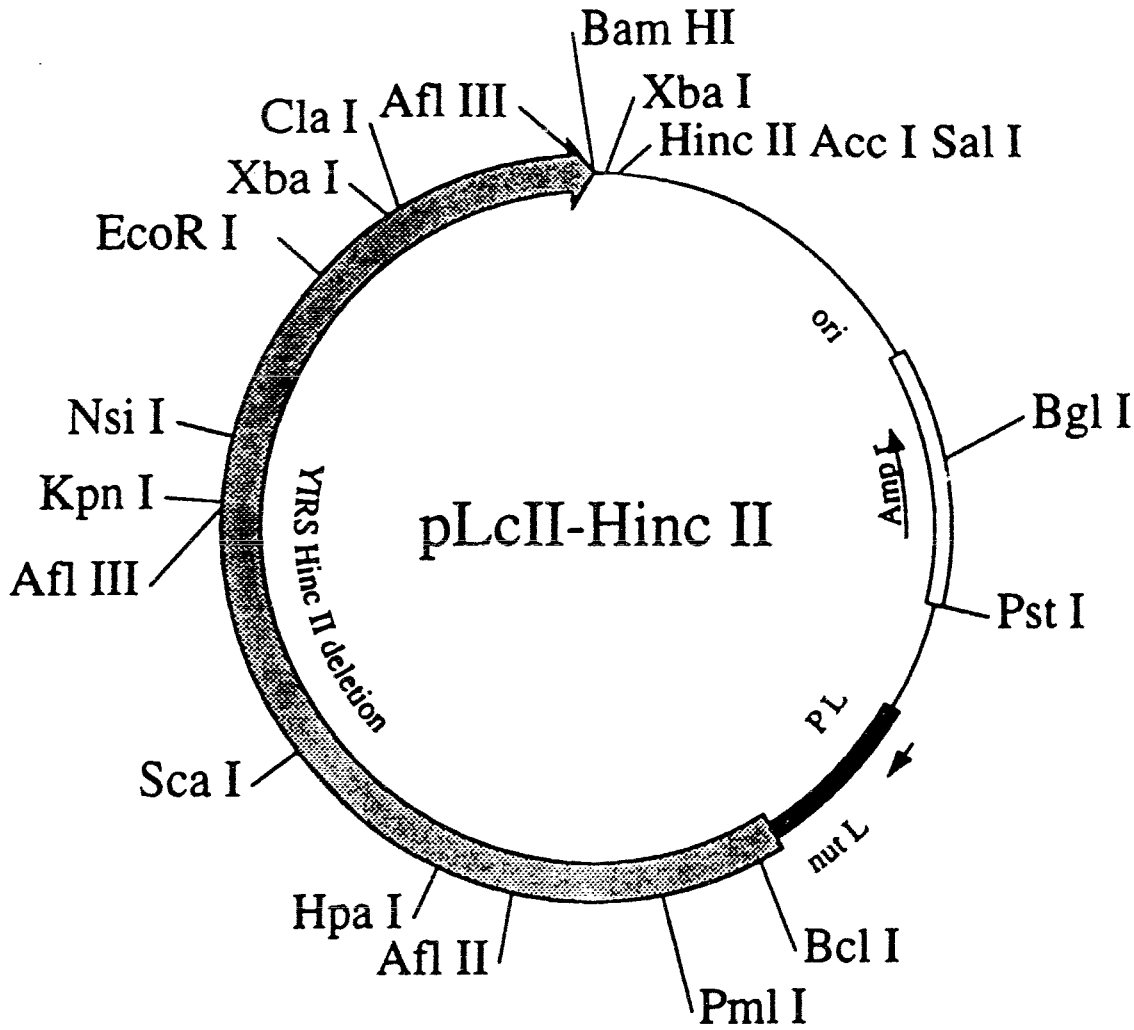
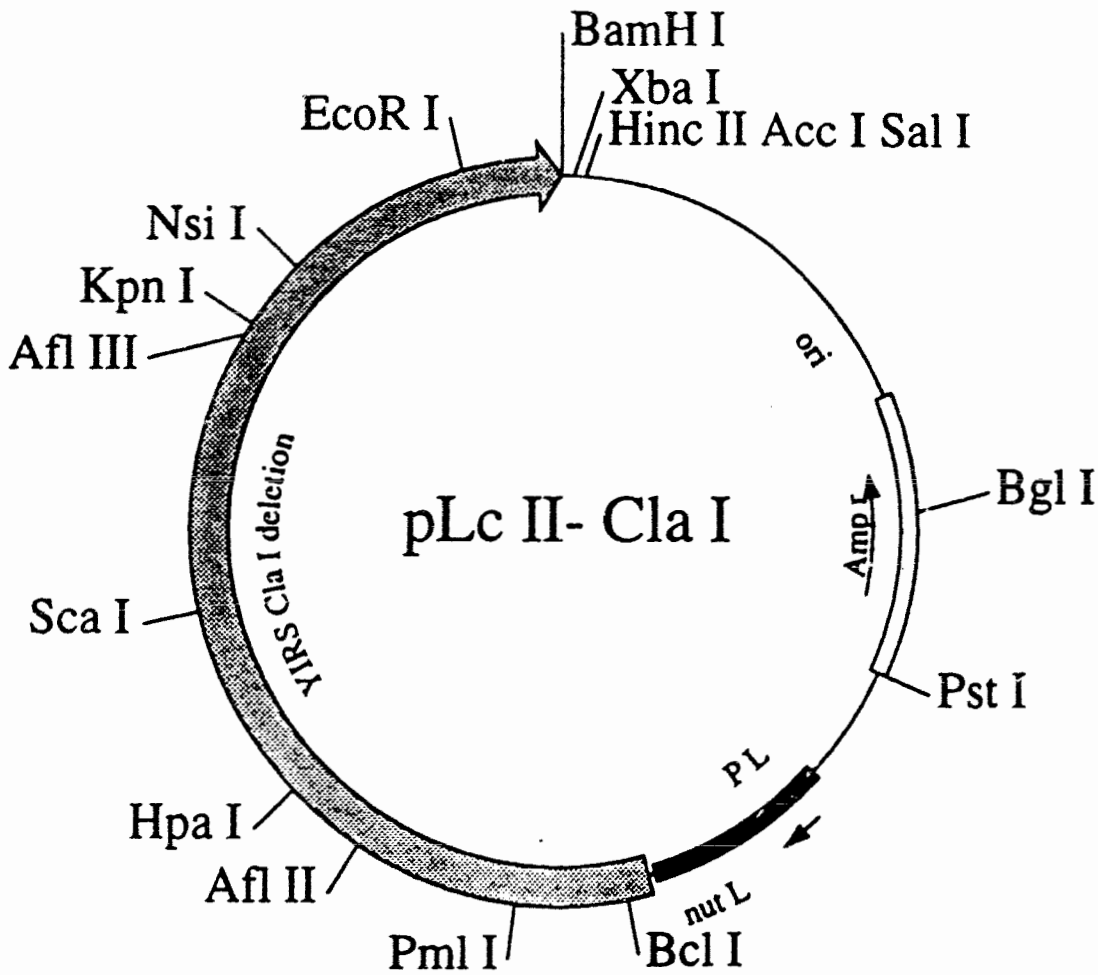

AatII
NdeI
SspI
NarI
XmnI
Pvu I
ScaI
EcoRI
HindIII
Lac Z
Bcl I
GsuI
Amp r
Cfr10I
PpaI
pEND-ITS restriction map.
ORI
Sca I
Afl III
SphI
PstI
SalI
XbaI
Kpn I
BamHI
TAG(3059)
EcoR I
TAA(3055)
TAA(3051)
Sma I *
Sac I

Figure A12: Regulatory Sequences and Partial Restriction Map of the Expression Vector, pLcII-Hinc II. The plasmid pLcII-Hinc II was derived from the vector pLmp10. The yIRS Hinc II deletion gene was cloned into this pLmp10 vector fragment and is expressed under the λ promoter. See appendix B3 for cloning details.



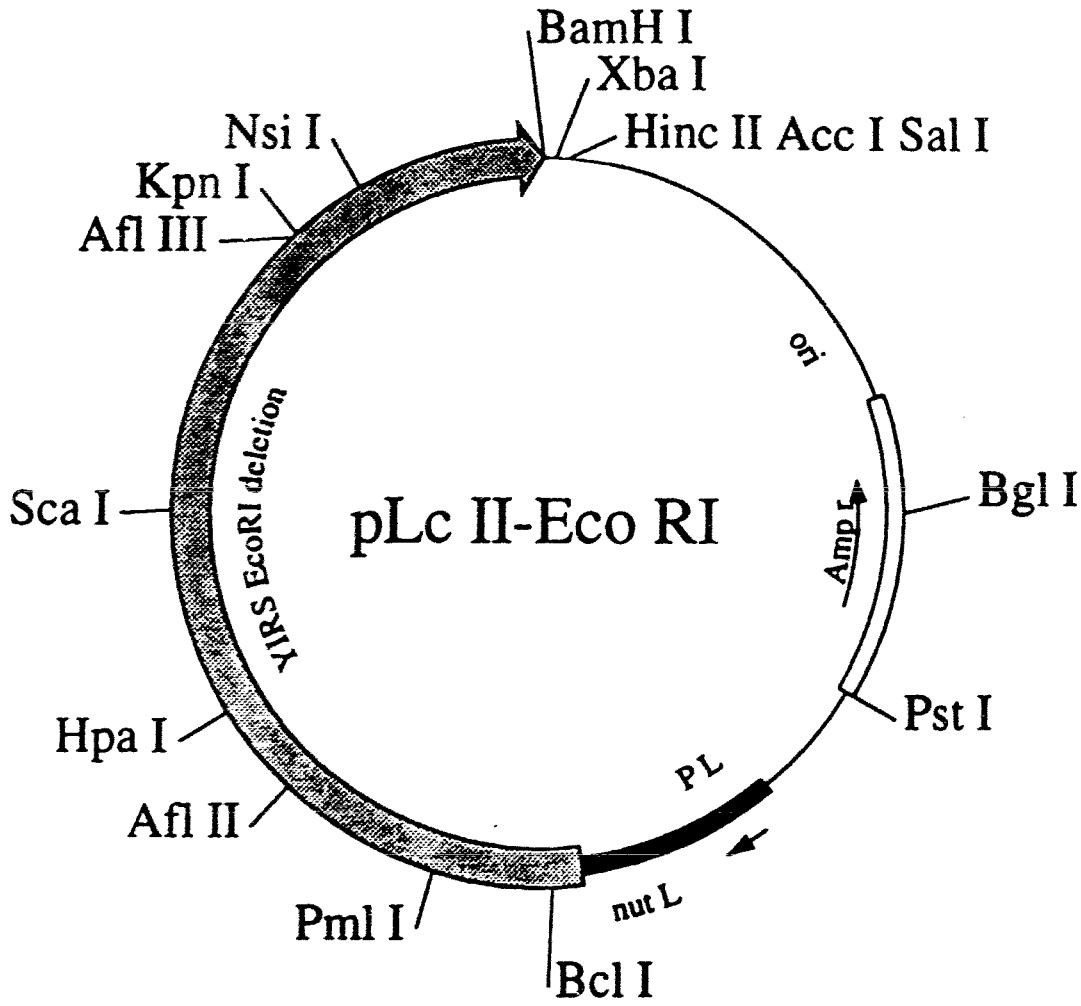
M_r value for yIRS Hinc II Deletion Protein: 113817 Da

Figure A13: Regulatory Sequences and Partial Restriction Map of the Expression Plasmid, pLcII-Cla I. Like pLcII-Hinc II, pLcII-Cla I is a derivative of the pLmp10 vector. The yIRS Cla I deletion gene was cloned into the pLmp10 vector as described in section Appendix B4.



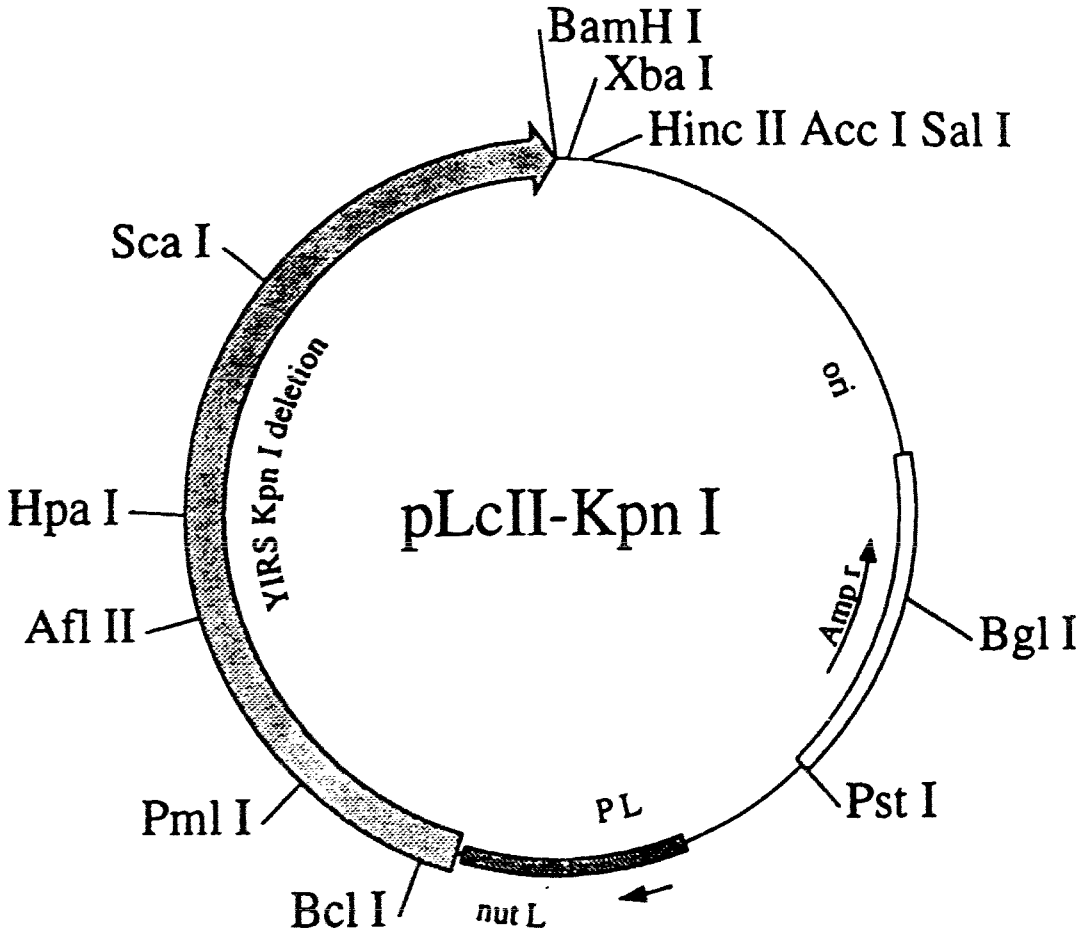
M_r value for yIRS Cla I Deletion Protein: 96167 Da

Figure A14: Regulatory Sequences and Partial Restriction Map of the Expression Vector, pLcII-Eco RI. A derivative of the pLmp10 vector containing the yIRS Eco RI deletion gene under the control of the λ promoter.



