Characterization and Regulation of the Mouse Insulin Receptor Substrate Gene Promoter

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To evaluate the potential for regulation of the insulin receptor substrate IRS-1, we have cloned the mouse IRS-1 gene, identified its promoter, and analyzed promoter activity in the basal state and in response to stimulation. The 5′-region of the mouse IRS-1 gene lacks typical CAAT and TATA boxes but contains nine potential Spl binding sites consistent with a housekeeping gene. The 5′-region of the IRS-1 gene also has significant regions of homology with the promoters of the progesterone receptor gene, the insulin-like growth factor I receptor gene, and the androgen receptor gene. Multiple transcription start sites were identified 0.4-1.2 kilobases (kb) upstream from the start codon. Using a chloramphenicol acetyl transferase assay in Chinese hamster ovary (CHO) cells, basal promoter activity was present in the 3.2 kb 5′-flanking region of IRS-1 gene. Within this region, there were 184-base pair and 60-base pair negative regulatory elements at −3.2 kb and −1.6 kb surrounded by positive elements. By gel shift assay, a nuclear factor was identified in CHO cells which binds to −1666 and −1666 sequence in the negative regulatory element and appears to be distinct from C/EBP, CREB, and AP-1. In 3T3-F442A adipocytes dexamethasone treatment significantly decreased IRS-1 mRNA and IRS-1 protein. This was due to a decrease in the half-life of IRS-1 mRNA, with no change in IRS-1 promoter-chloramphenicol acetyl transferase activity. Insulin also decreased IRS-1 protein by ∼60% within 9 h but did so without altering IRS-1 mRNA levels or chloramphenicol acetyl transferase activity. Thus, both insulin and dexamethasone down-regulate IRS-1 expression at the posttranscriptional level; with insulin this is probably due to an effect on protein half-life, whereas with dexamethasone the effect is due to a change in the half-life of IRS-1 mRNA. (Molecular Endocrinology 9: 1367-1379, 1995)

INTRODUCTION

After insulin stimulation, the insulin receptor undergoes autophosphorylation on tyrosine residues which, in turn, activates the tyrosine kinase activity of the receptor toward other substrates. Insulin receptor substrate-1 (IRS-1), initially identified as pp185 (2), is the major substrate for the insulin receptor tyrosine kinase in all cells and tissues studied. IRS-1 is a cytoplasmic protein that migrates between 165 and 185 kilodaltons (kDa) on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and is tyrosine phosphorylated by the insulin receptor tyrosine kinase immediately after insulin stimulation (1-3). Based on a number of studies using mutated insulin receptors, insulin receptor tyrosine kinase activity and the phosphorylation of IRS-1 appear to be essential steps in insulin signal transduction (4-11). In 1991, using partial peptide sequences from the purified protein, we cloned the rat liver IRS-1 cDNA (1, 12). More recently, we and others have cloned the human skeletal muscle IRS-1 cDNA (13), the human IRS-1 gene (13, 14), and the mouse IRS-1 gene (15). The human and mouse IRS-1 genes have been shown to contain the entire coding region in a single exon. All three IRS-1 proteins are highly homologous (15). Twenty potential tyrosine phosphorylation sites, nine of which are in Tyr-Met-X-Met (YMXM) or Tyr-X-X-Met (YXXM) motifs (16), are completely conserved, and most of the potential serine/threonine phosphorylation sites are also conserved among these three species (15). Based on peptides designed from rat IRS-1, ty-
rosine phosphorylation of IRS-1 by the insulin receptor tyrosine kinase probably occurs with high affinity on the YMXM and/or YXXM motifs (17), and this in binding of IRS-1 to the 85-kDa subunit of phosphati-
dylinositol 3-kinase and other SH2 domain-containing molecules, leading to further downstream signal trans-
duction (18, 19).

Like other components of the early insulin response, IRS-1 is subject to a variety of regulatory influences. In animal studies, we have shown that IRS-1 protein levels are altered in fasting and streptozotocin-diabetic and ob/ob mice, suggesting that insulin may play some role in IRS-1 regulation (20). In 3T3-L1 cells, IRS-1 protein increases during differentiation from fi-
broblast to adipocytes and is negatively regulated by dexamethasone (Dex) and insulin (21). In human fetal tissues, IRS-1 mRNA is detected in every tissue ex-
amined; however, the abundance of the mRNA varies with higher expression in brain, kidney, and small in-
estine (13). At present, the mechanisms of regulation of IRS-1 protein, mRNA, or gene transcription are un-
known. To begin to elucidate these processes, we have cloned and characterized the promoter region of the mouse IRS-1 gene, identified a nuclear factor that may be responsible for negative regulation, and used 3T3-F442A cells stably transfected with the IRS-1 pro-
moter-chloramphenicol acetyl transferase (CAT) con-
structs to examine the effects of insulin and Dex on regulation of IRS-1 protein, mRNA, and gene tran-
scriptional activity.

RESULTS
Cloning and Expression of the Mouse IRS-1 Gene

A 6.7 kilobase (kb) BamHI-EcoRI fragment from the mouse IRS-1 gene, which contained the 5'-flanking region and a part of coding region, was subcloned into pBluescript SK-II, and the nucleotide (nt) sequence of the 3.3-kb coding region and 3.4-kb 5'-flanking region was determined (Fig. 1). The nt sequence of the 5'-region of the mouse IRS-1 gene showed a high level of identity with that of the 5'-untranslated region of the rat IRS-1 cDNA (94.6%, Ref. 1) and human IRS-1 cDNA (82.2%, Ref. 13).

