Identification of Intron and Exon Sequences Involved in Alternative Splicing of Insulin Receptor Pre-mRNA*

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The human insulin receptor (IR)1 is encoded by a single gene that is located on chromosome 19 and composed of 22 exons. The mature IR consists of three isoforms, designated A and B, which result from alternative splicing of the primary transcript (1–3). The A isoform lacks exon 11, is expressed ubiquitously, and is the only isoform in lymphocytes, brain, and spleen; the B isoform contains exon 11 and is expressed predominantly in liver, muscle, adipocytes, and kidney (4–6). Exon 11 is composed of 36 nucleotides that encode a 12-amino acid segment (residues 717–728) of the carboxyl terminus of the α-subunit of IR. A number of investigators have suggested that the isoform ratio could be altered in non-insulin-dependent diabetes mellitus (7–10), but other studies have produced conflicting results (11–14). We have found that alterations in isoform ratio in skeletal muscle were associated with hyperinsulinemia rather than diabetes (15). Similar results have been found in the rhesus monkey (16). Along these lines, Sell and co-workers (17) have shown that alternative splicing of the IR gene is regulated by insulin in the Fao hepatoma cell line. Furthermore, we have shown that the alternative splicing is hormonally and developmentally regulated in both the HepG2 hepatoma and 3T3-L1 adipocyte cell lines (18). The changes in splicing were accompanied by increases in insulin sensitivity, as measured by a number of parameters (19). These data indicate that regulation of the alternative splicing of the IR is important for insulin sensitivity and responsiveness.

Splicing of pre-mRNA depends on the presence of relatively short RNA sequence elements, the 3′ splice site, the 5′ splice site, the branch point sequence, and the polypyrimidine tract. In alternative splicing, a given splice site may be selected or ignored depending on the cell type or physiological state. This apparent flexibility of the splicing machinery raises the question of molecular mechanisms involved in selection of certain splice sites over others. A number of factors have been implicated in the choice of alternative splice sites, including RNA secondary structure (20–23), size of the exon (24, 25), and relative strengths of the competing splice sites (26). Alterations in the splicing pattern of a number of genes have been demonstrated during cellular differentiation (27); however, the hormonal regulation of alternative splicing is not as common. We have shown that the alternative splicing of exon 11 of the IR gene is modulated by glucocorticoids in HepG2 cells, and, as mentioned above, insulin modulates splicing in Fao cells (17, 18). Insulin has also been shown to alter the splicing pattern of the COOH terminus of protein kinase C-β in L6 myotubes, and growth factors including epidermal growth factor, platelet-derived growth factor, and basic fibroblast growth factor alter the splicing of the COOH terminus of protein-tyrosine phosphatase 1B in human fibroblasts (28, 29). Chew and co-workers (30) have shown that splicing of the insulin-like growth factor-I mRNA is regulated by growth hormone in HepG2 cells. The molecular mechanisms involved in the hormonal regulation of this process are not understood. A prerequisite for mechanistic studies of hormonal regulation is a knowledge of the RNA sequences involved in the alternative splicing event. To that end, we have identified the regions of intron 10 and exon 11 involved in the alternative splicing of the IR gene.

EXPERIMENTAL PROCEDURES

Materials—Cell culture reagents were purchased from Life Technologies, Inc., and fetal calf serum was from Gemini Bioproducts (Calaba-
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Fig. 1. Structure of IR minigenes containing exon 11. Panel A, schematic of IR minigene. Basic minigene contains 110 nucleotides of exon 10, 2.3 kb of intron 10, 36 nucleotides of exon 11, and 103 nucleotides of exon 12. Intron 11 is greater than 8 kb in length; consequently, a large internal deletion was created leaving approximately 180 nucleotides at both the 5' and 3' ends. Corresponding amino acid residues are indicated below the minigene. Numbers above intron 10 indicate the positions used to create the internal deletions described in this paper. The two As indicate potential BPS upstream of the 3' splice site. Minigenes are subcloned into mammalian expression vector pSG5. Panel B, sequence of IR minigene. Sequence of basic minigene is shown divided into exons and introns. The end points of the internal deletions are indicated by numbers below the sequence of exon 10.