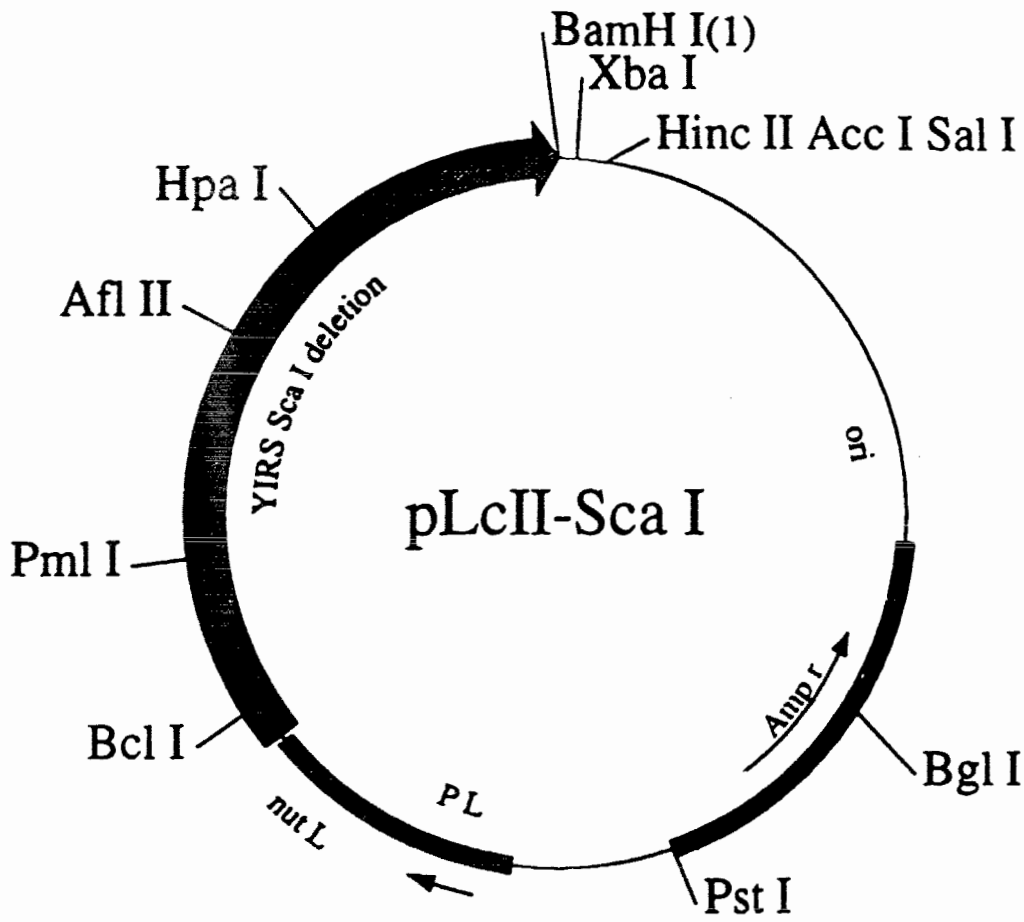
M_r value for yIRS Eco RI Deletion Protein: 87685 Da

Figure A15: Regulatory Sequences and Partial Restriction Map of the Expression Construct, pLcII-Kpn I. This plasmid is derived from the pLmp10 vector. This plasmid contains the yIRS Kpn I deletion gene under the λ promoter.



M_r value for yIRS Kpn I Deletion Protein: 66892 Da

Figure A16: Regulatory Sequences and Partial Restriction Map of the Expression Plasmid, pLcII-Sca I. The yIRS Sca I deletion gene was cloned into the pLmp10 vector. Expression of the Sca I gene is directed by the λ promoter of the pLmp10 vector.



M_r value for yIRS Sca I Deletion Protein: 44089 Da

Figure A17: Regulatory Sequences and Partial Restriction Map of the Expression Construct, pKR15Q. The plasmid pKR15Q was derived from the pMal c vector and pKK223-3. pKR15Q contains a single base mutation in lac I gene which causes the constitutive expression of the lac I gene (this mutated form of the lac I is called the lac I^q). The lac I^q and the *ptac* promoter was from the pMal c vector and the ori, rrnBT_1T_2 and the 5S RNA sequences were taken from pKK223-3.

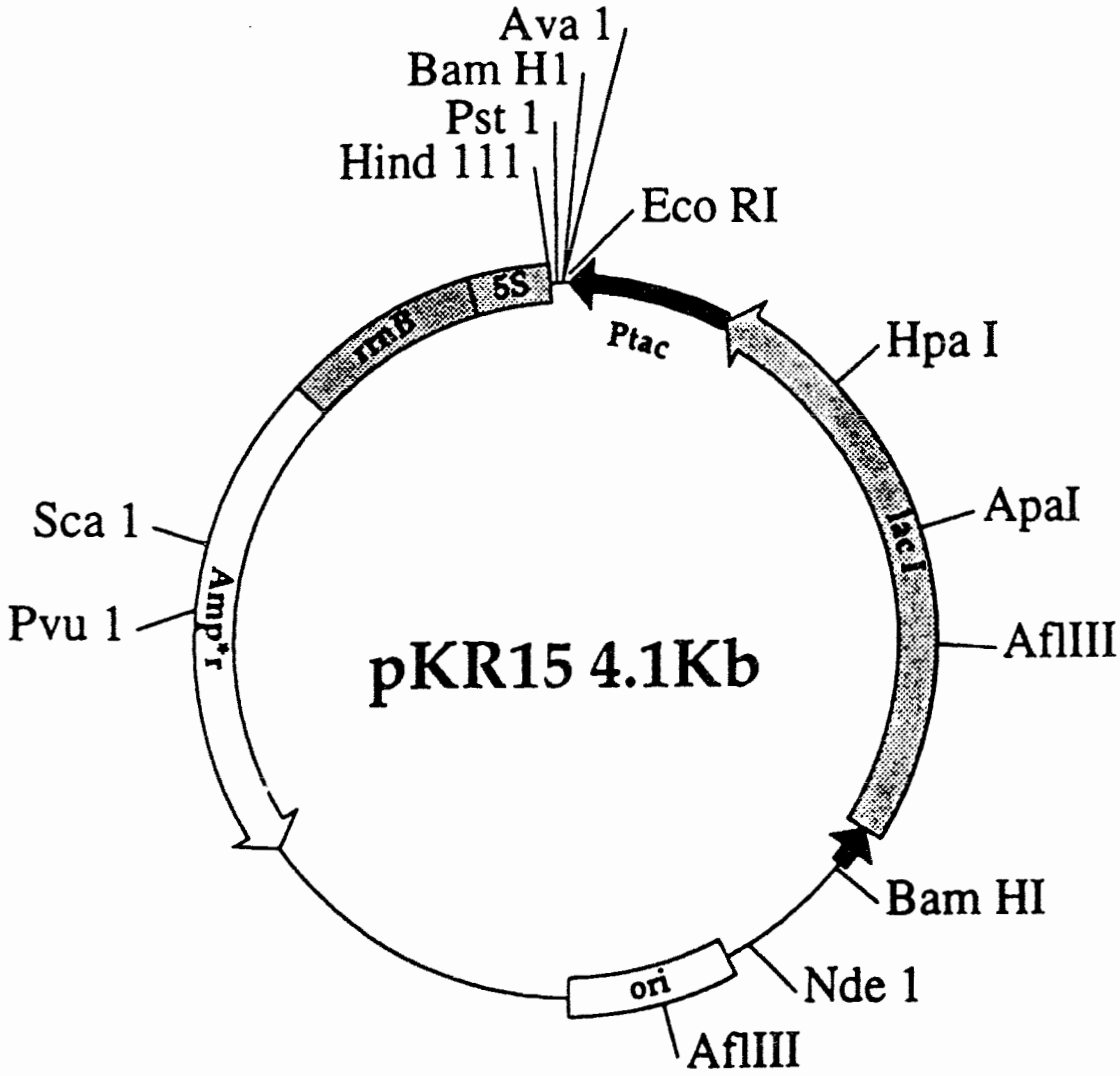
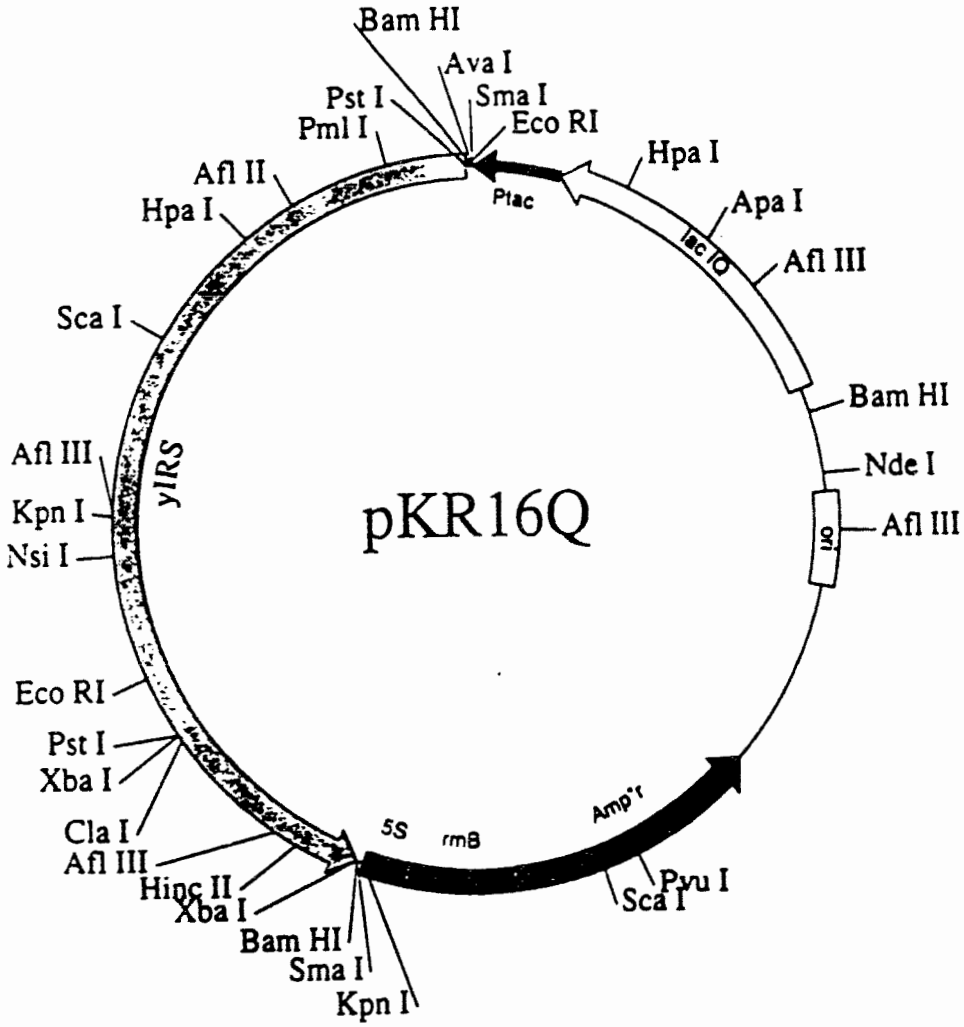
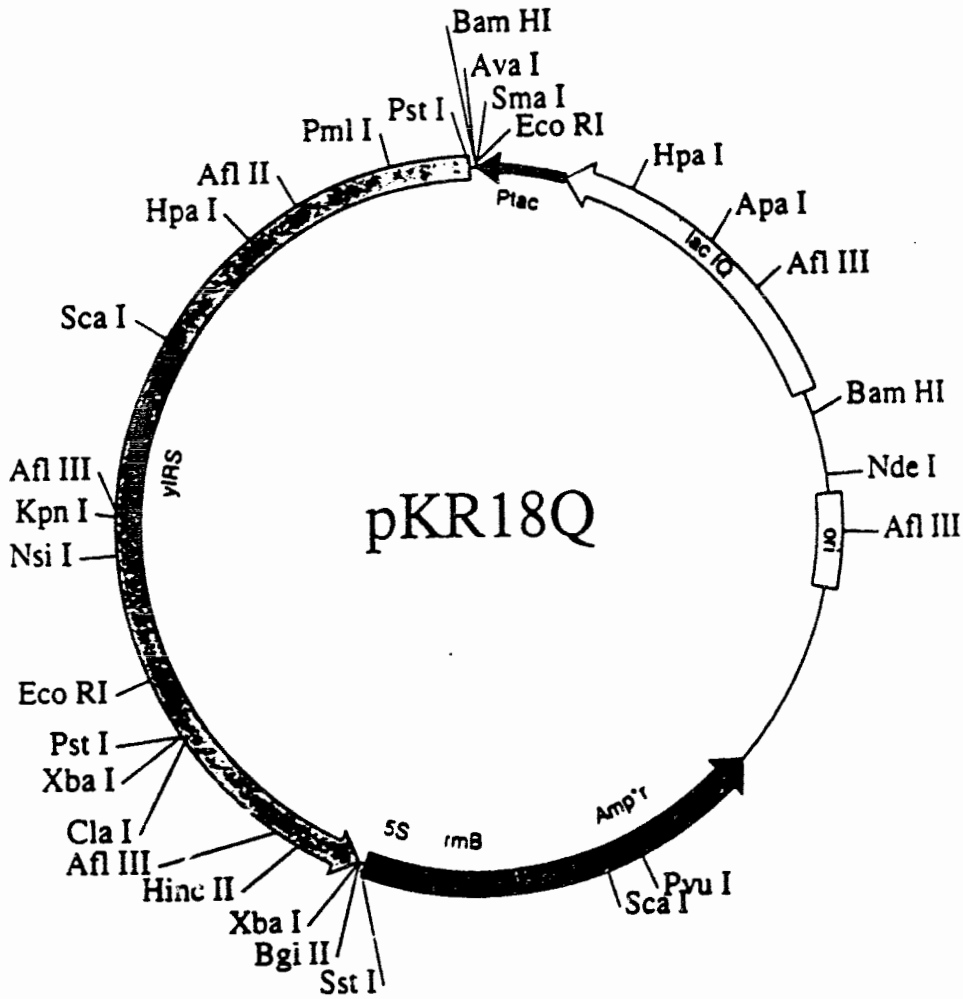