The expression of IRS-1 mRNA in different mouse tissues was assessed by Northern blot analysis using 2 µg poly(A)+ RNA prepared from mouse heart, brain, spleen, lung, liver, skeletal muscle, kidney, and testis. IRS-1 mRNA appeared as a single species of 9.5 kb in most tissues and could be detected in every tissue examined after 3 days exposure at −70 C with an intensifying screen (Fig. 2). The relative abundance of IRS-1 as compared with actin was greatest in brain, heart, lung, and kidney. We have previously shown that IRS-1 mRNA could be detected in human tissues in poly(A)+ RNA blot, but not in blots prepared with 10–50 µg total RNA, suggesting a low abundance of IRS-1 mRNA in both human and rodent tissues (13).

Identification and Characterization of the Promoter Region of Mouse IRS-1 Gene

To determine the transcriptional activity of the 5'-flanking region of the mouse IRS-1 gene, a 3.2-kb BamHI and Tth1111 I fragment [−3369 to −266 nt in Fig. 1] was inserted into the low background CAT vector pSV001CAT as described in Materials and Methods. The basal CAT activity of this vector in CHO

Fig. 1. Nucleotide Sequence of the Mouse IRS-1 Promoter

The numbers at the left are the nt number with -1 corre-
sponding to the A of the ATG codon. Nucleotides upstream of the ATG are shown as negative numbers. Representative restriction enzyme sites are indicated by arrows. Potential Sp1-binding sites are shown in the boxes. Potential AP-1-binding sites, AP-2-binding sites, C/EBP-binding sites, and sequences with 80% homology to the mammalian response elements are underlined and noted as AP-1, AP-2, C/EBP, GRE+/PRE, GRE−, ERE, IRE-1 and IRE-2, respectively (see Results). The nt corresponding to the cDNA start site of human IRS-1 and rat IRS-1 are indicated by the asterisk and circle, respectively. Primers used for the primer extension analysis are shown by arrows (p-1  p-5). Possible major and minor transcription start sites are shown in filled and open triangles under the nt, respectively.

![Fig. 1](https://academic.oup.com/mend/article-abstract/9/10/1367/2715073/1013672715073?force=1)
Northern Blot Analysis of Mouse IRS-1

Fig. 2. Northern Blot Analysis of Mouse IRS-1

A, A 2.6-kb portion of mouse IRS-1 gene coding region (16-2581 nt, in Ref. 15) (panel A) or β-actin cDNA fragment (48) (panel B) was isolated, 32P labeled, and used as a probe (10^6 cpm/ml) on a multiple tissue Northern (MTN) blot (Clontech), which contained 2 μg poly(A)+ RNA from eight different mouse tissues [heart (H), brain (B), spleen (S), lung (Lu), liver (L), skeletal muscle (SM), kidney (K), and testis (T)]. B, In the lower panel, the blot has been reprobed using a 32P-labeled β-actin probe (51). The arrows indicate the normal migration of the major β-actin species of 2 and 1.8 kb. The latter represents a major β-actin mRNA species in skeletal and cardiac muscle.

Based on sequence analysis of the promoter region, there was no typical TATA box or CAAT box, but there were nine potential Sp1 transcription factor-binding sites [GGGGCG, shown in shaded boxes in Fig. 1] (26), five potential AP-1 binding sites [(C/G)TGACT(C/A)A] (27), three potential AP-2 binding sites [CC(C/G)CCGCG] (28), and one potential C/EBP binding site [T(G/T)NNNG(T/C)AA(G/T)] (29). There were also two extremely purine-rich regions (947 to -888 and -529 to -492), one pyrimidine-rich region (830 to -783), and one CAG repetitive sequence (-630 to -608), which might have some roles in the transcriptional regulation. In addition, there were sequences with more than 80% homology to the glucocorticoid/progestosterone response elements [GGTACANNNTGTCT, (GRE+/PRE) and AT(C/T)ACNNTGAC(T/A), (GRE-)], estrogen response element [AGGTACANNNTGTCT, (ERE)] (30), insulin response elements from glyceraldehyde-3-phosphate dehydrogenase gene [CCCGCCTCTC, (IRE-I)] (31) and phosphoenolpyruvate carboxykinase [TGGTGT(T)TG, (IRE-2)] (32). In other genes, the GRE+/PRE, ERE, and IRE-1 have been reported to regulate gene expression positively (33), whereas GRE- and IRE-2 are associated with negative regulation (32, 34).

Analysis of the 5'-flanking region of the mouse IRS-1 gene also revealed an 814-nt region (-1241 to -410) with 52% identity to the 5'-region of chicken progesterone receptor gene (35), a 374-nt region (-1350 to -986) with 56% identity to the human insulin-like growth factor I receptor promoter (36) and 59% identity in a 170-nt overlap (-882 to -716) with the 5'-region of the human androgen receptor gene (37).