To measure exogenous minigene IR transcripts, the primer pair consisted of oligonucleotides spanning nucleotides 1022–1042 (sense primer, 5'-TAATACGACTCACTATAGGGC-3') and 1068–1088 (antisense primer, 5'-GCTGCAATAAACAAGTTCTGC-3'), which are unique sequences in the pSG5 vector just upstream and downstream of the cloning sites. To measure endogenous IR transcripts, the primer pair consisted of oligonucleotides spanning nucleotides 2140–2163 (sense primer, 5'-TAATACGACTCACTATAGGGC-3') and 2424–2447 (antisense primer, 5'-TTTCTACAAAGCGCTTGCCTTCTC-3') and 2424–2447 (antisense primer, 5'-TTTCTACAAAGCGCTTGCCTTCTC-3') of the IR cDNA (numbering as in Ref. 1), which lie outside the minigene and, thus, specific for the endogenous gene.

Five μl of the DNA synthesis reaction was used for PCR amplification in a 50-μl final reaction volume (0.5 μl each oligonucleotide primer, 10 μl Tris-HCl (pH 8.3), 50 mm KCl, 1.5 mm MgCl₂, 0.1 mm dNTPs, 2 units of Taq DNA polymerase, and 1 μCi of [α-32P]dCTP). Twenty-five cycles of amplification were performed using a Perkin-Elmer DNA thermal cycler (System 9600). Each cycle consisted of a 30-s denaturation at 94 °C, a 30-s annealing at 55 °C, and a 60-s extension at 72 °C. The number of cycles was optimized to ensure that the amplification lay within the exponential phase. The products of the PCR amplification were resolved by electrophoresis on 8% polyacrylamide gels. The gels were dried and exposed to film at room temperature. The band densities were quantitated using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

RESULTS AND DISCUSSION

Intron 10 Contains All the Sequence Information for Alternative Splicing of Exon 11—We have shown previously that the endogenous IR gene in HepG2 cells generates mRNA for both the A and B isoform IR. We used this cell line to investigate which sequences surrounding the alternatively spliced exon 11 are involved in the alternative splicing. A minigene was created from by amplifying each exon and the adjacent intron from the known exon/intron sequences (2). This minigene contained 110 nucleotides of exon 10, 2.3 kb of intron 10, 36 nucleotides of exon 11, 184 nucleotides of the 5' end of intron 11, 188 nucleotides of the 3' end of intron 11, and finally 103 nucleotides of...
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**FIG. 2. Transfection of minigenes into HepG2 cells.** Panel A, structure of basic minigene and four internal deletions of intron 10 in order of decreasing size. Deletions are indicated by dashed lines. Numbers above intron 10 refer to the end points of the deletions. The As indicate potential branch point sequences. Panel B, autoradiogram of representative gel showing splicing of transfected minigenes. Minigenes were transfected into HepG2 cells by the calcium phosphate co-precipitation technique. Forty-eight hours later, cells were harvested and total RNA isolated. RNA was subjected to RT-PCR as described under “Experimental Procedures.” PSG indicates cells transfected with the parental expression vector without a minigene insert. Letters A and B to left of the gel show the PCR products generated from A (– exon 11) and B isoform (+ exon 11) splicing (274 and 310 base pairs, respectively). Panel C, quantification of alternative splicing. Gels were quantified using a PhosphorImager. Results are from four independent experiments performed in duplicate and are expressed as percent of B isoform (+ exon 11) splicing and show the mean ± S.E. Lowercase letters indicate statistical significance: a, p < 0.01 vs. minigene A; B, p < 0.01 vs. minigene C; c, p < 0.01 vs. minigene B and C. Panel D, identification of intermediate splicing products. PCR amplification was performed for 40 cycles in the absence of [32P]dCTP and analyzed on a 1.5% agarose gel. Products from fully spliced mRNA, precursor RNA and an intermediate are indicated to the left of the gel.