Ava 1
Bam H1
Pst 1
Hind 111
Eco RI
5S
rrnB
Ptac
Hpa I
ApaI
AflIII
Bam HI
Nde 1
AflIII
ori
lacI
Amp*r
Sca 1
Pvu 1
pKR15 4.1Kb

Figure A18: Regulatory Sequences and Partial Restriction Map of the Expression Plasmid, pKR16Q. pKR16Q is a derivative of pKR15Q containing the yIRS gene. The yIRS gene, with its altered ribosome-binding site (see section 2.4.1.2), is expressed under the *ptac* promoter.



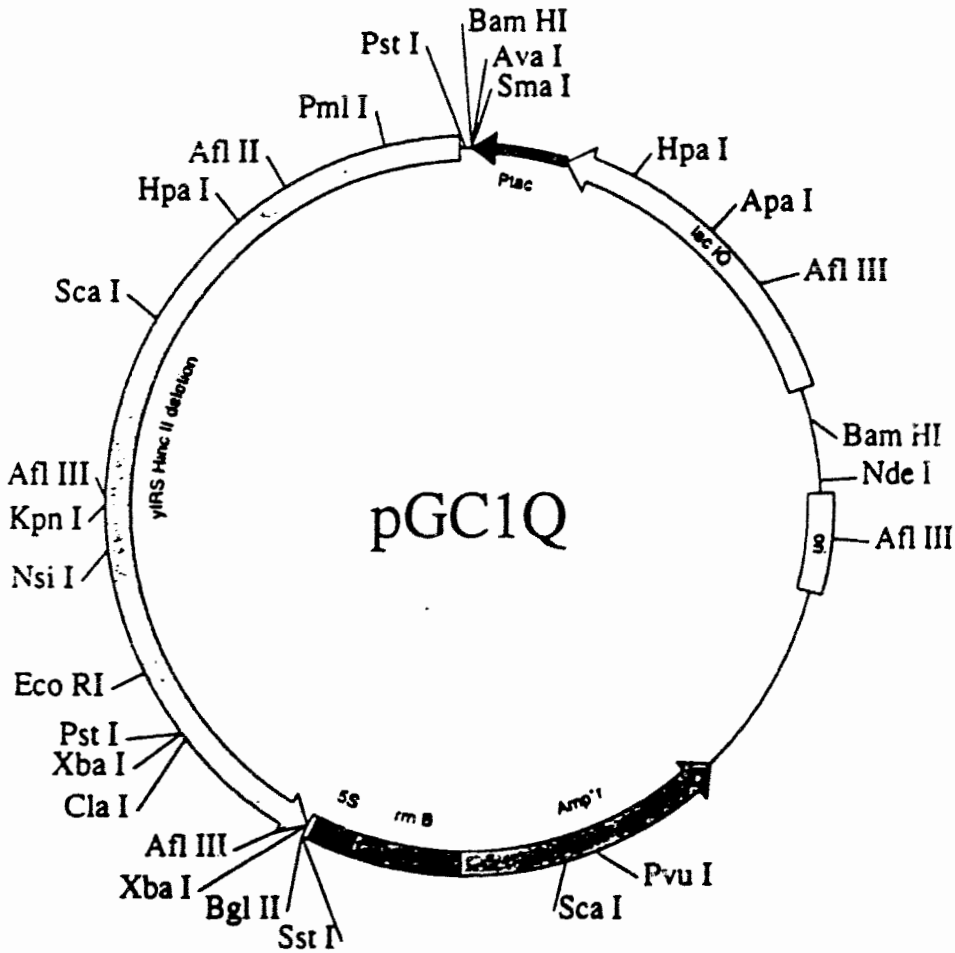
M_r value for yIRS: 122983 Da

Figure A19: Regulatory Sequences and Partial Restriction Map of the Expression Plasmid, pKR18Q. The plasmid pKR18Q is essentially identical to pKR16Q except for the changes in the polylinker site that is downstream from the yIRS gene. In the place of the restriction sites Bam HI, Sma I and Kpn I in pKR16Q, these sites were altered to Bgl II and Sst I restriction sites in pKR18Q.



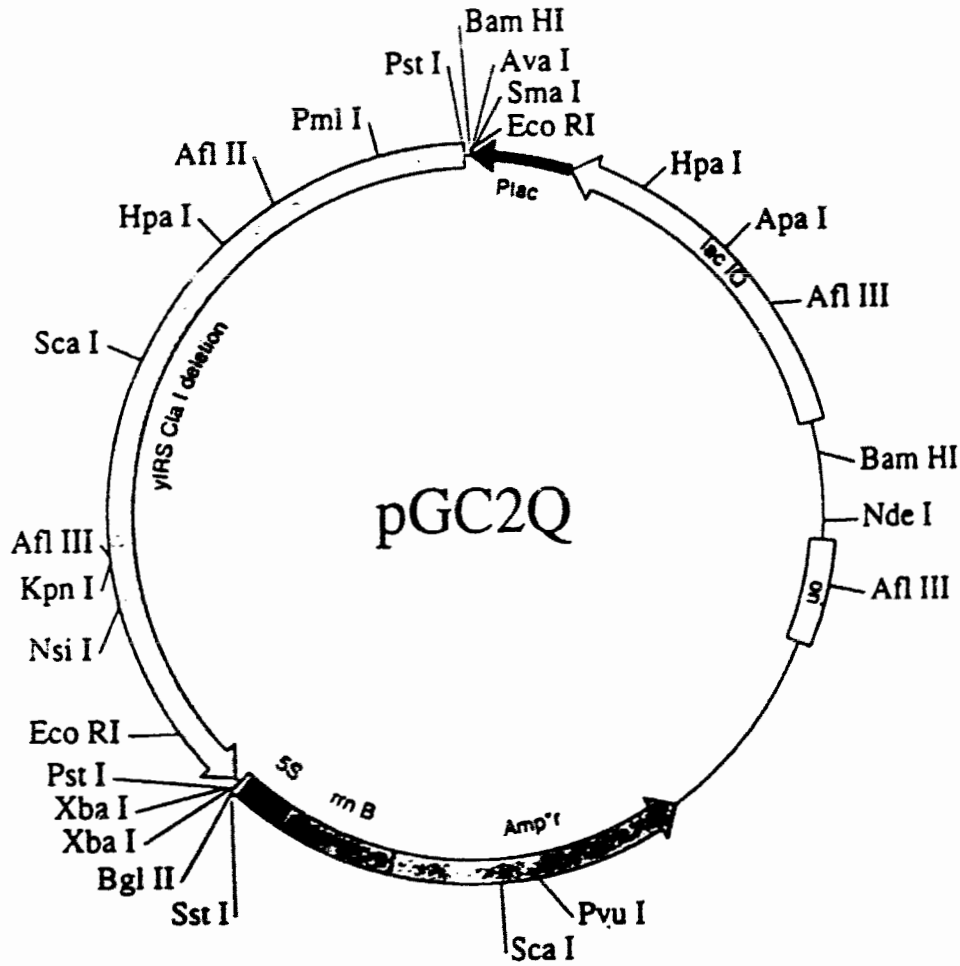
M_r value for yIRS: 122983 Da

Figure A20: Regulatory Sequences and Partial Restriction Map of the Expression Construct, pGC1Q. The yIRS Hinc II deletion gene from pLcII-Hinc II was subcloned into pKR18Q to create the plasmid pGC1Q. Expression of the protein is under the control of the constitutive lac I^q gene and the *ptac* promoter.



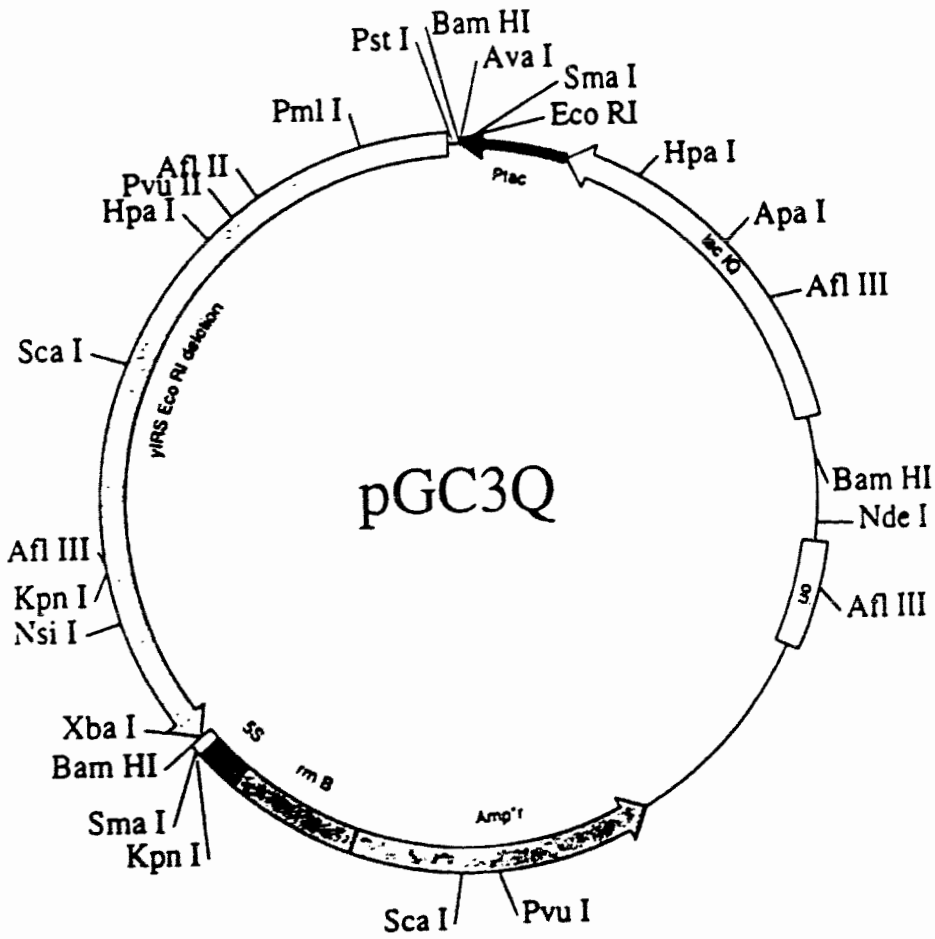
M_r value for yIRS Hinc II Deletion Protein: 113817 Da

Figure 21: Regulatory Sequences and Partial Restriction Map of the Expression Construct, pGC2Q. The yIRS Cla I deletion gene was subcloned to pKR18Q to improve the expression of mutant protein.