Primer Extension and Deletional Analysis of the IRS-1 Promoter

The transcription start site(s) of the IRS-1 gene were identified in primer extension experiments using five different primers (shown by arrows in Fig. 1). Seven major and nine minor potential transcription start sites could be found in the promoter region: all were located between 400 and 1200 base pairs (bp) upstream from ATG codon (Fig. 4 and shown by triangles under the sequence in Fig. 1). The start sites of rat (1) and human (13) IRS-1 cDNAs (shown by open circle and asterisk in Fig. 1, respectively) are close to the possible transcription start sites at positions -610, -1072, and -1079.

To determine the mechanisms of transcriptional regulation of the IRS-1 gene, 5'-deletion mutants of the promoter were constructed starting from pIRSBa-CAT, as described in Materials and Methods, and subjected to CAT assay after transient expression in CHO cells. Deletion of the promoter to -3185 (Kpnl site) produced an increase of the CAT activity by 113% (P < 0.01), while deletion from -3185 to -2425 (Sphl site) produced a significant (P < 0.01) decrease of the CAT as compared with the original level. These results indicate that there are elements that negatively and positively regulate the transcription between -3369 and -3185 and between -3185 and -2425, respectively. Further deletion (pIRSBg-CAT) to -1585 produced a second increase in CAT activity by ~120% (P < 0.05), indicating another negatively regulating element located between the Sphl and BgllI sites (-2425 to -1585). To further analyze this region another deletion was made from -2425 to -1646 using the BspEl site and named pIRSBs-CAT. The activity of the pIRSBs-CAT in CHO cells was slightly
A portion (3.2 kb) of the mouse IRS-1 gene 5'-region was inserted in the CAT vector pSV001CAT in the correct (pIRSBar-CAT) or reverse (pIRSBaR-CAT) orientation. 5'-Deletions of the mouse IRS-1 gene promoter were made by deletion of fragments from pIRSBaR-CAT as described in Materials and Methods. These plasmids, as well as negative (pSV001CAT) or positive controls (pHICAT and pSV2-CAT), were transiently transfected in the CHO cells, and CAT assays were performed and corrected for β-galactosidase activities as described in Materials and Methods. B, The promoter activity of another deletion mutant, pIRSBs-CAT, was analyzed along with pIRSSp-CAT and pIRSBg-CAT. C, Schematic structures of the plasmids and the means ± SE of three different CAT assay experiments (each in duplicate). The CAT activity of the pIRSBaR-CAT is arbitrarily defined as 100%.

- **: p < 0.01
- ***: p < 0.001
- n.s.: not significant

(n.s.; not significant, *; p < 0.05, **; p < 0.01, ***; p < 0.001)

Fig. 3. Analysis of the Mouse IRS-1 Gene 5'-Flanking Region Using a CAT Assay System

- Higher than that of pIRSSp-CAT but significantly lower than that of pIRSBg-CAT (Fig. 3B). Deleting up to the XhoI site (-666) produced a second decrease in CAT activity, probably as a result of deletion of some transcription start sites (Fig. 3C). Thus, there appear to be positively acting elements localized between -3185 and -2425 and between -1585 and -666 and negatively acting elements between...
Fig. 4. Primer Extension Analysis of the Mouse IRS-1 Gene Promoter

Five different synthetic oligonucleotides complementary to the 5'-region were end-labeled with [γ-32P]ATP and T4 polynucleotides kinase. Poly(A)⁺ RNA (5 μg) prepared from mouse liver or yeast tRNA (as a control) was mixed with 2 pmol labeled primer and subjected to the analysis. To directly compare the products with the genomic nt sequence, the sequence reaction was performed using the same primer that had been used for primer extension and electrophoresed in 6% urea-acrylamide gels with the product of primer extension. A representative result is shown. Large and small arrowheads show the major and minor transcription start sites, respectively.

-3369 and -3185 and between -1645 and -1585 in promoter region of the mouse IRS-1 gene (Fig. 3C).

Fig. 5. Gel Shift Assays Were Performed Using 1 fmol 32P-Labeled Probe Corresponding to -1606 and -1596 bp of Mouse IRS-1 Gene Promoter (Probe 1)

Lane 1, No extract; lane 2, 2 μg nuclear extract of CHO cells; lanes 3–6, 2 μg nuclear extract of CHO cells with 1 pmol of competitors (nonlabeled probe, C/EBP, CREB, and AP-1 binding sequences, respectively). Thin arrow shows the retarded band, and thick arrow indicates the nonretarded probe.

Gel Shift Assay of the Negative Regulatory Region

To further analyze the negatively acting cis-acting element between -1645 and -1585 bp of the IRS-1 gene, we performed gel shift assays. Using a probe that corresponded to the -1606 to -1586 bp region, a single retarded band was observed in the presence of the nuclear extract of CHO cells (Fig. 5, lane 2). This band was competed out by adding the excess of cold probe (Fig. 5, lane 3) but was not competed out by addition of the competitors with the consensus sequences of C/EBP, CREB, and AP-1 binding sites (Fig. 5, lanes 4–6). By comparison, no retarded band was observed in the gel shift assay using a probe corresponding to base -1645 to -1606 (data not shown). These data indicate that there is a nuclear factor in CHO cells that binds to the sequence between the -1606 and -1596-bp region of the mouse IRS-1 promoter, negatively regulates IRS-1 expression, and is distinct from C/EBP, CREB, and AP-1.

