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Human isoleucyl-tRNA synthetase: sequence of the cDNA, alternative mRNA splicing, and the characteristics of an unusually long C-terminal extension

(Gene cloning; polymerase chain reaction; autoantigen; interferon-stimulated response element; repeat motif; high-molecular-weight complex)

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SUMMARY

The human isoleucyl-tRNA synthetase (IRS)-encoding cDNA, whose primary structure we report here, has an open reading frame (ORF) which encodes a protein of 1262 amino acids (aa) with strong homology to IRS from yeast (53.5%) and *Tetrahymena* (51.0%) and contains all the major consensus motifs of class-I hydrophobic amino-acyl-tRNA synthetases (aaRS; MRS, LRS, VRS, IRS). However, the human enzyme has an unusually long C-terminal extension composed, in part, of a twice-repeated motif which shows no homology to any reported protein. We also report the presence of a coiled-coil-like motif in the C-terminal half of the protein. The mRNA has an additional exon in the 5′-untranslated region (*UTR*) which is alternatively spliced, giving rise to two types of mRNA, both of which are expressed in several human tissues. The longer of the two transcripts contains predicted secondary structure in the 5′-*UTR* which may reduce the translational efficiency of this mRNA. Two possible regulatory elements in the 5′-*UTR*, an interferon-stimulated response element (*ISRE*)-like sequence and a short ORF, have been identified. Because human IRS has previously been shown to be the target of antibodies in autoimmune disease, we discuss the role of protein structural features in the development of an autoimmune response to IRS.

INTRODUCTION

In the idiopathic human autoimmune diseases polymyositis and dermatomyositis, auto-Ab directed at one or another of several aaRS (ARS, GRS, HRS, IRS, TRS) may accompany the lymphocytic destruction of myocytes (Targoff et al., 1993). Although the mechanism of disease induction is not known, the appearance of auto-Ab precedes the onset of disease, and they mature in a manner suggesting that the native human enzyme itself drives the response (Miller et al., 1990). Since the targeted aaRS differ greatly in size, since they come from different

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Abbreviations: aa, amino acid(s); aaRS, aminoacyl-tRNA synthetase; Ab, antibody(ies); bp, base pair(s); cDNA, DNA complementary to mRNA; CP, cytoplasm; DMSO, dimethylsulfoxide; DTT, dithiothreitol; GCG, Genetics Computer Group (Madison, WI, USA); HMWC, high-molecular-weight complex; HPLC, high-performance liquid chromatography; IRS, isoleucyl-tRNA synthetase; *IRS*, gene (DNA) encoding IRS; *ISRE*, interferon-stimulated response element; kb, kilobase(s) or 1000 bp; LYB, 50 mM Tris pH 7.5/ 150 mM NaCl/1 mM PMSF/5 mM DTT/0.5 μg per ml aprotinin/0.25 μg per ml leupeptin; NP-40, Nonidet P-40; ORF, open reading frame; PAGE, polyacrylamide-gel electrophoresis; PMSF, phenylmethylsulfonyl fluoride; SDS, sodium dodecyl sulfate; SSPE, 3.0 M NaCl/0.2 M $NaPH_2PO_4$/0.02 M Na_2EDTA pH 7.8; TCA, trichloroacetic acid; *UTR*, untranslated region(s).

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classes, and since only one of them is from the high-molecular-weight complex (HMWC) of higher eukaryotic aaRS, it is of interest to determine whether the structures of the genes and of the aaRS proteins provide clues to the reasons they induce an autoimmune response. The primary structures of human TRS (Cruzen and Arfin, 1991) and human HRS (Raben et al., 1992), two of the targeted aaRS (both members of class IIa), have been determined. The aim of this study was to sequence and characterize the cDNA of human IRS, the class-I aaRS primarily targeted in myositis.