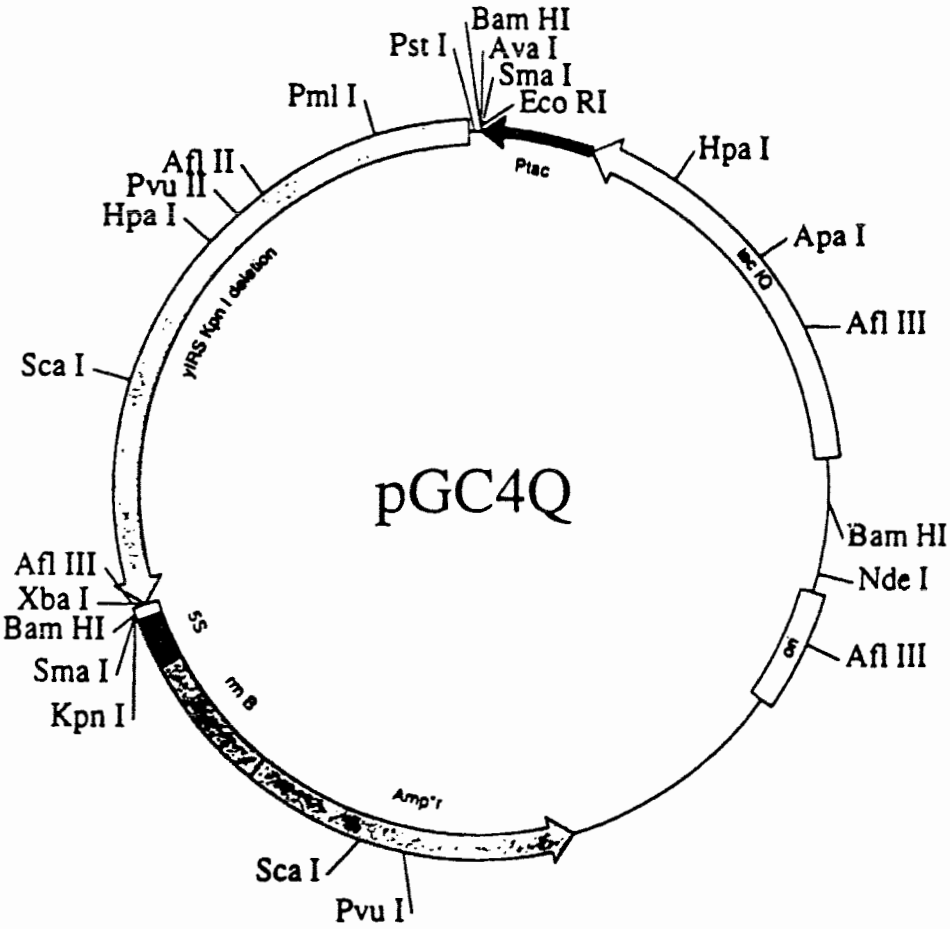
M_r value for yIRS Cla I Deletion Protein: 96167 Da

Figure A22: Regulatory Sequences and Partial Restriction Map of the Expression Construct, pGC3Q. The plasmid, pGC3Q, was derived from pKR16Q and contains the yIRS Eco RI deletion gene under the control of the lac I^q gene and the *ptac* promoter.



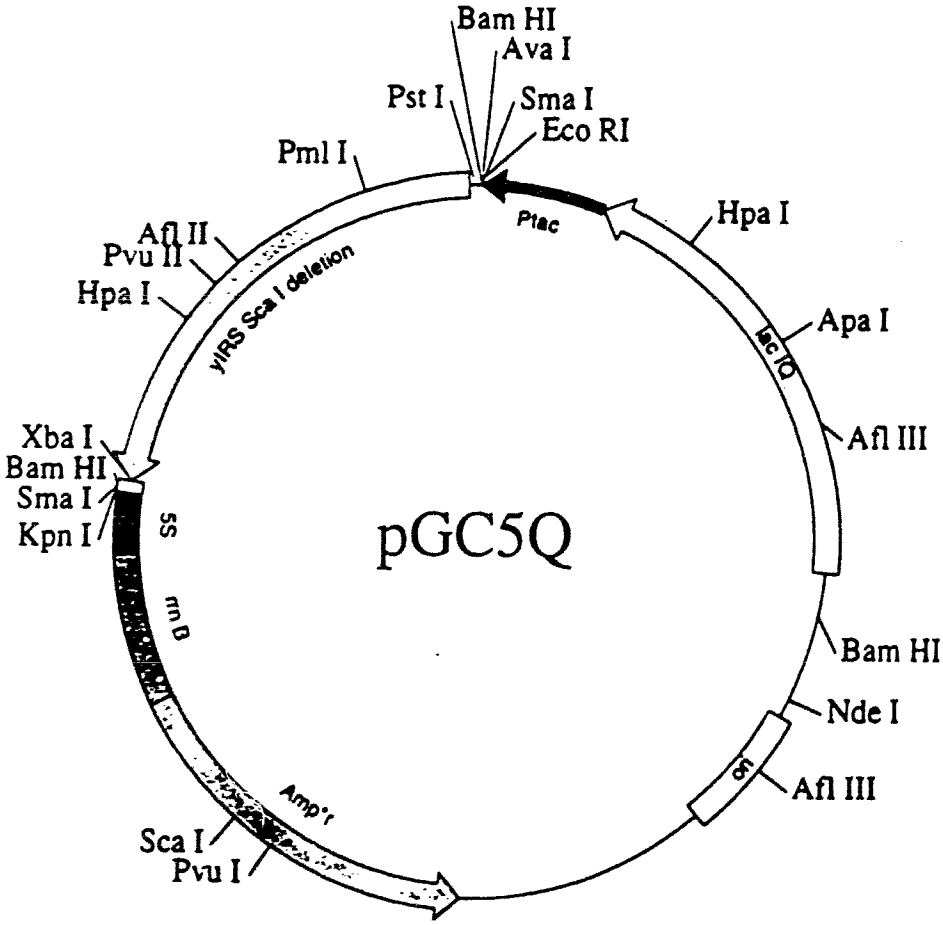
M_r value for yIRS Eco RI Deletion Protein: 87685 Da

Figure A23: Regulatory Sequences and Partial Restriction Map of the Expression Construct, pGC4Q. The yIRS Kpn I deletion gene was cloned into the expression vector pKR16Q.



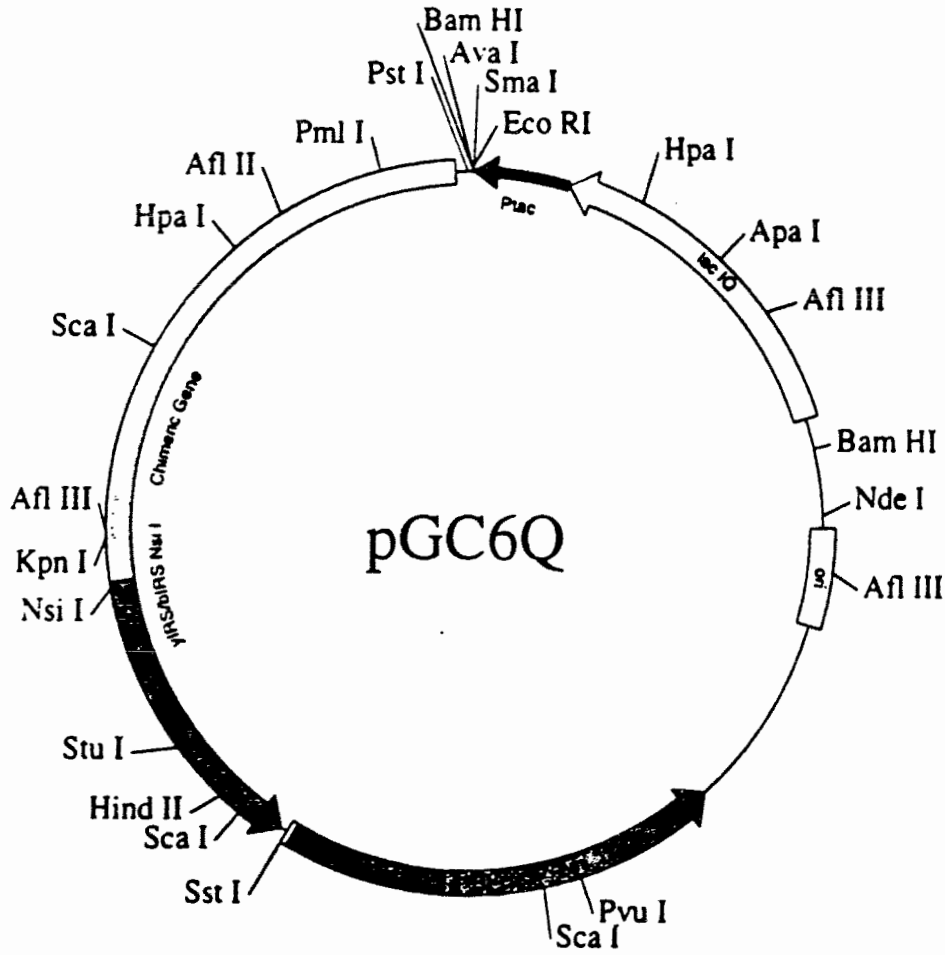
M_r value for yIRS Kpn I Deletion Protein: 66892 Da

Figure A24: Regulatory Sequences and Partial Restriction Map of the Expression Construct, pGC5Q. The plasmid, pGC5Q, is a derivative of pKR16Q containing the yIRS Sca I deletion gene.



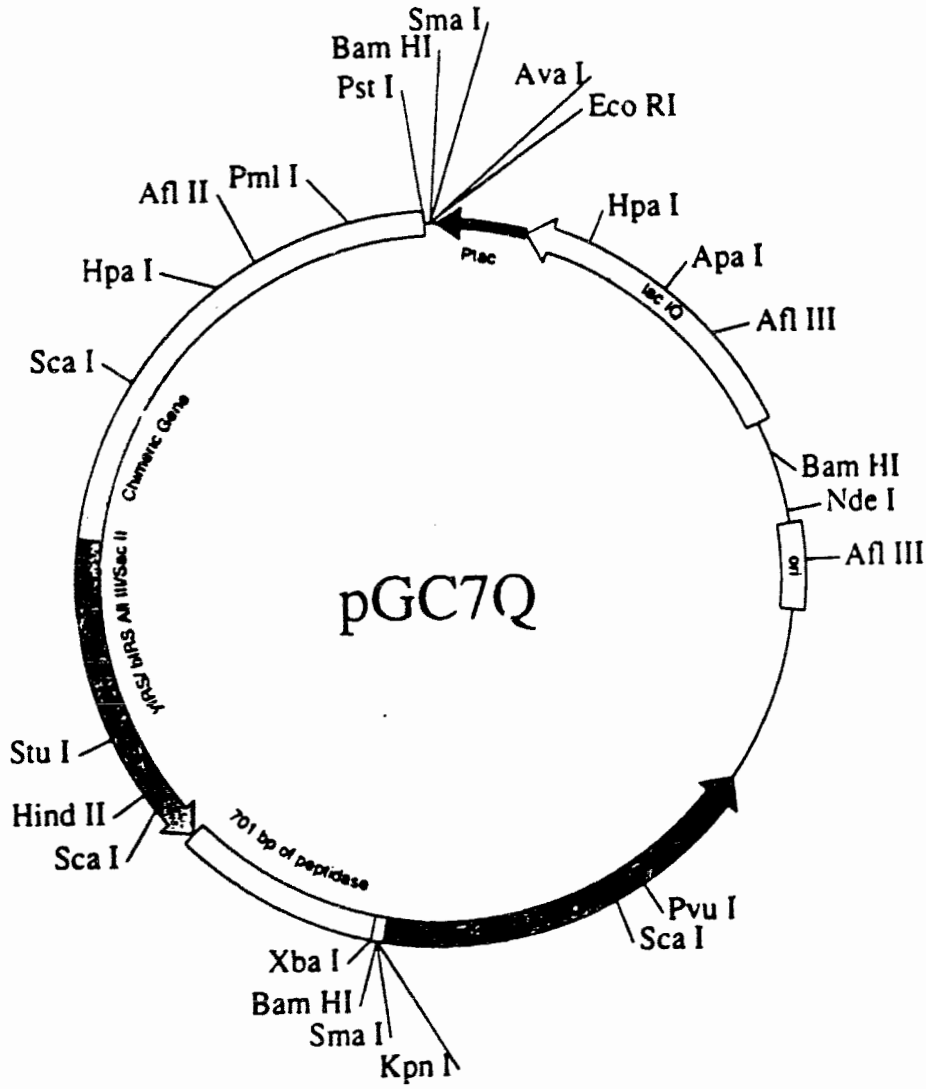
M_r value for yIRS Sca I Deletion Protein: 44089 Da

Figure A25: Partial Restriction Map and Regulatory Sequences of the Expression Construct, pGC6Q. The plasmid, pGC6Q, is derived from pKR16Q and contains the Nsi I chimeric gene. The light gray represents the yIRS portion of the chimeric gene and the dark gray represents the bIRS portion of the gene.



M_r value for Nsi I Chimera: 107254 Da

Figure A26: Partial Restriction Map and Regulatory Sequences of the Expression Plasmid pGC7Q. The Afl III/Sac II chimeric gene from the plasmid pKR7 was subcloned into pKR16Q to improve the expression of the chimera. The light gray represents the yIRS portion of the chimeric gene and the dark gray represents the bIRS portion of the gene.



M_r value for Afl III/Sac II Chimera: 107886 Da

APPENDIX B: PLASMID CLONING PROCEDURES

B.1 pEND (Cloning vector)

pEND was constructed by Kalmar (39) as follows: pUC19 was first digested with Sst I (Sac I) and Bam HI thereby eliminating the Sma I/Xma I and Kpn I sites from the plasmid and the termination sequence was added as shown below.

Poly-linker region of pUC19:

```
                        Xma I                   Acc I
          Sac I         Sma I           Xba I   Hinc II   Pst I              Hind III
     Eco RI        Kpn I        Bam HI                 Sal I           Sph I
5'~396GAATTCGAGCT CGGTACCCGGGG ATCCTCTAGAGTCGACCTGCAGGCATGCAAGCTTGGC453~3'
3'    CTTAAGC TCGAGCCATGGGCCCCTAGGA GATCTCAGCTGGACGTCCGTACGTTCGAACCG    ~5'
```

```
5'~GAGCTCCCGGGTAAGTAACTAGGATCC~3'
3'~CTCGAGGGCCCAATCATTGATCCTAGG~5'

  Sac I                   Bam HI
       Sma I
```

pUC19 bases are shown in plain text and the termination sequence is depicted in bold. The termination codons are doubly underlined. The Sac I and Bam HI sites are part of the poly-linker region of pUC19 and the Sma I site was re-introduced in the termination sequence.

B.2 pEND-ITS (Cloning Vector)

The pEND plasmid was cleaved at the single Hind III site and the sticky ends were filled in. Upon re-ligation of the plasmid, the Hind III site was destroyed, thus creating the intermediate plasmid pEND(Hind III$^-$). pEND(Hind III$^-$) was then digested with Eco RI, blunt ended and the 2.6 kb fragment of the yIRS gene, which was cut from the plasmid pKR3 with the restriction enzymes Pvu I and Cla I (then blunt ended), was subcloned into pEND(Hind III$^-$) to create the plasmid designated pEND-ITS.