Stable 3T3-F442A Transfectants with IRS-1 Promoter-CAT Constructs

To understand the mechanisms of regulation of IRS-1 expression, the protein, mRNA, and promoter activity of IRS-1 were analyzed in 3T3-F442A cells stably transfected with a mouse IRS-1 promotor-
CAT gene fusion plasmid. Two independent clones (3T3IRSCAT-5 and 3T3IRSCAT-14) were used for this analysis to avoid artifacts due to clonal variability and variability of the integration site of the transfected plasmid.

In 3T3-F442A cells, IRS-1 was detected as a single band of ~170 kDa on immunoblots after SDS-PAGE under reducing conditions. After acute insulin stimulation, the IRS-1 band was shifted to an apparent higher molecular weight, probably due to the increased phosphorylation of the protein (1, 21). Upon prolonged stimulation with 10^{-6} M insulin, IRS-1 protein decreased in a time-dependent manner with a ~50% reduction by 6 h (P < 0.05) (Fig. 6A and Fig. 8, filled circle). Dexamethasone (10^{-6} M) treatment also decreased the amount of IRS-1 significantly by up to ~60% in 9 h (P < 0.01) (Fig. 6B and Fig. 9, filled circle); however, the kinetics of the IRS-1 decrease were somewhat different with the two hormones. IRS-1 protein decreased rapidly after insulin stimulation with a significant reduction already detected at 30 min, whereas with Dex IRS-1 increased initially at 30 min and 1 h after stimulation and then started to decrease at 3 h (compare Figs. 8 and 9, filled circles). These differences in kinetics suggest different mechanisms of hormonal regulation of the IRS-1 protein.

Effects of Hormones on IRS-1 mRNA

Since IRS-1 mRNA is of very low abundance, mRNA levels were estimated in semiquantitative reverse transcriptase-polymerase chain reaction (RT-PCR) with labeled primers and autoradiography. Products of the expected sizes were observed for both IRS-1 (221 bp) and β₂-microglobulin (136 bp) used as an internal standard. Although the IRS-1 sequence is derived from a single exon, PCR in the absence of reverse transcription revealed negligible contamination of genomic DNA (data not shown). The amount of β₂-microglobulin mRNA was quite constant from experiment to experiment. After either acute or chronic insulin stimulation, there was no significant change in IRS-1 mRNA (Fig. 7A and Fig. 8, filled triangles). By contrast, IRS-1 mRNA decreased significantly upon stimulation with Dex, reaching 60% of basal levels within 3 h (P < 0.05) (Figs. 7B and 9, filled triangles). The decrease in IRS-1 mRNA with Dex began by 60 min and preceded the decrease in IRS-1 protein levels. Neither insulin (Fig. 8, filled squares) nor Dex (Fig. 9, filled squares) stimulation produced any significant change in CAT activity using the 3.2-kb 5' fragment of mouse IRS-1 gene in 3T3-F442A adipocytes.

Effect of Dex on Half-Life of IRS-1 mRNA

In view of the dissociation between IRS-1 mRNA and promoter activity after Dex stimulation, we measured the half-life of IRS-1 mRNA in cells with or without Dex treatment. Specifically, cells were treated or not treated with Dex for 8 h, and actinomycin D (5 μg/ml final concentration) was added for up to 6 h before the harvest of the cells. The changes of IRS-1 mRNA are shown in Fig. 10A and quantitated in Fig. 10B taking the amount of IRS-1 mRNA without Dex and actinomycin D treatment as 100%. In the control cells, the t½ for IRS-1 mRNA was 190 ± 28 min; this decreased to 70 ± 11 min after Dex treatment (P < 0.002). Thus, the decrease of IRS-1 mRNA produced by Dex appears to be due to a 2.5- to 3-fold decrease in the half-life of IRS-1 mRNA.
DISCUSSION

To understand the potential molecular mechanisms involved in regulation of the IRS-1 gene, we have isolated the mouse IRS-1 gene, characterized its promoter, and examined the expression of IRS-1 mRNA in mouse tissues. The promoter activity of the 3.2-kb mouse IRS-1 5'–fragment is approximately 25% of that of a human insulin receptor promoter (24, 25) and is active only if inserted in the correct orientation (pIRS Ba-CAT). The promoter region of the mouse IRS-1 gene lacks typical TATA box and CAAT boxes but contains nine potential Sp1-binding sites, five potential AP-1 binding sites, two potential AP-2 binding sites, and one potential C/EBP binding site. Similar sites have been suggested to play a role in regulation of transcription of other genes involved in insulin action including the insulin receptor (25, 38) and GLUT4 glucose transporter (39). Several potential steroid hormone receptor-binding sites (30) and two potential insulin response elements (31, 32) are also located in this region. Finally, the IRS-1 promoter contains two purine (AG)-rich regions, one pyrimidine (CT)-rich region, and a CAG repetitive sequence promoter region. AG-rich sequences have been reported in many genes, but their function is not known. In some genes, CT repetitive sequences have been shown to enhance the transcription (40). Interestingly, all of these regions are conserved in human IRS-1 gene (13), suggesting that they have functional roles in IRS-1 gene expression.