exon 12 (Fig. 2, minigene B). Transfection of minigene B into HepG2 cells gave RNA corresponding to both the A (– exon 11) and B (+ exon 11) isoform splicing patterns in a 40:60 ratio (Fig. 2, panels B and C). The endogenous gene in these cells expressed both isoform RNAs in a 50:50 ratio using the endogenous gene primer pair (data not shown), suggesting that the minigene contained most of the information for correct splicing. A second minigene (minigene A), containing a large internal deletion of 2.0 kb in intron 10 (between positions 3 and 5 in the minigene), caused an increase in the percent of B splicing isoform (Fig. 2, compare minigenes A and B). A further deletion (to position 7) in minigene C caused a complete loss of B isoform splicing suggesting that sequences in this region (between positions 5 and 7) may be important for skipping of exon 11.

Two potential branch point sequences (BPS) were identified in the intron upstream of exon 11 (indicated by A in Fig. 2A). Removal of the upstream potential BPS by deletion to position 9 (minigene D) caused a large (80%) decrease in the amount of B isoform splicing, suggesting that this BPS may be involved in splicing site selection. These deletions suggested that the regulatory sequences might lie in the region upstream of exon 11. Therefore, intron 10 was sequenced from the 3’ end, and an Alu repetitive sequence was found approximately 300 nucleotides upstream of exon 11. A further minigene was created (minigene E) deleting the central portion of the intron up to, but no further than, the Alu repeat. Transfection of minigene E into HepG2 cells gave a splicing ratio that was identical to minigene A; therefore, the extra sequence up to the Alu repeat did not affect splicing.

The PCR amplification was performed for 40 cycles in an attempt to identify low abundance intermediate splice products for these five minigenes (Fig. 2D). The mature, fully spliced products (mature mRNA) were observed as well as the unspliced precursor RNAs (pre-mRNA) that differed in size due to the internal deletions in the minigenes. No precursor RNA was observed for minigene B as it is predicted to be >3 kb and is not amplified under standard conditions. A product of ~700 base pairs was observed for all minigenes regardless of the size of the internal deletion in intron 10. This PCR product was subcloned, sequenced, and found to be a splicing intermediate lacking intron 10 but still containing intron 11. No intermediates containing intron 10 but lacking intron 11 were isolated. Thus, removal of intron 10 preceeds removal of intron 11 in agreement with the processive model of RNA splicing and suggests that the 3’ splice site of exon 11 is subject to selection.

Intron 10 Contains a Splicing Enhancer Sequence and an Inhibitory Region That Causes Exon Skipping—The increase in B isoform splicing by the internal deletions in minigenes A, C, and E in Fig. 2 could be explained by alterations in the spacing of the splice sites. If this were the case, then larger deletions should have an even greater effect. Consequently, minigenes K, L, and M were constructed with larger deletions starting 26 nucleotides downstream of the 5’ splice site of exon 10 (Fig. 3, position 1) and extending to the end points of minigenes A, C, and D (Fig. 3, minigenes K, L, and M, respectively). Surprisingly, transfection of minigene K into HepG2 cells gave <10% B isoform splicing in contrast to minigene A which gave >85%.
FIG. 3. Identification of splicing enhancers and inhibitors in intron 10. Left panel, structure of minigenes used in these experiments. Deletions are indicated by dashed lines. Numbers above intron 10 refer to the end points of the deletions. The As indicate potential branch point sequences. Right panel, quantification of alternative splicing by RT-PCR. Minigenes were transfected into HepG2 cells by the calcium phosphate co-precipitation technique. Forty-eight hours later, cells were harvested and total RNA isolated. RNA was subjected to RT-PCR as described under “Experimental Procedures.” Gels were quantified using a PhosphorImager. Results are from four independent experiments performed in duplicate and are expressed as percent of B isoform (+ exon 11) splicing and show the mean ± S.E. The vertical dashed line indicates the degree of exon incorporation observed with the parental minigene. ns, not significantly different from the parental minigene B.