B.3 pLcII-Hinc II (yIRS C-terminal Deletion Protein Expression Vector)

A 3' deletion of the yIRS gene at the Hinc II site at base #2976 of the yIRS gene was engineered in the following manner: The pEND vector was initially digested with Eco RI and Sma I, thereby excising the Sac I site from the plasmid. pKR3 was cleaved at the Eco RI and Hinc II sites and a 0.7 kb fragment containing bases #2266-#2976 of the yIRS gene was isolated. This 0.7 kb fragment was then subcloned into the cleaved pEND vector. This plasmid was designated pEND(Eco RI/Hinc II).

pEND(Eco RI/Hinc II) was later cut at the Cla I and Bam HI sites and a 0.5 kb fragment was recovered. pKR3 was digested at the Cla I and Bam HI sites and the 0.5 kb fragment from pEND(Eco RI/Hinc II), which contains bases #2493-#2976 of the yIRS gene. was subcloned into the pKR3 vector fragment to reconstruct the yIRS 3' Hinc II deletion gene. This plasmid (pEND-Hinc II) contains the intact 5' consensus ribosome-binding region and the 5' coding region of the yIRS gene up to the Hinc II deletion at base #2976. The transcription stop signal codon for this 3' mutant gene was provided by the termination sequence of the pEND vector (see below).

3' end of the Hinc II deletion mutant gene:

Native base sequence:

```
        glu  leu  val  asn  arg
5'~2968GAGCTGGTC ACCAGA2982~3'
3'~     CTCGACCAG TGGTCT     ~5'
              Hinc II
```

pEND termination sequence:

```
5'~CCC GGGTAAGTAACTAG~3'
3'~GGG CCCAATCATTGATC~5'
       Sma I
```

```
        glu  leu  val  gly  stop
5'~2968GAGCTGGTCGGGTAAGTAACTAG~3'
3'~     CTCGACCAGCCCAATCATTGATC~5'
```

Reconstructed 3' end of the Hinc II deletion mutant gene.

pEND-Hinc II was then cleaved at the Hind III sites to excise the 3 kb 5' non-coding and the coding region of the yIRS Hinc II deletion mutant gene. This 3 kb fragment was then digested with Bam HI to yield a Bam HI sticky end at the 3' end of Hinc II deletion mutant gene. The plasmid, pLmp10 was first digested with Eco RI, blunt ended and digested with Bam HI. This excised the nut R, tR1 and a portion of the cII genes from the plasmid and a vector fragment was recovered. The 3 kb Hinc II deletion gene was then subcloned as a cassette into the pLmp10 vector fragment to create pLcII-Hinc II.

B.4 pLcII-Cla I (yIRS C-terminal Deletion Protein Expression Vector)

A 3' deletion of the yIRS gene at the single Cla I site at base #2492 was constructed as follows: pKR3 was initially cut with Hind III and Cla I and blunt ended. A 2.5 kb fragment containing the 3' deleted yIRS gene was isolated. pEND-ITS was cut with Hind III and Sma I and the pEND-ITS vector fragment was isolated (without the yIRS gene fragment). The 2.5 kb fragment of the yIRS gene from pKR3 was then ligated to the pEND vector fragment to create pEND-Cla I. pEND-Cla I contains the 5' non-coding consensus ribosome-binding region and the coding region of the yIRS gene up to the Cla I site at base #2942. The transcription stop signal for the Cla I deletion mutant gene was provided by the termination sequence of the pEND vector (see below).

3' end of the Cla I deletion mutant gene:

Native base sequence:

ser val ile asp leu
5'~^{2485}TCCGTTAT CGATTTG^{2499}~3'
3'~ AGGCAATAG CTAAAC ~5'
Cla I

fill in sticky end

5'~^{2485}TCCGTTATCG
3'~ AGGCAATAGC

pEND termination sequence:

5'~CCC GGGTAAGTAACTAG~3'
3'~GGG CCCAATCATTGATC~5'
Sma I

ser val ile gly val ser asn stop
5'~^{2485}TCCGTTATCGGGGTAAGTAACTAG~3'
3'~ AGGCAATAGCCCCAATCATTGATC~5'

Reconstructed 3' end of the Cla I deletion mutant gene.

pEND-Cla I was subsequently digested with Hind III, blunt ended and then digested with Bam HI to excise the 2.5 kb 3' Cla I deletion mutant gene. pLmp10 was cleaved with Eco RI, blunt ended and cut Bam HI as described in section 2.4.1.12. The 2.5 kb fragment was subcloned into the cleaved pLcII plasmid to create pLcII-Cla I.

B.5 pLcII-Eco RI (yIRS C-terminal Deletion Protein Expression Vector)

The third 3' deletion mutant of the yIRS gene, named the Eco RI deletion mutant, was constructed in the following manner: The plasmid, pKR3, was cleaved at the Eco RI site and the sticky end were filled in with T4 polymerase, followed by cutting at the Hind III site. This resulted in the 2.2 kb 3' Eco RI deletion mutant gene which was cloned into the pEND-ITS vector fragment (see section 2.4.1.13) to create pEND-Eco RI. pEND-Eco RI contains the coding region of the yIRS gene from base $^{\#}$1-$^{\#}$2269 and the 5' consensus ribosome-binding sequence. The transcription termination signal was provided by the termination sequence of the pEND vector as shown below:

3' end of the Eco RI deletion mutant gene:

Native base sequence:

ala leu asn ser leu
5'~^{2260}GCATTG AATTCTTTA^{2275}~3'
3'~ CGTAACTTAA GAAAT ~5'
Eco RI

pEND termination sequence:

5'~CCC GGGTAAGTAACTAG~3'
3'~GGG CCCAATCATTGATC~5'
Sma I

fill in sticky end

5'~^{2260}GCATTGAATT
3'~ CGTAACTTAA

ala leu asn trp val ser asn stop
5'~^{2260}GCATTGAATTGGGTAAGTAACTAG~3'
3'~ CGTAACTTAACCCAATCATTGATC~5'

Reconstructed 3' end of the Eco RI deletion mutant gene.

pEND-Eco RI was then cleaved with Hind III and blunt ended, followed by digestion with Bam HI to excise the 2.2 kb 3' Eco RI deletion mutant gene. pLmp10 was digested as described in section 2.4.1.13 and the 2.2 kb Eco RI deletion mutant gene was inserted into the pLcII vector fragment to yield the plasmid pLcII-Eco RI.

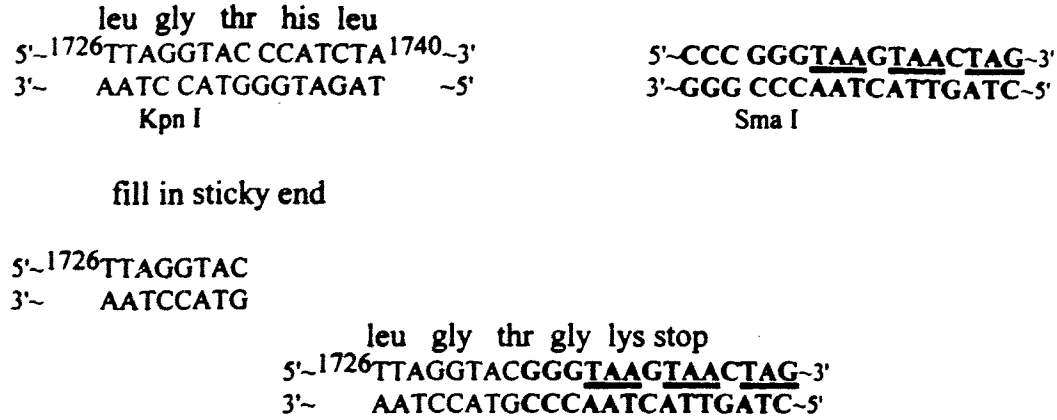
B.6 pLcII-Kpn I (yIRS C-terminal Deletion Protein Expression Vector)

The 3' Kpn I deletion mutant gene of yIRS was engineered in the following manner: pKR3 was initially cleaved at the Kpn I site, blunt ended and then cleaved at the Hind III site. A 1.8 kb fragment of the yIRS gene from bases #1-#1734 and the 5' ribosome-binding sequence was isolated and subcloned into the pEND-ITS vector fragment (see section 2.4.1.13) to create pEND-Kpn I. The transcription stop signal was provided by the termination sequence of the pEND vector.

3' end of the Kpn I deletion mutant gene:

Native base sequence:

pEND termination sequence:



Reconstructed 3' end of the Kpn I deletion mutant gene.

pEND-Kpn I was later digested with Hind III, blunt ended and digested with Bam HI to isolate the reconstructed 3' Kpn I deletion mutant gene with the stop codon. This 1.8 kb fragment was subsequently cloned into the pLcII vector fragment (see section 2.4.1.13) to create pLcII-Kpn I.

B.7 pLcII-Sca I (yIRS C-terminal Deletion Protein Expression Vector)

The smallest yIRS 3' deletion mutant was derived from the following: pKR3 was digested at the Sca I and Hind III sites and a 1.2 kb fragment of the yIRS gene which includes the 5' consensus ribosome-binding sequence and bases 1-1141 of the yIRS gene coding sequence was isolated. This 1.2 kb fragment was subcloned into the pEND-ITS vector fragment (see section 2.4.1.13) to create pEND-Sca I. The termination signal was provided by the pEND vector's termination sequence (see below):

3' end of the Sca I deletion mutant gene:

Native base sequence:

```
        ile  lys  tyr  leu  thr
5'~1135ATTAAGT ACTTAACT1149~3'
3'~     TAATTCA TGAATTGA     ~5'
           Sca I
```

pEND termination sequence:

```
5'~CCC GGGTAAGTAACTAG~3'
3'~GGG CCCAATCATTGATC~5'
      Sma I
```

```
        ile  lys  trp  val  ser  asn  stop
5'~1135ATTAAGTGGGTAAGTAACTAG~3'
3'~     TAATTCACCCAATCATTGATC~5'
```

Reconstructed 3' end of the Sca I deletion mutant gene.

pEND-Sca I was cleaved at the Hind III site, blunt ended and cut at the Bam HI site to isolate the 1.2 kb fragment of the yIRS 3' Sca I deletion mutant along with the termination sequence. This 1.2 kb fragment was then cloned as a cassette into the pLcII vector fragment (see section 2.4.2.13) to create pLcII-Sca I.

APPENDIX C: BIO-RAD Affi-Gel Hz IMMUNO-AFFINITY KIT PROCEDURE

APPENDIX C: BIO-RAD Affi-Gel Hz IMMUNO-AFFINITY KIT PROCEDURE

Buffer Exchange - The FPLC purified antibody sample was subjected to a buffer exchange with 1X Coupling Buffer (100 mM NaOAc and 150 mM NaCl, pH 5.5) using the Econo-Pac 10DG Desalting column provided by the Bio-RAD Affi-Gel Hz Immunoaffinity Kit (catalog #153-6060). The column was first washed with 5 column volumes of 1X Coupling Buffer before the application of 3 mL of concentrated, purified monoclonal antibody. The first 3.0 mL was drained into the column and the first 3.0 mL eluted from the column was collected. The antibody was eluted using the 1X Coupling Buffer in 0.5 mL fractions and the absorbance of each fraction was monitored by UV. Spectroscopy at 280 nm. Fractions containing protein were pooled. The desalting column was then washed with 5 column volumes of 1X Coupling Buffer and stored in 5 mL of 1X Coupling Buffer supplemented with 0.02% w/v sodium azide at 4°C.

Oxidation of Monoclonal Antibody - Oxidation of the monoclonal antibody was accomplished using sodium periodate. 25 mg of $NaIO_4$ was dissolved in 1.2 mL ddH_2O. In the absence of light, 450 µL of this periodate solution was added to 4.5 mL of antibody in a 15 mL falcon tube wrapped with tin foil. The antibody/periodate mixture was mixed using a rotating end-over-end shaker for 1 hour at room temperature.