Northern blot analysis revealed that IRS-1 mRNA is expressed in every tissue mRNA, supporting the notion that the IRS-1 gene is a housekeeping gene. However, expression of IRS-1 mRNA varied between tissues and was highest in brain, heart, lung, and kidney, which interestingly, except for the heart, are tissues not viewed as classic metabolic targets for insulin action. We have previously analyzed the abundance of the IRS-1 mRNA in human fetal tissues by a quantitative RT-PCR method (13). Human IRS-1 mRNA was most abundant in brain, kidney, and small intestine and was less abundant in liver and skeletal muscle. Thus, the tissue distribution in mice is similar to that in humans. Studies using immunocyto-
Effect of Insulin on IRS-1 Expression

![Graph showing the effect of insulin on IRS-1 expression in 3T3-F442A adipocytes.](image)

Fig. 6. Effect of Insulin on IRS-1 Expression in 3T3-F442A Adipocytes

The mean ± SD of IRS-1 protein (filled circle), IRS-1 mRNA (filled triangle), and CAT activity (filled square) after insulin stimulation (10^{-6} M) are plotted as a percent of the initial value. The data are the mean of four separate experiments. Asterisks indicate the statistic significance compared with the initial level (" = P < 0.05 and "" = P < 0.01).

Effect of Dexamethasone on IRS-1 Expression

![Graph showing the effect of dexamethasone on IRS-1 expression in 3T3-F442A adipocytes.](image)

Fig. 9. Effect of Dex on IRS-1 Expression in 3T3-F442A Adipocytes

The mean ± SD of IRS-1 protein (filled circle), IRS-1 mRNA (filled triangle), and CAT activity (filled square) after Dex stimulation (10^{-6} M) of four separate experiments are shown. Asterisks show the statistic significance compared with the initial level (" = P < 0.05 and "" = P < 0.01).

Chemistry suggests that within these tissues IRS-1 is not uniformly distributed but occurs in specific cell types (F. Folli and C. R. Kahn, manuscript in preparation). The differences of IRS-1 gene expression in various tissues may be due to tissue-specific transcription factors that regulate IRS-1 gene expression. Analysis of IRS-1 promoter activity using different cell lines should give us some idea as to the mechanism of tissue-specific regulation. In human and rats, we detected two species of IRS-1 mRNA (6.9 and 6 kb in human and 8.5 and 5.4 kb in rat) (13), whereas in most mouse tissues, excepting skeletal muscle, a single 9.5-kb IRS-1 mRNA was detected. The significance of the different size IRS-1 mRNA species is not known. The small amount of IRS-1 mRNA in mouse tissues agrees with the relatively low promoter activity of the mouse IRS-1 gene.
there are two overlapping inverted repeats and one possible glucocorticoid response element which has been reported to regulate the expression negatively (GRE−, Ref. 30). By deletion analysis, we could also map a 60-bp region between −1845 and −1586 bp involved in negative regulation of the mouse IRS-1 promoter. In this region, there is one AP-1 and one C/EBP putative binding site (see Fig. 1). To identify the possible transcription factor(s) that may be responsible for the negative regulation of the IRS-1 gene transcription, we performed gel shift assay using probes corresponding to −1606 to −1586 bp (probe 1) and −1645 to −1607 bp (probe 2) of this region. In this assay, we could identify a nuclear factor that binds to the probe 1. Since this binding was not competed for by addition of excess amounts of C/EBP, CREB, and AP-1 consensus sequences, this factor seems to be distinct from these factors. Further analysis is necessary to characterize this factor, which appears to be responsible for the negative regulation of the IRS-1 gene transcription.

Computer analysis of the 5'-flanking region of the mouse IRS-1 gene revealed an 814-nt region with 52% identity to the 5'-region of chicken progesterone receptor gene (35), a 374-nt overlap with 56% identity to the human insulin-like growth factor I receptor promoter (36) and a 59% identity in a 170-nt overlap with the 5'-region of the human androgen receptor gene (37). The homology domain of the mouse IRS-1 gene (−1248 to −466) and of the chicken progesterone receptor gene (35) (−212 to +583) includes one AG-rich region and several possible transcription start sites. The chicken progesterone receptor gene has been reported to code for two forms (A and B) of the receptor using two different ATG codons (19, 35, 43). The ATG for form B of the chicken progesterone receptor gene is included in this homology domain; thus the sequence of chicken progesterone receptor gene shown here represents a part of the promoter for the A form of the receptor (35, 43, 44). The high homology of the 5'-region of the mouse IRS-1 gene with those of chicken progesterone receptor gene and other hormone receptor genes may indicate that these genes are regulated in a similar manner. Further analysis of this point is necessary.

By primer extension analysis, six major and nine minor possible transcription start sites were identified. Multiple transcription start sites have also been reported for the human epidermal growth factor (EGF) receptor (41) and insulin receptor genes (42), which also lack typical TATA and CAAT boxes but possess multiple Sp1-binding sites. Although it was not possible to confirm these transcription start sites by S1 mapping analysis due to the low abundance of the IRS-1 mRNA, the primer extension experiments were reproducible. The fact that the start sites of rat and human IRS-1 cDNAs mapped among these possible transcription start sites also supports these results.