EXPERIMENTAL AND DISCUSSION

(a) Human IRS isolation

By immobilizing immunoglobulins from patients with auto-Ab to human IRS on protein A agarose beads, the IRS-containing HMWC can be isolated from cytoplasm (CP). The complex is composed of about half of the 20 aaRS and three non-aaRS proteins. The IRS band (Fig. 1) has an estimated molecular mass of 144 kDa. To obtain partial aa sequence the band corresponding to IRS was excised and analyzed by tryptic digestion, HPLC separation and peptide sequencing (Keck Foundation Biotechnology Resource Laboratory, New Haven, CT, USA). Three peptides were sequenced (ambiguous or uncertain aa are in parentheses) and compared to predicted IRS sequence: fragment 1: N(I/V)(I/D)(F/V)DN(D)YK; IRS aa 141–149, WIIFDNDYK; fragment 2: (F/G)GNWLK; IRS aa 545–550, FGNWLK; fragment 3: YIIEELNV(R); IRS aa 869–877, YIIEELNVR. All three peptide fragments are flanked by Lys or Arg. That the excised band was likely to be human IRS was confirmed by the aa sequence of fragment 3 which was identical to an aa sequence of yeast IRS (aa 871–879; Martindale et al., 1989).

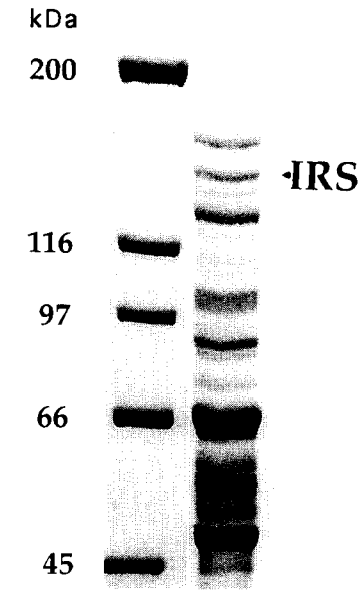

Fig. 1. SDS-PAGE of human HMWC proteins immunoprecipitated with patient antisera covalently bound to Sepharose beads. **Methods:** HeLa cells were homogenized in LYB lysis buffer with 1% NP-40 and diluted to 0.1% NP-40 with LYB. The HMWC was immunoprecipitated from the $100\,000 \times g$ supernatant CP using patient anti-OJ Ab (which recognizes and inhibits human IRS; Targoff, 1990) covalently cross-linked by dimethylpimelimidate to Protein A agarose beads (Targoff, 1990). Bound proteins were eluted with 0.1 M HCl, neutralized with NaOH, brought to 50 mM Tris pH 7.0/1 mM PMSF/1 mM DTT/0.5 μg per ml aprotinin/0.25 μg per ml leupeptin and concentrated by ultrafiltration (Centricon 30, Amicon, Beverly, MA, USA). Proteins were separated on 0.1% SDS-6% PAGE gels and stained with Coomassie Blue. Molecular mass markers were myosin (200 kDa), β-galactosidase (116 kDa), phosphorylase *b* (97.4 kDa), bovine serum albumin (66 kDa) and ovalbumin (45 kDa).

(b) Sequence analysis of human *IRS*

The complete sequence of human *IRS* is contained in the 4508-bp sequence submitted to GenBank (accession No. U04953). Of three in-frame ATG codons, the first (nt 20–22) begins a very short ORF (20-ATGAGTTGCTTTTAG-34). The regions flanking the second ATG (nt 244–246) lack any similarity to the eukaryotic start-site consensus sequence (GCCRCCATGG, where R is A or G; Kozak, 1991). The third ATG (nt 256–258) has an adenine at the most critical −3 position and we presume that this is the translation start site, with an ORF which codes for a 1262-aa protein (144 469 Da).