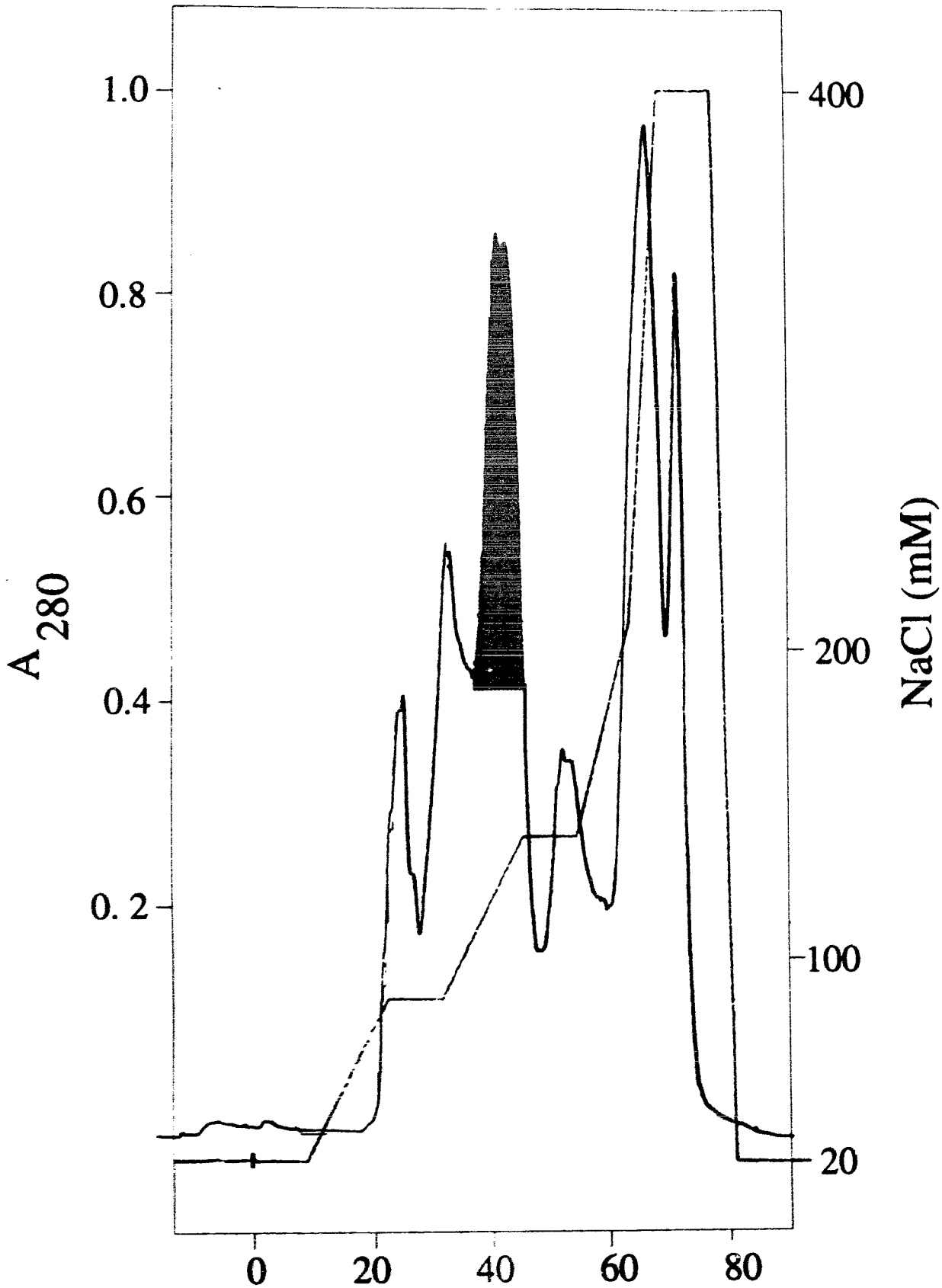
Buffer-Exchange of Oxidized Antibody - Immediately after oxidation, the oxidized antibody solution was subjected to a buffer exchange to remove the periodate. The oxidized antibody was loaded onto the Econo-Pac 10DG column (which was previously washed with 5 column volumes of 1X Coupling Buffer) in 3.0 mL volumes (see section 2.10.5.1.1). The 3.0 mL sample was drained into the column and the elutent was collect in 0.5 mL fractions. The sample was eluted from the column with 1X Coupling Buffer and collected in 0.5 mL fractions. The elution of protein was monitored by UV. spectroscopy at 280 nm and fractions containing

protein up to the protein peak were pooled. Fractions collected after the protein peak was discarded to prevent periodate contamination of antibody sample.

Immobilization of the Antibody to the Hydrazine-Activated Agarose Matrix - The Affi-gel Hz agarose beads were washed with 1X Coupling Buffer to remove the isopropanol. The oxidized desalted antibody was added to the agarose and the tube was sealed using parafilm. The reaction solution was incubated at room temperature for 24 hours using an end-over-end rotating shaker. After 24 hours, the slurry was poured into a 1.0 cm X 10 cm Econo column. The column was washed with 1 column volume of buffer D which contains 0.5 M NaCl. The elutent from the column was collected to determined the efficiency of antibody binding to the matrix.

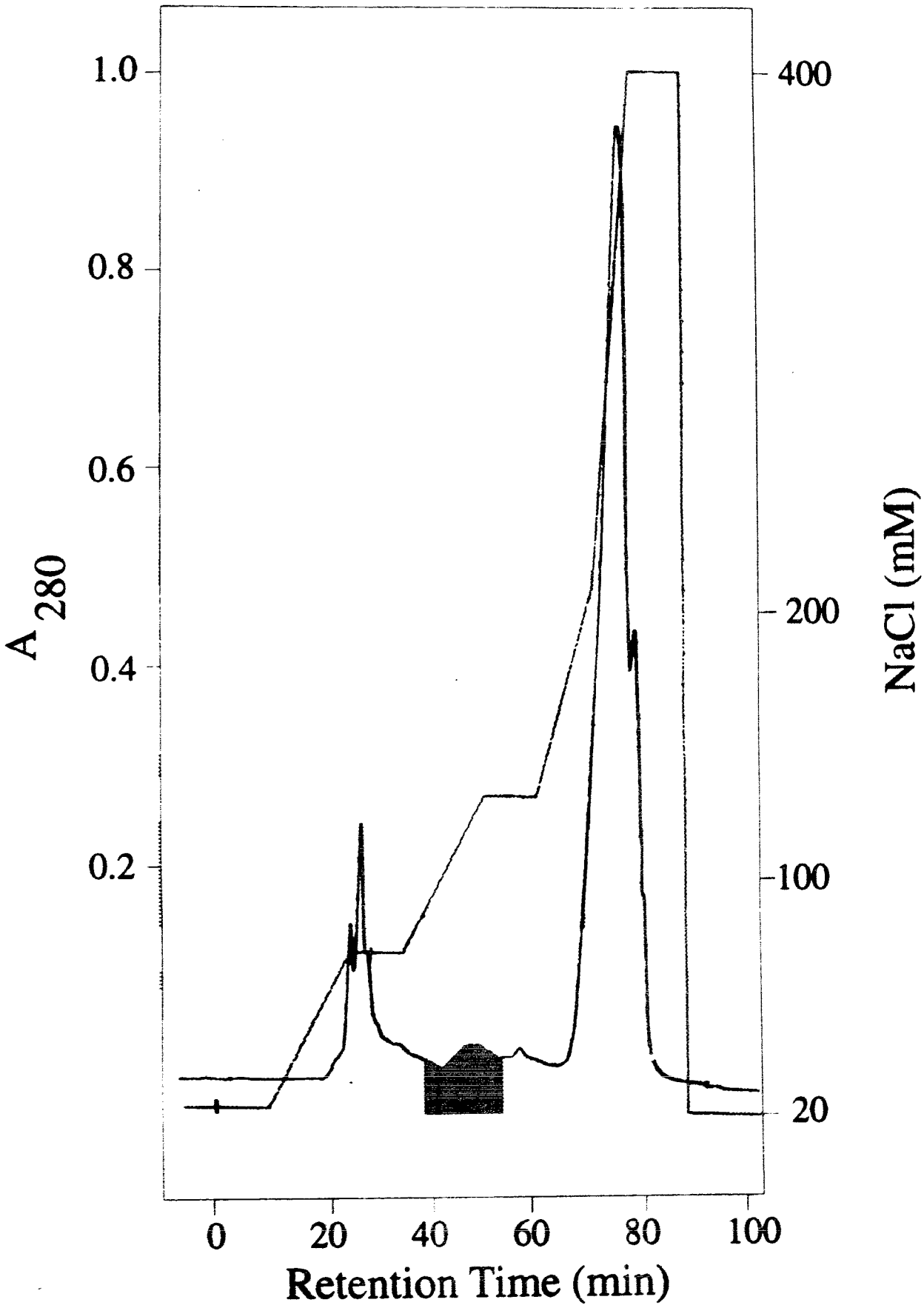
APPENDIX D: MONOCLONAL ANTIBODY PURIFICATION - ELUTION PROFILES

Figure A27: Purification of Monoclonal Antibody Y1 by FPLC. Ascitic fluid containing the monoclonal antibody, Y1, was first subjected to 50% ammonium sulphate precipitation, followed by dialysis (as described in section 2.10.4), prior to loading onto a Waters Protein-Pak 8HR AP-1 column. The peaks containing active antibody were identified by ELISA. Active Y1 antibody was eluted with 100 mM NaCl. Shaded peaks show the fractions collected.



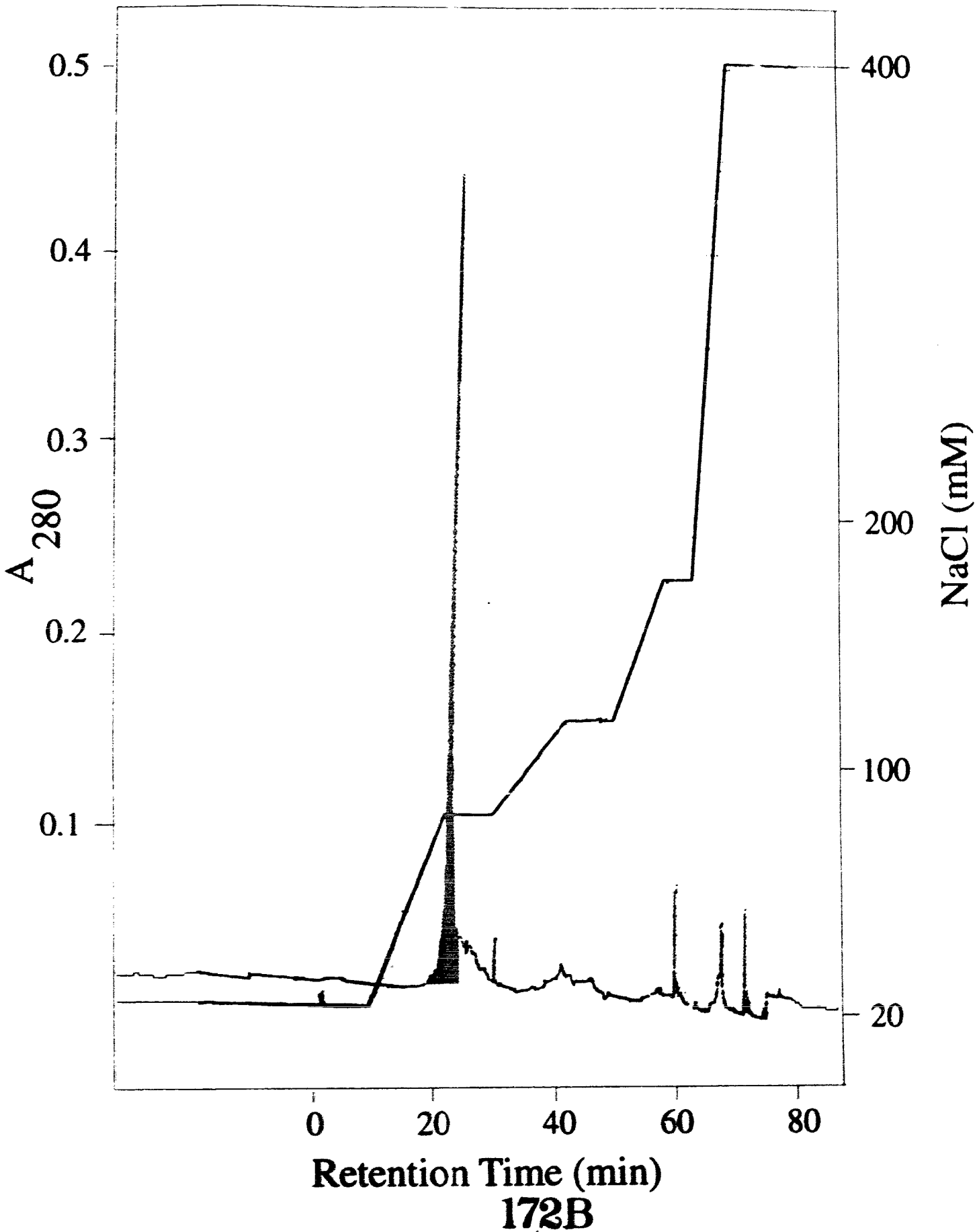
170B

Figure A28: Purification of Monoclonal Antibody Y11 by FPLC. A sample of Y11 antibody ,which was previously precipitated with 50% ammonium sulphate and dialyzed to remove the salt, was loaded onto a Waters Protein-Pak Q 8HR AP-1 column. The peaks containing active Y11 antibody were identified by ELISA. The shaded peaks denotes the fractions pooled. Y11 eluted earlier in the purification than Y1, with approximately 90 mM NaCl.


1.0
0.8
0.6
0.4
0.2
A 280
400
200
100
20
NaCl (mM)
0
20
40
60
80
100
Retention Time (min)