The analysis of the transcriptional activity of the 5'-region was performed employing deletion analysis of pIRS3a-CAT transiently expressed in CHO cells. Two negatively and two positively acting regions could be mapped by this analysis. In one of the negatively acting region localized between −3369 and −3185, there are two overlapping inverted repeats and one possible glucocorticoid response element which has

![Fig. 10. Effect of Dex on IRS-1 mRNA in 3T3-F442A Adipocytes](https://example.com/fig10)

A, 3T3-F442A adipocytes were treated with 10−6 M Dex or vehicle only (−Dex) for 8 h. Actinomycin D (5 μg/ml final concentration) was added 45, 120, and 240 min before the harvest of the cells for RNA. IRS-1 mRNA was measured using RT-PCR as described in Materials and Methods. A representative result of three separate experiments is shown. B, Time courses (mean ± so) of the IRS-1 mRNA from non-Dex-treated (open circles) or Dex-treated (closed circles) for three experiments are shown. Data are plotted as the percent of the initial level of IRS-1 mRNA from the cells without Dex treatment and without actinomycin D treatment.
Insulin significantly decreased IRS-1 protein in 3T3-F442A cells in a relatively short time (30 min) but did not change the amount of IRS-1 mRNA or promoter activity as measured in the CAT assay. Thus, the decrease of IRS-1 protein is likely due to decreased half-life of IRS-1 protein and/or increased degradation of IRS-1 possibly due to posttranslational modification of the protein. Regulation of IRS-1 in 3T3-L1 adipocytes by insulin has also been studied by Hice et al. (21, 46), who have found that insulin stimulates the degradation of IRS-1 protein in 3T3-L1 adipocytes. They have shown that the half-life of IRS-1 protein is about 25 h in the basal state and decreases to 2.5 h in the presence of 1 μM insulin (46). They have also shown that the insulin-induced down-regulation of IRS-1 protein occurs in the presence of cycloheximide. Our data are consistent with these observations and further support that insulin has no effect on IRS-1 mRNA level and IRS-1 gene promoter activity.

Dexamethasone also decreased the level of IRS-1 protein; however, the mechanism of the down-regulation in this case appears to be different. Dexamethasone decreased the IRS-1 mRNA, as well as protein, without changing the IRS-1 promoter activity. Furthermore, Dex decreased IRS-1 mRNA half-life to almost one third of control levels. The decrease in IRS-1 mRNA started at 1 h after stimulation with Dex and preceded the decrease in IRS-1 protein, which was first detected after 2 h of stimulation. This delay could be explained by the time required for IRS-1 translation from mRNA to protein. Taken together, the down-regulation of IRS-1 produced by glucocorticoids is due mainly to a decrease in IRS-1 mRNA half-life. Although the sequencing analysis of IRS-1 promoter region suggests potential GREs and IREs, the promoter activity as measured in the CAT assay in 3T3-F442A adipocytes did not confirm a significant regulation by insulin or Dex. Further analysis, including cotransfection with glucocorticoid receptor cDNA and use of other cell types, will be needed to characterize these potential cis-acting elements.

MATERIALS AND METHODS

Cloning and Sequencing of the 5'-Region of the Mouse IRS-1 Gene

Mouse IRS-1 gene was isolated from a mouse genomic library as described (15). A 6.7-kb BamHI-EcoRI fragment which contained 3.4 kb of the 5'-flanking region and 3.3 kb of the coding region of mouse IRS-1 gene was isolated from λMO2 phage DNA (15) and subcloned into pBlueScript II (Stratagene, La Jolla, CA). The nt sequence of the insert was determined by the dideoxy chain termination method (47) using a Sequenase DNA sequencing kit (United States Biochemical Corp., Cleveland, OH).

Northern Blot Analysis of Mouse IRS-1

A portion (2.6 kb) of mouse IRS-1 gene coding region (16-2581 nt, in Ref. 15) was isolated, 32P labeled using an oligo-labeling kit (Pharmacia, Piscataway, NJ), and used as a probe (106 cpm/ml). A multiple tissue Northern (MTN) blot (CLON-TECH, Palo Alto, CA) on which 2 μg of poly(A)+ RNA from eight different mouse tissues were subjected to analysis was used following recommendations of the manufacturer. The final blot was reprobed with the β-actin cDNA (48) as a control.

Analysis of the Transcription Start Sites

Poly(A)+ RNA was prepared from mouse liver using the RNAzol B (Biotex Laboratories, Houston, TX) followed by oligo (dT) cellulose chromatography. Five different oligonucleotides synthesized complementary to the 5'-region of the gene were end labeled with [γ32P]ATP using T4 polynucleotide kinase. Five micrograms of poly(A)+ RNA or yeast RNA (as a control) were mixed with 2 pmol labeled primer and heated at 95 C for 2 min, chilled on ice, and annealed at 37 C for 30 min and then subjected to a reverse transcription reaction. To directly compare these products with the genomic nt sequence, the sequence reaction was performed as described above using the same primer that had been used for primer extension and electrophoresed in a 6% ureacylamide gel with the products of the primer extension reaction.