Two clones (Y-61 and Y-8) of human *IRS* cDNA contained sequence in the *5′-UTR*. The Y-61 clone (long transcript) contained 170 bp (nt 79–248; see Fig. 2) not found in clone Y-8 (short transcript). To determine if both long and short transcripts are normally present in human tissues and are not an aberration, we analyzed by PCR the cDNA from three tissues: HeLa cells, HepG2 liver cells, and mixed blood lymphocytes. A primer (**A**; Fig. 2) upstream from the 170-bp fragment was used with either a primer within the 170-bp fragment (**B**), or a primer downstream from the 170-bp fragment (**C**). PCR products of the expected sizes were obtained and they hybridized with radiolabeled probes specific for each transcript (Probes **1** and **2** for long and short transcripts, respectively; Fig. 2). Identity of the PCR products was confirmed by sequencing. To determine if the 170-bp fragment was an intron, genomic DNA was PCR-amplified using primers flanking the 170-bp fragment. A 4.0-kb product was generated using conditions favoring large products (T4 gene *32* protein, Boehringer-Mannheim, Germany; or Taq Enhancer, Stratagene, La Jolla, CA, USA; 5 min extension time). The product hybridized to a specific probe by Southern analysis under stringent conditions (63°C, 0.1 × SSPE/0.1% SDS). To determine the splice-site sequences of the intronic DNA, the PCR pro-

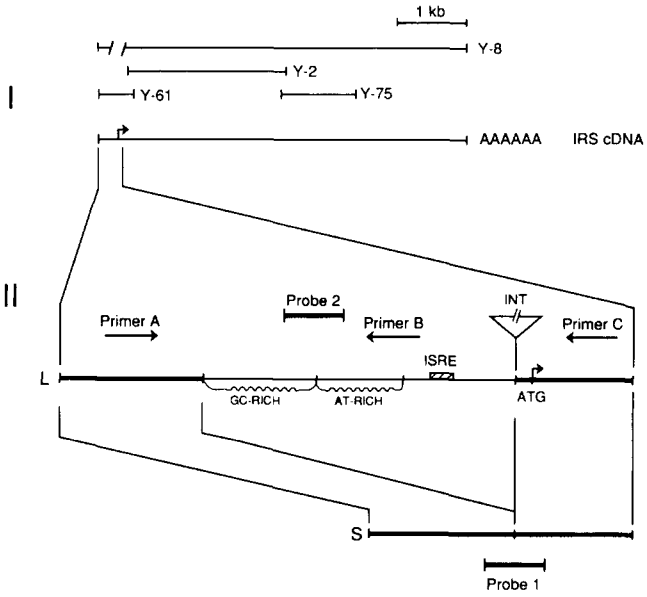

Fig. 2. Structure of human *IRS* and the 5′-*UTR*. (**I**) Four clones were obtained from λZAP (Y-2, Y-8) and λgt10 (Y-61, Y-75) cDNA libraries and both strands of each clone were sequenced. All clones overlapped by at least 200 bp and were identical where they overlapped. Clone Y-8 lacked 170 bp found in clone Y-61. (**II**) Structural elements identified in the 170-bp region of clone Y-61 include: (a) a G+C-rich region (nt 82–134; 68% G+C), (b) an A+T-rich region (nt 146–188; 72% A+T) and (c) an *ISRE*-like motif. Also shown are the 5′-*UTR* intron (INT) and the presumptive ATG start codon (nt 256–258). Primers **A**, **B** and **C** and Probes **1** and **2** were used to characterize long (L) and short (S) transcripts as described in section **b**. In the 3′-*UTR*, a polyadenylation signal (AGTAAA) was found at nt 4483–4489. **Methods:** Two human cDNA libraries (HepG2 human liver hepatoma in λZAP (Stratagene, Menasha, WI, USA); HeLa S3 in λgt10 (Clontech, Palo Alto, CA, USA)) were obtained, and $>2\times10^6$ plaques screened with yeast *IRS* cDNA (gift of Dr. C. Csank) probes. Two λZAP clone (Y-2, Y-8) had strong predicted aa sequence homology to yeast IRS. Y-2 insert DNA was used to obtain λgt10 clones Y-61 and Y-75. pBluescript plasmids were rescued from λZAP vectors following the manufacturer's instructions and sequenced using Sequenase (US Biochemical, Cleveland, OH, USA). Purified λgt10 DNA was sequenced by direct sequencing of PCR-generated single-stranded DNA (Nichols and Raben, 1994).