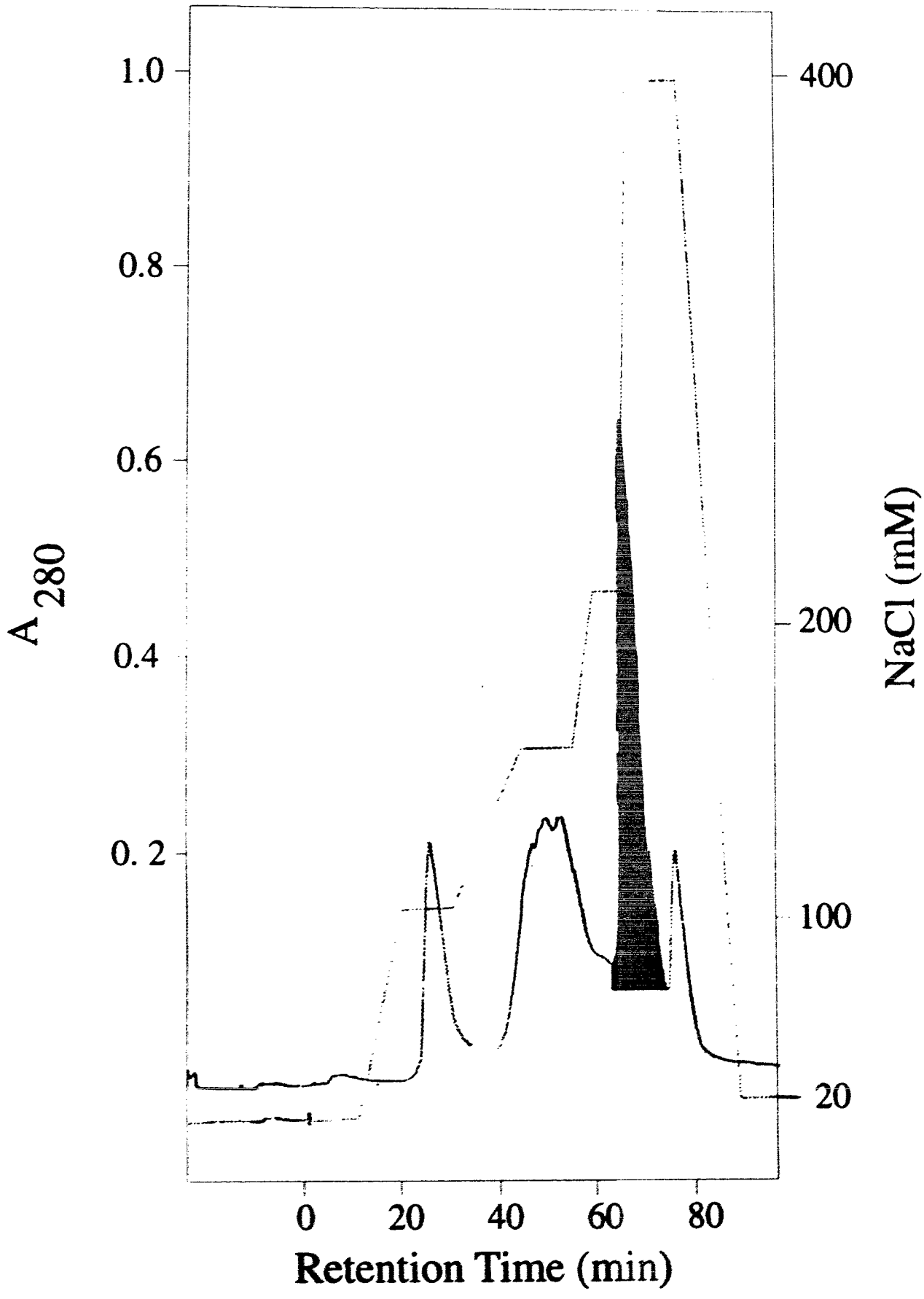
171B

Figure A29: Purification of Monoclonal Antibody Y12 by FPLC. Ascites fluid containing Y12 antibody was first precipitated with 50% ammonium sulphate as described in section 2.10.4. The dialyzed sample was loaded onto a Pharmacia MonoQ column for purification. A single sharp peak was eluted with approximately 90 mM NaCl. Subsequent ELISA assays identified this peak (shaded in the figure) as the active antibody peak. Fractions containing the antibody were pooled.


0.5
0.4
0.3
0.2
0.1
A280
400
200
100
20
NaCl (mM)
0
20
40
60
80
Retention Time (min)

172B

Figure A30: Purification of Monoclonal Antibody B1B2 by FPLC. A sample of B1B2 antibody isolated from tissue culture was first dialyzed to remove the DME media. After dialysis, the sample was loaded onto a Water Protein-Pak Q 8HR AP-1 column. Protein peaks were assayed by ELISA to identify active B1B2 antibody. The active B1B2 antibody was found to elute later in the purification scheme than the previous three monoclonal antibodies, with 210 mM NaCl. Pooled peak fractions are shaded in black.


1.0
0.8
0.6
0.4
0. 2
A 280
400
200
100
20
NaCl (mM)
0
20
40
60
80
Retention Time (min)

173B

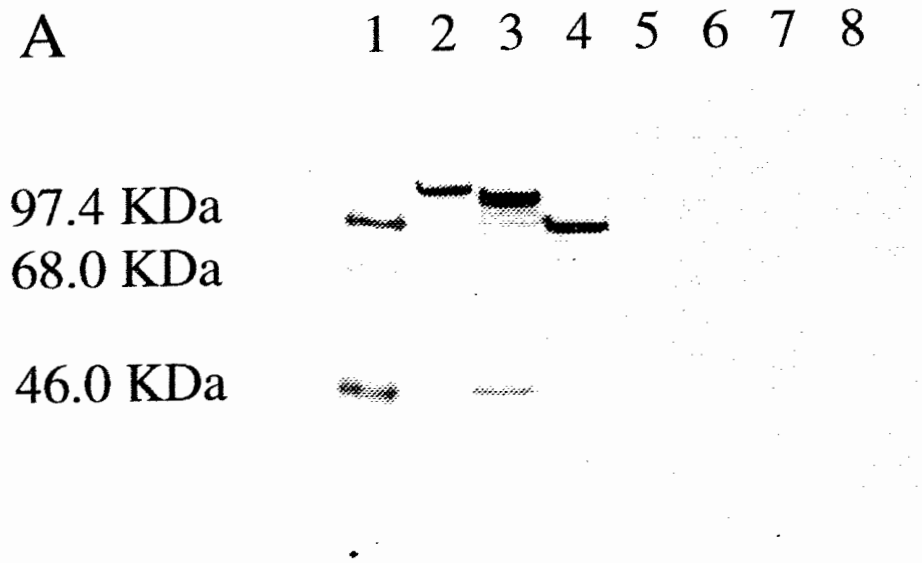
APPENDIX E: WESTERN IMMUNOBLOTS USING MONOCLONAL ANTIBODIES

Figure A31: Western Immunoblot Analysis of the Binding Site of Monoclonal Antibody Y1. The following proteins were probed with monoclonal antibody Y1. Note that the plasmid name is in square brackets after the protein it expresses.

A: Crude cells lysates of *E. coli* strain DH5α expressing the yIRS C-terminal deletion mutants. The lanes designations are as follows: E.C.L. (Amersham) Molecular Weight Standard (lane 1), purified yIRS (lane 2), Hinc II deletion protein [pGC1Q] (lane 3), Cla I deletion protein [pGC2Q] (lane 4), Eco RI deletion protein [pGC3Q] (lane 5), Kpn 1 deletion protein [pGC4Q] (lane 6), Sca I deletion protein [pGC5Q] (lane 7) and purified bIRS (lane 8).

B: Crude cell lysates of *E. coli* strain TG2 and DH5α expressing the chimeric IleRS enzymes. The lane assignments are as follows: purified yIRS (lane 1), Hpa I/Hpa I Chimera [pKR5] (lane 2), Afl III/Sac II Chimera [pKR7] (lane 3), Nsi I Chimera [pGC6Q] (lane 4), E.C.L. (Amersham) Molecular Weight Standard (lane 5), Bgl II/Pvu II [pKR8] (lane 6), Stu I/Cla I [pKR9] (lane 7) and purified bIRS (lane 8).

A



B

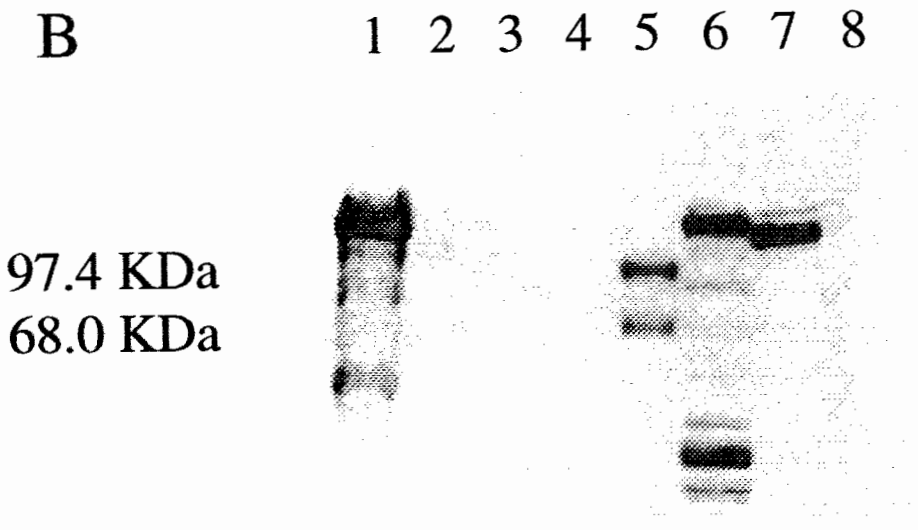
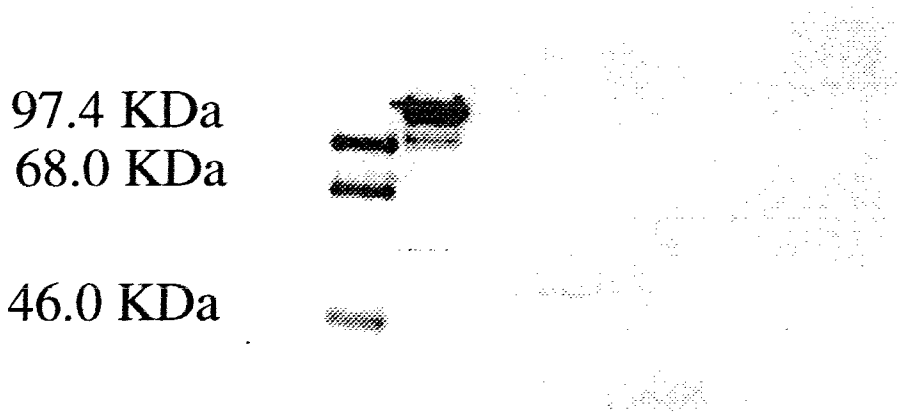


Figure A32: Western Immunoblot Analysis of the Binding Site of Monoclonal Antibody Y11. The following proteins were probed with monoclonal antibody Y11. Note that the plasmid name is in square brackets after the protein it expresses.

A: Crude cells lysates of *E. coli* strain DH5α expressing the yIRS C-terminal deletion mutants. The lanes designations are as follows: E.C.L. (Amersham) Molecular Weight Standard (lane 1), purified yIRS (lane 2), Hinc II deletion protein [pGC1Q] (lane 3), Cla I deletion protein [pGC2Q] (lane 4), Eco RI deletion protein [pGC3Q] (lane 5), Kpn 1 deletion protein [pGC4Q] (lane 6), Sca I deletion protein [pGC5Q] (lane 7) and purified bIRS (lane 8).

B: Crude cell lysates of *E. coli* strain TG2 and DH5α expressing the chimeric IleRS. The lane assignments are as follows: purified yIRS (lane 1), Hpa I/Hpa I Chimera [pKR5] (lane 2), Afl III/Sac II Chimera [pKR7] (lane 3), Nsi I Chimera [pGC6Q] (lane 4), E.C.L. (Amersham) Molecular Weight Standard (lane 5), Bgl II/Pvu II [pKR8] (lane 6), Stu I/Cla I [pKR9] (lane 7) and purified bIRS (lane 8).

A



B

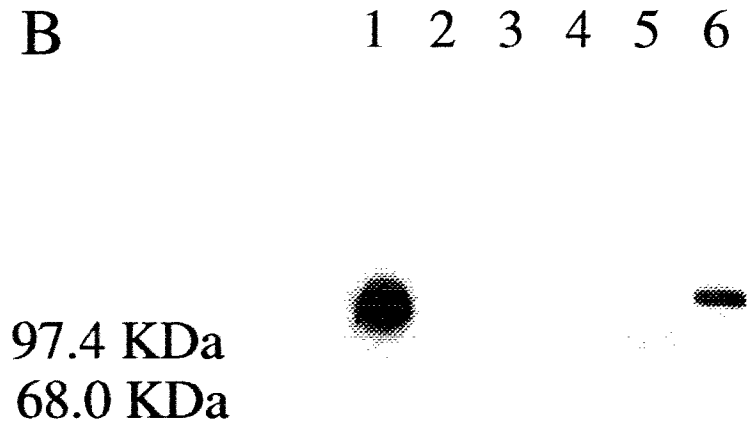


Figure A33: Western Immunoblot Analysis of the Binding Site of Monoclonal Antibody Y12. The following proteins were probed with monoclonal antibody Y12. Note that the plasmid name is in square brackets after the protein it expresses.

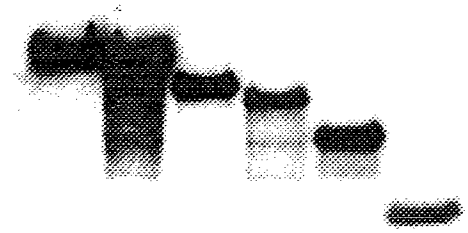
A: Crude cells lysates of *E. coli* strain DH5α expressing the yIRS C-terminal deletion mutants. The lanes designations are as follows: E.C.L. (Amersham) Molecular Weight Standard (lane 1), purified yIRS (lane 2), Hinc II deletion protein [pGC1Q] (lane 3), Cla I deletion protein [pGC2Q] (lane 4), Eco RI deletion protein [pGC3Q] (lane 5), Kpn 1 deletion protein [pGC4Q] (lane 6), Sca I deletion protein [pGC5Q] (lane 7) and purified bIRS (lane 8).

B: Crude cell lysates of *E. coli* strain TG2 and DH5α expressing the chimeric IleRS enzymes. The lane assignments are as follows: purified yIRS (lane 1), Hpa I/Hpa I Chimera [pKR5] (lane 2), Afl III/Sac II Chimera [pKR7] (lane 3), Nsi I Chimera [pGC6Q] (lane 4), E.C.L. (Amersham) Molecular Weight Standard (lane 5), Bgl II/Pvu II [pKR8] (lane 6), Stu I/Cla I [pKR9] (lane 7) and purified bIRS (lane 8).