Construction of the IRS-1 Promoter CAT Gene Fusion Plasmids

For the analysis of the 5'-flanking region of the mouse IRS-1 gene, a low background promoterless CAT plasmid with multicing sites (5'-SacⅠ-3', HindⅢ-XhoⅠ-SphⅠ-KpnⅠ-BglⅡ-ecOHV-3') named pSVU01 CAT was constructed. Two synthetic oligonucleotides (5'-AGCGAGCTCTCGCAAGCCTTCTCGAACATCCGGTACGATCGTCTGTAATC3' and 5'-AGCGAGCTCTCGCAAGCCTTCTCGAACATCCGGTACGATCGTCTGTAATC3') were annealed and inserted at the HindⅢ site of pSV0001 CAT (23). A 3.2-kb fragment was isolated by BamHI and HInfl digestion from the 5'-region of the IRS-1 gene, blunt-ended using the Klenow fragment of DNA polymerase I, and inserted at the EcoRV site of pSV0001 CAT. The resultant plasmids, which contained the 5'-region fragment in the correct orientation and reverse orientation, were named pIRSBa-CAT and pIRSBar-CAT, respectively. 3'-Deletions of the mouse IRS-1 gene were made by excising the fragments from pIRSBa-CAT using restriction enzymes that cut the polynucrinder and mouse IRS-1 gene insert fragment (KpnⅠ, SphⅠ, BglⅡ and XhoⅠ); the resulting constructs were named pIRSelp-CAT, pIRSSp-CAT, pIRSBg-CAT, and pIRSh-CAT, respectively. For the construction of pIRSbs-CAT, pIRSps-CAT was digested with HindⅢ (in the polynucrinder of pSV001 CAT) and BspEⅠ, blunt-ended using Klenow fragment of DNA polymerase I, and self-ligated.

Cell Culture and Transient CAT Assay

To test the activity of the CAT plasmid constructs, CHO cells grown in F12 medium with 10% FBS were used for transfection. Cells (5 x 105) were plated in 100-mm dishes 24 h before transfection. Transfections were carried out by the calcium phosphate method using 10 μg plasmid DNA followed by 12.5% glycerol shock after 4 h of transfection. Cells were harvested 48 h later and subjected to CAT assay as previously described (23, 25, 49). As an internal control, 2 μg pAC-lacZ plasmid, which contains the lacZ gene under the control of chicken β-actin promoter (gift from Dr. J. Miyazaki [49]), was cotransfected with the CAT plasmid, and CAT
activities were normalized by β-galactosidase activities. For positive controls, pHIR-CAT, which carries the human insulin receptor promoter (pSphl-00CAT in Ref. 25), and pSV2-CAT, which carries the SV40 early promoter, were used. All experiments were performed in duplicate, and a representative result is shown. CAT assays were done three times, and each set of experiments was carried out using the different preparations of plasmid DNAs. The radioactivity of each spot of the CAT assays was determined by Cerenkov counting. Statistical significance was determined by one-way analysis of variance followed by the Newman-Kuels post hoc test.

**Gel Shift Assay**

Two fragments corresponding to the sequence −1606 to −1586 (5'-GATCAGAAATCTTTCAAAAAATGTA-3' and 5'-GATCTACATTCTTTGAAGATTTCT-3', probe 1) and −1646 to −1626 (6'-CCCGAGATCTTGAAGAGATGCTCACTAACCTTGGTG- 3' and 5'-CCCGAGATCTTGAAGAGATGCTCACTAACCTTGGTG-3', probe 2) of the IRS-1 gene were used as probes. Both probes were end labeled with [$\gamma$-32P]ATP using T4 polynucleotide kinase before analysis. Nuclear extract was purified from the CHO cells as previously described (50). Competitors corresponding to the C/EBP, CREB, and AP-1 binding sites were designed according to the previously reported consensus sequences for C/EBP, 5'-GATCCAAATGTAGTCATATGCAATACCCACG-3' and 5'-GATCTCCTTTGCTGACTCAAGAGAC-5' (52), and for AP-1, 5'-GATCCAAAAAGATTCAAGGT-3' and 5'-GATCTCCTTTGCTGACTCAAGAGAC-5' (52), for C/EBP, 5'-GATCCAAAAAGATTCAAGGT-3' and 5'-GATCTCCTTTGCTGACTCAAGAGAC-5'. Approximately 1 fmol 32P-labeled probe was added to the nuclear extract, and the preparations were incubated with 1 μg poly(dI-dC) in binding buffer (25 mM Hepes (K+), pH 7.9, 50 mM KCl, 0.5 mM EDTA, 10% glycerol, 0.5 mM dithiothreitol, and 0.05 mM phenylmethyl sulfonyl fluoride (PMSF)) on ice for 30 min. One micromole (1000-fold excess) of nonlabeled competitor DNA fragment was added at the same time as the labeled probe. DNA-protein complexes were resolved on a 3.4% polyacrylamide gel with 0.4 TBE buffer, and 0.05% SDS. The DNA was dried and autoradiographed with intensifying screen at −70°C.

**Construction of the 3T3-F442A Cells That Carry the Fusion Plasmid of Mouse IRS-1 Promoter and CAT**

3T3-F442A cells were cotransfected with the fusion plasmid of the 3.2-kb mouse IRS-1 promoter and CAT gene (pirSBA- CAT) and pcDNANeo (Invitrogen, San Diego, CA) which carries a neomycin resistance gene as described previously (5). Seven of 12 neomycin-resistant clones expressed levels of CAT activity sufficient for analysis. Two of these (3131HSCL15 and 3131HSCL21-4) were used for further analysis.