duct was cloned into a pCRII plasmid (Invitrogen, San Diego, CA, USA). The acceptor splice-site sequence (cagC) agrees well with the consensus acceptor sequence (yagG). The sequence of the short transcript donor site (CAGguacgc) agrees with the consensus sequence (C/AAGguragu) in seven of nine positions, and the long transcript donor site (GAGguagug) agrees in five of nine positions. We conclude that two human *IRS* mRNAs are transcribed in several tissues and that the transcripts probably result from alternative usage of donor sites in the 5′-*UTR*.

These results suggest that mRNA alternative splicing in the 5′-UTR may be a regulatory mechanism of human *IRS* gene expression. The long and short transcripts result from a simple but relatively rare form of intron splicing which utilizes alternative 5′-donor sites (Smith et al., 1989; McKeown, 1992). In one model (Mattox et al., 1992), gene expression may be regulated by alternatively spliced transcripts under the control of splicing factor(s). Selection of 5′-donor sites by a splicing factor (ASF/SF2) has been characterized for SV40 large-T/small-t splicing (Ge and Manley, 1990) and β-globin mRNA splicing (Kozak, 1991). Regulation of a housekeeping gene like human *IRS* at the level of mRNA could be a mechanism for controlling an as yet undetermined non-ligase IRS activity.

Based on the Kozak (1989) scanning model of protein translation, three considerations suggest that the short transcript may be more efficient for translation. (*i*) The shorter transcript contains one less ATG start site and would have fewer false starts. (*ii*) The shorter leader sequence of the short transcript should require less ribosomal scanning. (*iii*) The 170-bp alternatively excised fragment has G+C and A+T regions (see Fig. 2) predicted to form multiple hairpin loops which can block ribosomal scanning. A more efficiently translated short transcript could compete with the long transcript for ribosomal binding and increase the synthesis of IRS.

Several regulatory consensus sequences were identified in the 255-bp 5′-*UTR* and in the 3′-*UTR*. A sequence which strongly resembles an *ISRE* was found at nt 204–213. This sequence, GTTCGTTTCC, matches the *ISRE* consensus sequence, TTTC(N/−)NTTTCC (Williams, 1991), in eight of nine nt. Interferon-γ regulation of another aaRS (WRS) which contains *ISRE* elements has recently been reported (Fleckner et al., 1991). Another possible regulatory mechanism in the 5′-*UTR* is the short ORF at nt 20–34 which may effect the efficiency of translation. In yeast, translation may be up- or down-regulated by short ORF in the upstream region of the transcript (Abastado et al., 1991).

(c) Comparison of human IRS with other aaRS

A search was performed for homologous aa sequences deposited in the GenBank and EMBL databases using TFASTA (GCG). The most similar sequences were other aaRS in the class-I hydrophobic aa family. Sequence comparisons with reported IRS, VRS, MRS and LRS were performed using PILEUP (GCG). The three primary consensus regions common to class-I aaRS (Martindale et al., 1989; Csank and Martindale, 1992) are present in human IRS at aa 55–58 (HYGH), 455–459 (WTISR) and 600–604 (KMSKR). Two other motifs, GTG (Martindale et al., 1989) and PXXP (Despons et al., 1991) are located at aa 318–320 and 771–774, respectively. Both connective peptide loops (CP1 and CP2, see Burbaum and Schimmel, 1991) are present, and both domains are very similar in size to homologous regions in other species. CP1 is 277 aa (shortest: *Methanobacter*, 264 aa; longest: *Tetrahymena*, 287 aa) and CP2 is 112 aa

(shortest: *E. coli*, 106 aa; longest: *Methanobacter* and *Tetrahymena*, 114 aa).