FIG. 4. Mutation of the 3′ splice site. Left panel, sequence of the intron 10 splice site mutations used in these experiments. Deletions are indicated by dashed lines. Point mutations are indicated by letters, and dots indicate identity with the parental minigene. Numbers above the sequence refer to the end points of the deletions. The 3′ splice site is indicated by the colon. Right panel, quantification of alternative splicing by RT-PCR. Minigenes were transfected into HepG2 cells by the calcium phosphate co-precipitation technique. Forty-eight hours later, cells were harvested and total RNA isolated. RNA was subjected to RT-PCR as described under “Experimental Procedures.” Gels were quantified using a PhosphorImager. Results are from four independent experiments performed in duplicate and are expressed as percent of B isoform (+ exon 11) splicing and show the mean ± S.E. The vertical dashed line indicates the degree of exon incorporation observed with the parental minigene.
It is very striking that elimination of intronic sequences in the 5' end of intron 10 (between positions 1 and 3) caused a dramatic (75%) decrease in exon 11 inclusion. This suggests that this region contains a sequence that enhances inclusion of exon 11. Deletion of the sequences between positions 5 and 7 (mini-gene L) caused a large increase in the amount of B isoform splicing consistent with the results from minigenes A and C (Fig. 3). The change in splice site usage is even more dramatic for minigenes K and L than for minigenes A and C, as a result of the absence of the upstream enhancer that favors exon 11 inclusion. These results confirm that sequences between positions 5 and 7 cause exon skipping. Elimination of an additional 26 nucleotides to position 9 (minigene M) caused complete loss of B isoform splicing. A similar loss in B isoform splicing was associated with deletion of this region in minigenes C and D. However, minigene D still showed approximately 20% B isoform splicing, suggesting that this deletion severely weakens the 3' splice site but partial recognition is possible in the presence of the upstream enhancer.

This upstream splicing enhancer was identified by comparing minigenes A and K. Both of these minigenes contain large internal deletions within intron 10, which brings the enhancer closer to the 3' splice site. What is the function of this region in the context of the full intron? This region was deleted in the full intron (Fig. 3, minigene W). Deletion of this region caused a 20% decrease in B isoform splicing. Consequently, this region does function as a splicing enhancer but its effect is less pronounced when it is >2 kb upstream of the splice site (compare minigenes W, A, and K). The enhancer region contains a purine-rich sequence, so two additional deletions were created to assess the contribution of this purine-rich region. Minigene Y lacks the purine-rich sequence, whereas minigene X lacks the remaining sequence in this region. Only minigene Y showed a statistically significant decrease in B isoform splicing. The purine-rich region is 75% GA over a stretch of 60 nucleotides. Repeats of the motif GARGAR have been shown to function as splicing enhancers in other genes (31). The sequence in intron 10 does not contain such repeats, but is very GA-rich and may function in a similar manner.

The region between positions 5 and 7 that causes exon skipping was identified, as was the enhancer sequence, using minigenes with large internal deletions in intron 10. What is the role of this inhibitory region in the context of the full intron? A minigene was constructed which lacked only the inhibitory region between positions 5 and 7 (Fig. 3, minigene F). Transfection of minigene F into HepG2 cells gave >95% B isoform