For the studies of hormonal regulation of IRS-1, 3T3-F442A cells were grown in subconfluence in Dulbecco’s modified Eagle’s medium (DMEM) with 10% calf serum and then differentiated to adipocytes with DMEM with 10% fetal calf serum containing 10−6 M insulin for 8–12 days. The differentiated cells were detached with 0.05% trypsin/0.2 mM EDTA, and equal numbers of cells were plated in a six-well dish (8 × 104 cells per well) and allowed to recover in DMEM with 10% calf serum for 16 h without insulin. The cells were then washed three times with serum-free medium and incubated in serum-free DMEM overnight. For each experiment, cells were incubated with vehicle only, 10−6 M insulin, or 10−6 M Dex for the indicated times and used for Western blot, mRNA measurement, and CAT assay.

**Western Blot Analysis of IRS-1**

After hormonal treatment, 3T3-F442A cells were lysed with 0.5 ml extraction buffer (100 mM Tris, pH 8.0, containing 100 mM sodium fluoride, 4 mM EDTA, 2 mM sodium vanadate, 3.4 mg/ml PMSF, 100 mg/ml aprotinin, and 1% Triton X-100). The cells were scraped from the dishes, and insoluble material was removed by centrifugation at 15,000 rpm for 15 min in a microcentrifuge (DuPont, Wilmington, DE). The supernatant was treated with Laemmli sample buffer with 100 mM dithiothreitol and heated in a boiling water bath for 5 min. Equal amounts of protein (80–150 mg) for each set of experiments were subjected to SDS-PAGE (6% bis-acrylamide) in a Bio-Rad miniature slab gel apparatus (Bio-Rad Laboratories, Richmond, CA). Electrotransfer of proteins from the gel to nitrocellulose (Schleicher & Schuell, Keene, NH) was performed for 1 h at 90 V (constant) in a Bio-Rad miniature transfer apparatus as described by Towbin et al. (54), except that 0.02% SDS was added to the transfer buffer to enhance elution of high molecular mass proteins. The blots were incubated with the α-CT-IRS1 (a polyclonal antibody against a peptide designed from the C-terminal sequence of rat IRS-1) (13, 20), washed, and incubated with [125I]protein A (ICN Nutritional Biochemicals, Cleveland, OH). Autoradiography was performed as described previously (20), and the band intensities were quantitated by scanning densitometry (Molecular Dynamics, Sunnyvale, CA).

**Measurement of IRS-1 mRNA**

Due to their very small quantities, IRS-1 mRNA levels were measured using the RT-PCR method as described previously (66) with β2-microglobulin mRNA as an internal control. Primers for the RT-PCR were designed from the mouse IRS-1 gene sequence and mouse β2-microglobulin sequence (56). For IRS-1, the 5’-primer was AGTGGCCATGGCTCCAC (corresponding to nt 1177–1193), and the 3’-primer was TTGCACCATGCAGAT (corresponding to nt 1381–1397); for β2-microglobulin, the 5’-primer was TTGCACGAGTCCGG (corresponding to nt 3139–3156 in Ref. 56), and the 3’-primer was CTGCTTCTACATCTGCAGAT (corresponding to nt 3785–3802 in Ref. 56). RNA was prepared from the cells using RNAzol B, then treated with RNase-free DNase followed by phenol/chloroform extraction and ethanol precipitation. One microgram of each RNA sample was reverse transcribed using two 3’-primers (from IRS-1 and β2-microglobulin) 1 pmol each primer per reaction, then used for PCR. For each PCR reaction, one-twentieth of the total cDNA was used as a template. Before the PCR reaction, the primers were 32P end labeled as described by Wang et al. (55) omitting the step to remove the unincorporated nt. The final PCR conditions were 10 pmol of each primer per reaction, denaturing at 94°C for 30 sec, annealing at 57°C for 30 sec, and extension at 72°C for 1 min. Linear amplification of IRS-1 and β2-microglobulin mRNA was achieved using 50 ng total RNA per reaction and 21 to 27 cycles; amplification was also linear in both RT-PCR and PCR methods over a wide range of total RNA per PCR reaction (0.0156–0.25 μg) and 1–500 attomoles of IRS-1 cDNA per PCR reaction (data not shown). The final PCR products were separated on a 5% acrylamide gel in 0.5 TBE buffer, and gels were dried and subjected to autoradiography. The appropriate bands were cut from the gel, and the radioactivity was determined by Cerenkov counting. The amount of IRS-1 mRNA was normalized to the amount of β2-microglobulin mRNA. To determine the amount of IRS-1 PCR product derived from the contamination of genomic DNA in the RNA sample, the H1–H14 assay was performed omitting the reverse transcriptase.
CAT Assays

Cell lysates for the CAT assay were prepared as described previously (23, 25). The reaction mixture for the CAT assay contained 0.5 μCi [3H]chloramphenicol and 20 μl of lysates in a final volume of 75 μl. The labeled chloramphenicol and acetylated derivatives were separated by ascending TLC on silica gel plates (CHCl3/methanol, 90:10) and visualized by autoradiography. The radioactivity of each spot of the CAT assays was determined by Cerenkov counting.

Half-Life Studies of IRS-1 mRNA

Cells were treated with 10^{-6} μM Dex or vehicle only for 8 h. Actinomycin D (5 μg/ml final concentration) was added 45, 120, and 240 min before the harvest of cells for RNA. The quantification of IRS-1 mRNA was performed using the RT-PCR method described above.

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