A predicted protein secondary structure found in many autoantigens is the coiled-coil motif which contains unusually long stretches of amphipathic α-helix with a high percentage of charged aa and multiple heptad repeats. Analysis for predicted coiled-coil structure using the algorithm of Lupas et al. (Lupas et al., 1991, Dohlman et al., 1993) identified a region of coiled-coil-like structure in human IRS (aa 862–899). A HELICALWHEEL (GCG) diagram of the first 18 aa in the motif predicts that the sequence forms an amphipathic α-helix (Fig. 3). Although the probability of coiled-coil formation for the motif is low ($P=0.05$), the sequence is very long (38 aa) and is rich in charged aa (42%).

(d) C-terminal extension in human IRS

The most distinctive feature of human IRS is the C-terminal extension of about 180 aa. Within this region is a repeated hexapeptide, LLLENP (aa 1098–1103, 1194–1199). Comparison of aa sequence in the flanking regions suggests that the hexapeptides lie within repeated regions. When the first motif (1078–1149) was analyzed by TFASTA, the best fit in the GenBank/EMBL database was to the second motif. In a 54-aa overlap, 35% of aa were identical and 59% were similar (Fig. 4). Following

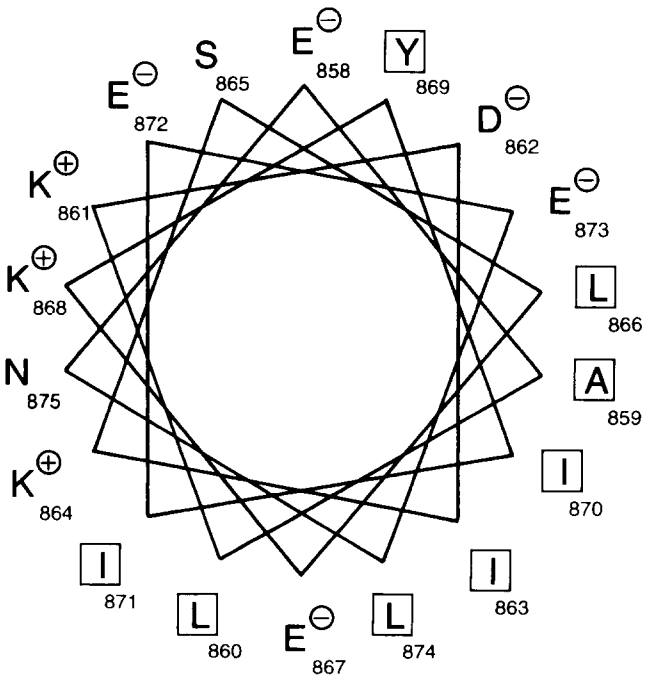

Fig. 3. HELICALWHEEL representation of the coiled-coil-like motif in human IRS. Secondary structural analysis (Lupas et al., 1991) of human *IRS* predicted one region (aa 858–895) to form a coiled-coil-like structure. The initial 18 aa (858–875) were plotted using HELICALWHEEL (GCG). The strong amphipathic character of this predicted structure is illustrated by the hydrophobic (boxed residues) and charged faces.

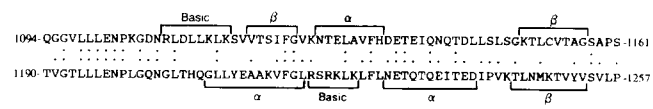

Fig. 4. Repeated motif in the C-terminal extension of human IRS. The aa sequences in the unique C-terminal extension in the region of the hexapeptide repeat (LLLENP) are aligned. Identical and similar aa are marked (similarity according to Eriani et al., 1990): hydrophobic = A,F,I,L,M,V,W,Y; positively charged = H,K,R; negatively charged = D,E,N,Q; small = P,G,S,T). Secondary structure predictions in which Chou-Fasman and Robson-Garnier algorithms agree are shown above the first motif and below the second motif. Also noted are two clusters of basic aa, one in each motif.