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**Fig. 5. Effect of mutation of exon 11 sequences.** Left panel, sequence and structure of minigenes used in these experiments. Deletions are indicated by dashed lines. In the upper panel, the exon is indicated by the boxed sequence, point mutations by letters below the sequence and an insertion by three Ts in minigene AB. In the lower panel, numbers above intron refer to the end points of the deletions. The As indicate potential branch point sequences. The letters in exon 11 indicate the four constitutive point mutations. Panel B, quantification of alternative splicing by RT-PCR. Minigenes were transfected into HepG2 cells by the calcium phosphate co-precipitation technique. Forty-eight hours later, cells were harvested and total RNA isolated. RNA was subjected to RT-PCR as described under “Experimental Procedures.” Gels were quantified using a PhosphorImager. Results are from four independent experiments performed in duplicate and are expressed as percent of B isoform (+ exon 11) splicing and show the mean ± S.E. The vertical dashed line indicates the degree of exon incorporation observed with the parental minigene.
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RNA compared with 65% for minigene B with the full intron. Thus, removal of 70 nucleotides from the 2.3-kb intron causes an increase in B isoform splicing. The inhibitory region was further localized to a 43-nucleotide sequence between positions 6 and 7 (minigene N). Deletion of the 27 nucleotides between positions 5 and 6 had no effect on splicing (minigene S). Removal of another 26 nucleotides (minigene G) or 52 nucleotides to the 3′ splice site of exon 11 (minigene H) dramatically weakens the splice site as would be expected. Similarly, small deletions of 26 or 13 nucleotides (minigenes O and T) caused a dramatic drop in exon 11 inclusion, suggesting that this 13-nucleotide sequence is necessary for the efficient use of the 3′ splice site.

Mutation of the 3′ Splice Site in Intron 10—Previous internal deletions in intron 10 had suggested that elimination of the upstream series of adenines between positions 7 and 9 in the minigene impaired the use of the downstream splice site and caused skipping of exon 11 (Fig. 4, minigene O). However, all deletions eliminated other regulatory regions as well. Adenine residues have been identified as the branch point nucleotides in many but not all introns, so elimination of these adenines might explain the alteration in splicing. Mutation of the four adenine residues in this region had no effect on splicing (Fig. 4, minigene I), indicating that this sequence cannot be the functional BPS. However, deletion of the three adenine residues proximal to the splice site (minigene V) gave <5% exon 11 inclusion. Although this result does not specifically identify the branch point residue, it is likely that one of these adenines is the functional BPS. Alignment of the most distal of the three adenines UCUCAA with the consensus branch point sequence UNCURAC indicates that this residue could be the branch point, however, accurate identification of the branch point residue will require in vitro branch point mapping. So why does B isoform splicing decrease when the region containing the four upstream adenines is deleted (minigene O)? One possible explanation is that the deletions may have impaired the function of the putative downstream BPS. A deletion from position 8 to 9 between the two stretches of adenines gave a similar reduction in exon inclusion (minigene T). The 3′ end of this deletion was 2 nucleotides 5′ to the BPS. All of the previous deletions were constructed by engineering BamHI restriction sites. Therefore, a minigene was constructed with four nucleotide substitutions to create a BamHI site at a position analogous to the deletion mutants (minigene U). This minigene gave <5% exon inclusion, indicating that mutation of four residues GUUCUCAAAGG to GGAUCCAAAGG could result in exon skipping, presumably by impairing the function of the BPS described above. Conversely, mutation of four nucleotides downstream of the triple adenines had little effect on splicing (minigene AE). The 3′ splice site in this intron contains a single G residue in the middle of a stretch of nine pyrimidines. Purine residues in the center of the poly pyrimidine tract have been shown to have a detrimental effect on RNA splicing (32). Mutation of the guanine residue to thymidine had little effect on exon inclusion (minigene AA). However, increasing the length of the py tract to 14 pyrimidines caused the exon to be spliced constitutively (minigene AF). Thus, the alternative splicing of exon 11 requires a weak 3′ splice site and strengthening the site renders the exon constitutive similar to results for other genes (33).

Exon 11 Sequences Are Involved in Splice Site Selection—To investigate whether the alternatively spliced exon itself might be involved in splice site recognition, we introduced mutations into exon 11 in the parental minigene B. Introduction of four point mutations in the middle of the exon caused the exon to be spliced constitutively (Fig. 5, minigene J). An overlapping four point mutations, however, had no effect (minigene AC). A deletion of eight nucleotides or insertion of three thymidine residues caused an almost complete loss of exon inclusion (Fig. 5, minigenes Z and AB). The point mutations that rendered the exon constitutive were tested in combination with deletions of the inhibitory region identified earlier (Fig. 3). There was no additional effect when combined with minigenes F and N that are deleted for the 3′ inhibitory region (Fig. 5, minigenes P and Q). Interestingly, when these exon mutations were introduced into minigene O, which contained a deletion that impaired function of the putative BPS, there was an increase in the amount of B isoform splicing. Thus, the weakened BPS is able to function, albeit weakly, in the presence of mutations in the exon. These results indicate that the exon 11 sequences play an active role in determining the degree of exon inclusion in both a positive and negative manner.