A

1 2 3 4 5 6 7 8

97.4 KDa
68.0 KDa

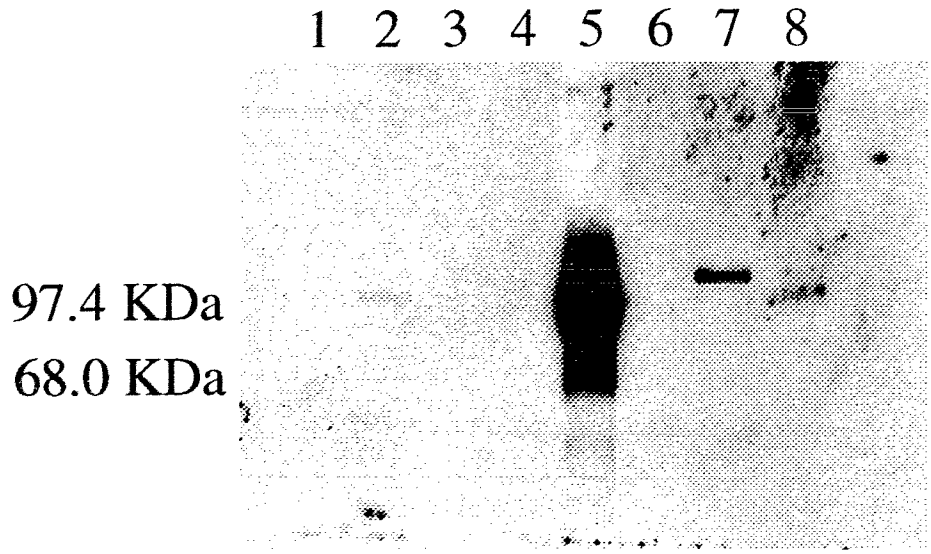


B

1 2 3 4 5 6 7 8

97.4 KDa
68.0 KDa

Figure A34: Western Immunoblot Analysis of the Binding Site of Monoclonal Antibody B1B2. The following proteins were probed with monoclonal antibody B1B2. Note that the plasmid name is in square brackets after the protein it expresses. Crude cell lysates of *E. coli* strain TG2 and DH5α expressing the chimeric IleRS enzymes. The lane assignments are as follows: purified yIRS (lane 1), Hpa I/Hpa I Chimera [pKR5] (lane 2), Afl III/Sac II Chimera [pKR7] (lane 3), Nsi I Chimera [pGC6Q] (lane 4), E.C.L. (Amersham) Molecular Weight Standard (lane 5), Bgl II/Pvu II [pKR8] (lane 6), Stu I/Cla I [pKR9] (lane 7) and purified bIRS (lane 8).


1 2 3 4 5 6 7 8
97.4 KDa
68.0 KDa

APPENDIX F: PROTEIN SEQUENCE ALIGNMENT OF YEAST ISOLEUCYL-tRNA SYNTHETASE AND *E. coli* ISOLEUCYL-tRNA SYNTHETASE

Primary protein sequence alignment between yeast and *E. coli* isoleucyl-tRNA synthetases using PALIGN from PCGENE program. Using this pre-defined matrix, the sequence homology between the yIRS and bIRS enzymes was determined to be approximately 17% as opposed to the 27% reported by Clarke *et al.* (17). Note that the difference in the method of alignment is different due to the difference between the parameters used.

```
****************************************
* ALIGNMENT OF TWO PROTEIN SEQUENCES. *
****************************************

The two sequences to be aligned are:

PYITS1.
DE   YEATS ILSI GENE FOR ISOLEUCYL-TRNA SYNTHETASE (EC 6.1.1.5)
OS   SACCHAROMYCES CEREVISIAE (YEAST)
Total number of residues: 1072.

BITS.
DE   E. COLI ISOLEUCYL-TRNA SYNTHETASE FROM P.SCHMIMMEL(MAY15/91)PMTS51
Total number of residues: 939.

Comparison matrix  : Structure-genetic matrix.
Open gap cost      : 7
Unit gap cost      : 1

The character to show that two aligned residues are identical is '|'


PYITS1     - MSESNAHFSFPKEEEKVLSLWDEIDAFHTSLELT---------------- -34
             ||                                | |
BITS       - MSD-----------------------YKSTLNLPETGFPMRGDLAKREPG -27

PYITS1     - ------------------KDKPEFSFFDGPPFATGTPHYGHILASTIKDI -66
                               | |  |   ||||  | |  | ||      |||
BITS       - MLARWTDDDLYGIIRAAKKGKKTFILHDGPPYANGSIHIGHSVNKILKDI -77

PYITS1     - VPR----------YATMTGHHVERRFGWDTHGVPIEHIIDK--------- -97
                          |             ||| || |||
BITS       - IVKSKGLSGYDSPYVP----------GWDCHGLPIELKVEQEYGKPGEKF -117

PYITS1     - ------------------------------------------------- -97

BITS       - TAAEFRAKCREYAATQVDGQRKDFIRLGVLGDWSHPYLTMDFKTEANIIR -167

PYITS1     - ------------------------------------------------- -97

BITS       - ALGKIIGNGHLHKGAKPVHWCVDCRSALAEAEVEYYDKTSPSIDVAFQAV -217

PYITS1     - ------------------------------------------------- -97

BITS       - DQDALKAKFAVSNVNGPISLVIWTTRRGLCLPTAQSLLHQISTMRWWQID -267

PYITS1     - ----------------KLGITGKDDVFKYGLENYNNECRSIVMTYASDWR -131
                             | |
BITS       - GQAVILAKDLVESVMQRIGVTD--------------------------- -289

PYITS1     - KTIGRLGRWIDFDNDYKTMYPSFMESTWWAFKQLHEKGQVYRGFKVMPYS -181

BITS       - ------------------------------------------------- -289
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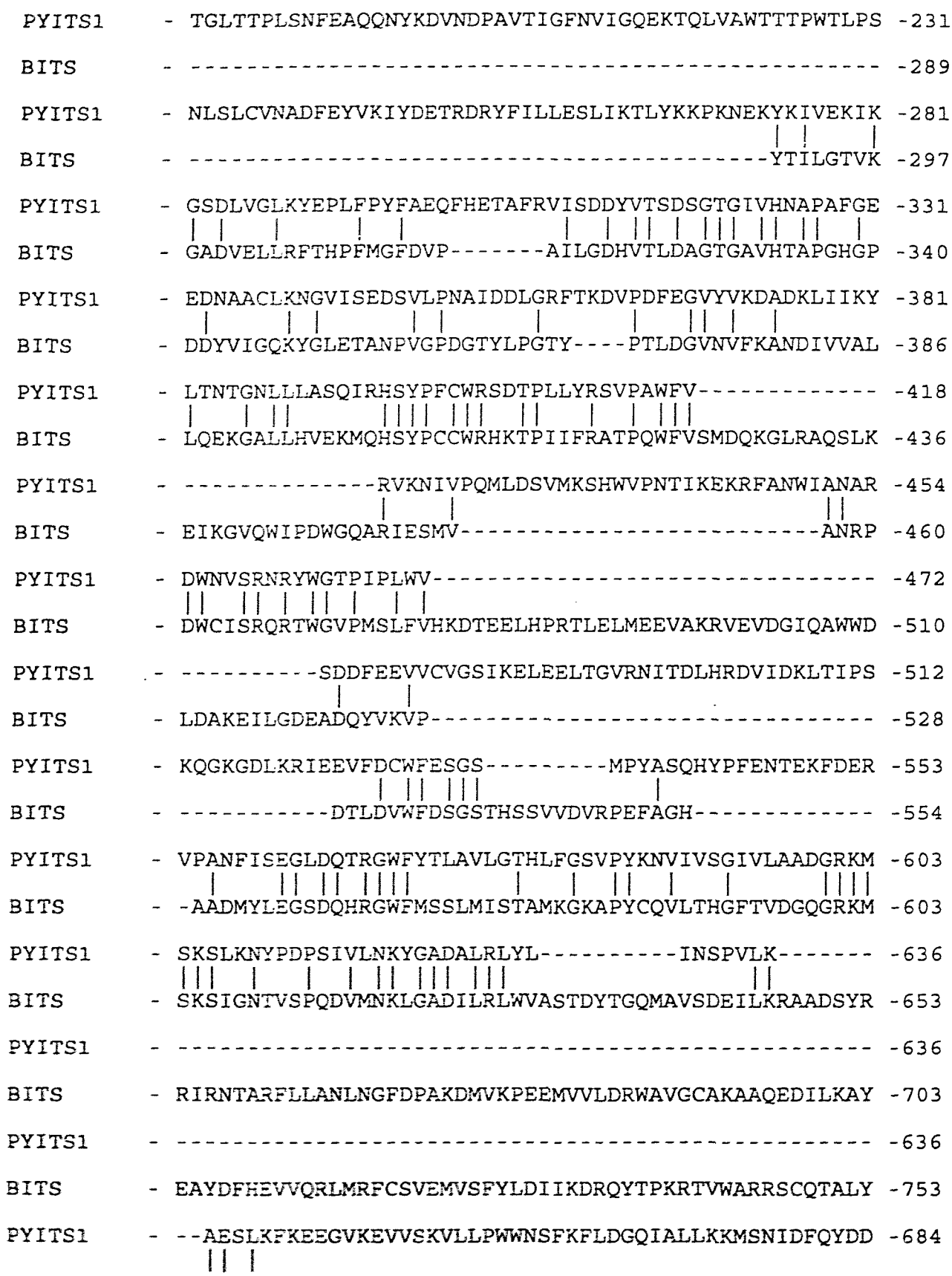
PYITS1 - TGLTTPLSNFEAQQNYKDVNDPAVTIGFNVIGQEKTQLVAWTTTPWTLPS -231
BITS - ---------- -289
PYITS1 - NLSLCVNADFEYVKIYDETRDRYFILLESLIKTLYKKPKNEKYKIVEKIK -281
BITS - ----------YTILGTVK -297
PYITS1 - GSDLVGLKYEPLFPYFAEQFHETAFRVISDDYVTSDSGTGIVHNAPAFGE -331
BITS - GADVELLRFTHPFMGFDVP-------AILGDHVTLDAGTGAVHTAPGHGP -340
PYITS1 - EDNAACLKNGVISEDSVLPNAIDDLGRFTKDVPDFEGVYVKDADKLIIKY -381
BITS - DDYVIGQKYGLETANPVGPDGTYLPGTY----PTLDGVNVFKANDIVVAL -386
PYITS1 - LTNTGNLLLASQIRHSYPFCWRSDTPLLYRSVPAWFV---------- -418
BITS - LQEKGALLHVEKMQHSYPCCWRHKTPIIFRATPQWFVSMDQKGLRAQSLK -436
PYITS1 - ----------RVKNIVPQMLDSVMKSHWVPNTIKEKRFANWIANAR -454
BITS - EIKGVQWIPDWGQARIESMV----------ANRP -460
PYITS1 - DWNVSRNRYWGTPIPLWV---------- -472
BITS - DWCISRQRTWGVPMSLFVHKDTEELHPRTLELMEEVAKRVEVDGIQAWWD -510
PYITS1 - ----------SDDFEEVVCVGSIKELEELTGVRNITDLHRDVIDKLTIPS -512
BITS - LDAKEILGDEADQYVKVP---------- -528
PYITS1 - KQGKGDLKRIEEVFDCWFESGS---------MPYASQHYPFENTEKFDER -553
BITS - ----------DTLDVWFDSGSTHSSVVDVRPEFAGH---------- -554
PYITS1 - VPANFISEGLDQTRGWFYTLAVLGTHLFGSVPYKNVIVSGIVLAADGRKM -603
BITS - -AADMYLEGSDQHRGWFMSSLMISTAMKGKAPYCQVLTHGFTVDGQGRKM -603
PYITS1 - SKSLKNYPDPSIVLNKYGADALRLYL----------INSPVLK------- -636
BITS - SKSIGNTVSPQDVMNKLGADILRLWVASTDYTGQMAVSDEILKRAADSYR -653
PYITS1 - ---------- -636
BITS - RIRNTARFLLANLNGFDPAKDMVKPEEMVVLDRWAVGCAKAAQEDILKAY -703
PYITS1 - ---------- -636
BITS - EAYDFHEVVQRLMRFCSVEMVSFYLDIIKDRQYTPKRTVWARRSCQTALY -753
PYITS1 - --AESLKFKEEGVKEVVSKVLLPWWNSFKFLDGQIALLKKMSNIDFQYDD -684

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BITS      - HIAEAL---------------------------------------------- -759

PYITS1    - SVKSDNVMDRWILASMQSLVQFIHEEMGQYKLYTVVPKLLNFIDELTNWY -734

BITS      - ---------------------------------------------------- -759

PYITS1    - IRFNRRRLKGENGVEDCLKALNSLFDALFTFVRAMAPFTPFLSESIYLRL -784
                                           || |||
BITS      - ------------------------------VRWMAP-------------- -765

PYITS1    - KEYIPEAVLAKYGKDGRSVHFLSYPVVKKEYFDEAIETAVSRMQSVIDLG -834

BITS      - ---------------------------------------------------- -765

PYITS1    - RNIREKKTISLKTPLKTLVILHSDESYLKDVEALKNYIIEELNVRDVVIT -884

BITS      - ---------------------------------------------------- -765

PYITS1    - SDEAKYGVEYKAVADWPVLGKKLKKDAKKVKDALPSVTSEQVREYL----- -930
                                             | |   |  ||
BITS      - --------------------------------ILSFTADEVWGYLPGER -782

PYITS1    - ----------------------------------------------------E -931

BITS      - EKYVLTGEWYEGLFGLADSEAMNDAFWDELLKVRGEVNKVIEQARADNKV -832

PYITS1    - SGKLEVAGIELVKGDLNAIRGLPESAVQAGQETRTDQDVLIIMDTNIYSE -981
              | ||| |          | |
BITS      - GGSLEAAVTLYAEPELSA-------------------------------- -850

PYITS1    - LKSEGLARELVNRIQKLRKKCGLEATDDVLVEYELVKDTIDFEAIVKEHF -1031

BITS      - ---------------------------------------------------- -850

PYITS1    - DMLSKTCRSDIAKYDGSKTDPIGDEEQSINDTIFKLKVFKL           -1072
                                                 ||
BITS      - ----------------------------------------KLTALGDELRF -861

BITS      - VLLTSDRRYVADYNDAPADAQQSEVLKGLKVALSKAEGEKCPRCWHYTQD -911

BITS      - VGKVAEHAEICGRCVSNVAGDGEKRKFA -939
```

Identity : 159 (16.93%)
Number of gaps inserted in PYITS1: 9
Number of gaps inserted in BITS: 11