each LLLENP sequence is a region of predicted secondary structure including α-helix and β-sheet. A cluster of basic aa is found in each motif. The first cluster (RLDLLKLK, aa 1108–1115) lies between the first LLLENP hexapeptide and the first β-sheet sequence. The second basic cluster (RSRKLK, aa 1221–1226) lies between two α-helices (aa 1209–1220, 1230–1240). The first motif contains six heptad repeats (aa 1117–1158) with Val (three times), Leu (two times) or Ile (one time) at the initial position.

A possible function of the C-terminal extension may relate to the association of human IRS with the HMWC. aaRS are known to associate with cellular constituents, including cytoskeleton (Mirande et al., 1985), components of the protein translation system (Ivanov et al., 1993) and with other aaRS in higher eukaryotes (Mirande, 1991). Association of aaRS in the HMWC is thought to involve hydrophobic interactions (Cirakoglu and Waller, 1985), perhaps mediated by lipids (Sivaram and Deutscher, 1990). Rabbit IRS was shown to be associated with a core structure of two aaRS, ERS (probably EPRS; Cerini et al., 1991) and IRS, which was resistant to biochemical disaggregation (Norcum, 1991). Expression studies with truncated variants may shed light on the role of the C terminus in the association of IRS with the HMWC.

(e) Inhibition of IRS activity by human antisera

To understand the nature of the antigenic stimulus in autoimmune myositis we have performed preliminary experiments to identify the epitope(s) recognized by the myositis-associated Ab. As shown in Fig. 5, Ab to human IRS did not inhibit IRS activity of *E. coli* or yeast CP. By contrast, pre-treatment with patient antisera inhibited both mouse and human IRS activity (90% and 82%, respectively). Cytoplasmic IRS from *Drosophila* and *Spodoptera frugiperda* were inhibited 20% and 36%, respectively. As in similar studies with human HRS (Miller et al., 1990), the disease-related auto-Ab inhibit the mammalian enzyme better than the enzymes from more distantly related higher eukaryotic species, and the