Models for the Regulation of the Alternative Splicing of Exon 11—The alternative splicing of exon 11 of the IR gene is consistent with the models depicted in Fig. 6. The GA-rich splicing enhancer at the 5′ end of intron 10 favors inclusion of exon 11. This could be due to a direct effect on the 3′ splice site. Alternatively, as the enhancer is >2 kb upstream of the BPS, the effect of the enhancer could be on the adjacent 5′ splice site. The proximal site (TAG:GUAGA) differs significantly from the consensus (CAG:GUAGA) so the effect of the enhancer may be to strengthen the interaction of the U1 small nuclear ribonucleoprotein particle with the 5′ splice site (34). How this might affect alternative splicing of the downstream exon is not clear, as this site is used whether or not the exon is included, but it may allow the use of a suboptimal splice acceptor site. SR proteins that recognize purine-rich enhancer sequences are known to favor use of proximal splice sites (35–42). SF2/ASF is a member of the SR protein family and can bind to GA-rich splicing enhancer sequences similar to that identified in the 5′ end of intron 10. Overexpression of SF2/ASF has been shown to promote inclusion of alternatively spliced exons in the rat clathrin light chain B and rat β-tropomyosin genes (41, 42). This is due to the ability of SF2/ASF to promote the use of a proximal splice site, either 5′ or 3′, over a distal site. Interestingly, this activity is antagonized by the hnRNP-A1 splicing
factor, which favors the use of distal splice sites over proximal. The observed choice of splice sites reflects a balance between SF2/ASF and hnRNP-A1 activities. Both SF2/ASF and hnRNP-A1 are RNA-binding proteins. In contrast to the SR proteins, hnRNP-A1 binds to sequences containing the motif UAGGGG or UAGGGOU (43). The 5’ end of intron 10 also contains the sequence CTAGGGACC, which includes an hnRNP-A1 binding site (underlined). Whether hnRNP-A1 binds to sequences containing the motif hnRNP-A1 is RNA-binding proteins. In contrast to the SR proteins, hnRNP-A1 binds to sequences containing the motif SF2/ASF and hnRNP-A1 activities. Both SF2/ASF and hnRNP-A1 are RNA-binding proteins. In contrast to the SR proteins, hnRNP-A1 binds to sequences containing the motif.

At the 3’ end of the intron, there are two regions that influence exon skipping. One is inhibitory and located in the intron upstream of the BPS. Interestingly, secondary structure prediction programs based on the Zuker algorithm indicate that this region has the potential to form a stable stem-loop structure. Whether such a structure is formed in vivo is not known. The role of secondary structure in alternative splicing has been reviewed recently (23). The polyuridine tract at the 3’ splice site is only 9 nucleotides with a central guanine residue. Mutation of the guanine to give a stretch of 10 contiguous pyrimidines did not have an effect on exon recognition; however, increasing the length of the polyuridine tract to 14 nucleotides rendered the exon constitutive. Thus, exclusion of the exon requires a suboptimal splice acceptor site. Similar effects have been seen with the growth hormone and other genes (33). The second regulatory region is contained within the alternative spliced exon itself and appears to have both positive and negative effects. On the one hand, introduction of four point mutations caused the exon to be constitutive, suggesting a negative role for the exon. On the other hand, insertion of three thymidines or deletion of a stretch of eight pyrimidines caused the exon to be constitutive, suggesting a positive role. Interestingly, the constitutive point mutations had little effect when combined with deletions of the inhibitory region upstream. Thus these regulatory regions could function independently to modulate recognition of the 3′ splice site (Fig. 6) or, alternatively, both sequences could be involved in a larger regulatory region involving secondary structure around the 3′ splice site. Further studies are planned to test these models.

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