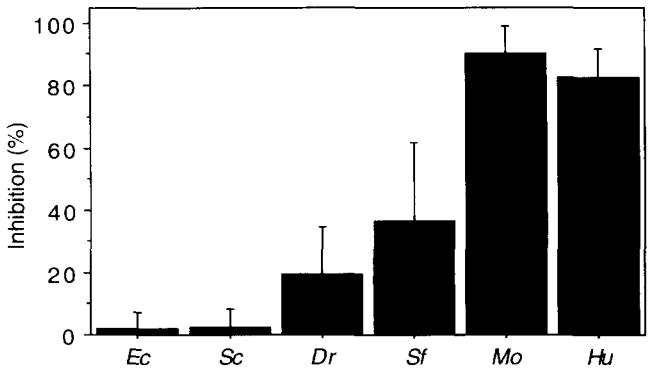

Fig. 5. Inhibition of IRS activity by antisera. CP prepared from cells of six species (*E. coli* (*Ec*), *Saccharomyces cerevisiae* (*Sc*), *Drosophila* (*Dr*), *Spodoptera frugiperda* (*Sf*), mouse C2C12 (Mo and human (Hu)) was incubated for 20 min at 4°C with diluted antisera from polymyositis patients with active disease or from non-diseased controls. Inhibition of IRS activity was measured as the reduction in [^{3}H]Ile incorporation into tRNA in samples treated with patient antisera as compared to samples treated with normal sera (Targoff, 1990). Percent inhibition = 100 × ((avg of two normal controls) − (patient))/(avg of two normal controls). *E. coli* cells were a gift of Dr J. Yancy. *Drosophila* embryos were a gift of Dr. J. Kassis. C2C12 mouse myoblasts were provided by Dr. J. Sherman. *Saccharomyces cerevisiae* CP was a gift of Dr. E. Cabib. **Methods:** Amino-acylation by IRS was measured as TCA-precipitable [^{3}H]Ile incorporated into tRNA. Inhibition was measured as the decrease in [^{3}H]Ile incorporation as compared to samples treated with normal human sera. Because concentrated CP can inhibit enzyme activity, samples were diluted as required to give enzyme activities which were within a linear range. CP from HeLa cells was prepared as described in the legend to Fig. 1. CP from other species was prepared as follows: *E. coli* cells were stored as a frozen pellet. Cells were suspended (1:2, w/v) in buffer (100 mM NaCl/50 mM Tris·HCl pH 7.4/10 mM $MgCl_2$/1 mM EDTA/5 mM DTT/1 mM PMSF/5% glycerol/0.5 μg/ml aprotinin/0.25 μg/ml leupeptin) and homogenized at 0°C by sonication. *Drosophila* embryos were homogenized in LYB/1% NP-40 by Dounce. C2C12 mouse myoblasts and *Spodoptera frugiperda* (Sf21) insect cells were disrupted in LYB/1% NP-40. All homogenates were centrifuged at 15 000 × *g* and the supernatants were brought to 5% glycerol/5 mM DTT and stored at −80°C until assayed.

yeast and bacterial enzymes were unaffected by the antisera. Recently the 60-aa N-terminal domain of human HRS has been shown to contain the autoantigenic epitope recognized by human auto-Ab (Raben et al., 1994). Like the C-terminal region of human IRS, the N-terminal region of HRS is thought to be unique to higher eukaryotes. It is tempting to speculate that Ab to human IRS are directed at a region present in the human IRS and absent in the yeast and bacterial IRS, and, therefore, the C-terminal extension is a plausible candidate epitope. The structure of the IRS from the two insect species is unknown, so the partial inhibition cannot be interpreted at all at present. It is possible, however, that the major epitope is located in the more conserved core of IRS – for example in the region predicted to assume a coiled-coil-like configuration – but still different among the species. Again, studies with recombinant human IRS and truncated mutants should provide a clearer picture of why this protein has been selected for immune recognition in inflammatory muscle disease.

(f) Conclusions

Four features newly recognized in human IRS are reported here:

(*1*) Comparison of human IRS with lower eukaryotic IRS sequences reveals that there is very little change in the overall size of domains previously identified (Martindale et al., 1989; Csank and Martindale, 1992). The most distinctive difference in the human IRS structure is the 180-aa C-terminal extension. In this region we have identified a repeated hexapeptide sequence, LLLENP, found within two motifs which share significant homology.

(*2*) Immunological analysis using human auto-Ab inhibition of enzyme activity suggests that the epitope in human IRS is shared among some higher eukaryotes but is not found in lower eukaryotes or prokaryotes. The C-terminal extension is unique to higher eukaryotes and is a region which may contain the epitope. In addition, human IRS contains a predicted coiled-coil-like region which is a common feature of autoantigens and may be the epitope recognized by auto-Ab.

(*3*) Two human *IRS* transcripts have been identified in several human tissues. The two transcripts apparently result from alternative splicing at the 5′-donor site in the 5′-*UTR* and differ markedly in size and predicted secondary structure. These results suggest that *IRS* protein synthesis could be regulated by a differential splicing mechanism acting on alternative 5′ splice sites to produce transcripts of unequal translational efficiency.

(*4*) Possible *ISRE* and short ORF regulatory elements have been identified. An *ISRE*-like consensus sequence in the 5′-*UTR* of human *IRS* suggests that regulation of this protein may be under the control of this cytokine.

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NOTE ADDED IN PROOF

While this manuscript was in press, Shiba et al. reported the human IRS cDNA sequence and the unique C-terminal extension (Proc. Natl. Acad. Sci. USA 91 (1994) 7435–7439).

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