Human apolipoprotein A-II (apoA-II) intron 2/exon 3 junction shows a peculiar tract of alternating pyrimidines and purines (GU tract) that makes the acceptor site deviate significantly from the consensus. However, apoA-II exon 3 is constitutively included in mRNA. We have studied this unusual exon definition by creating a construct with the genomic fragment encompassing the whole gene from apoA-II and its regulatory regions. Transient transfections in Hep3B cells have shown that deletion or replacement of the GU repeats at the 3′ splice site resulted in a decrease of apoA-II exon 3 inclusion, indicating a possible role of the GU tract in splicing. However, a 3′ splice site composed of the GU tract in heterologous context, such as the extra domain A of human fibronectin or cystic fibrosis transmembrane conductance regulator exon 8, resulted in total skipping of the exons. Next, we identified the exonic cis-acting elements that may affect the splicing efficiency of apoA-II exon 3 and found that the region spanning from nucleotide 87 to 113 of human apoA-II exon 3 is essential for its inclusion in the mRNA. Overlapping deletions and point mutations (between nucleotides 91 and 102) precisely defined an exonic splicing enhancer (ESEwt). UV cross-linking assays followed by immunoprecipitation with anti-SR protein monoclonal antibodies showed that ESEwt, but not mutated ESE RNA, was able to bind both alternative splicing factor/splicing factor 2 and SC35. Furthermore, overexpression of both splicing factors enhanced exon 3 inclusion. These results show that this protein-ESE interaction is able to promote the incorporation of exon 3 in mRNA and suggest that they can rescue the splicing despite the noncanonical 3′ splice site.

Pre-mRNA splicing is the process by which introns are removed and exons are joined together by a two-step trans-esterification reaction carried out by the spliceosome, a dynamic 60 S ribonucleoprotein particle (1). Formation of the spliceosome at particular splice junctions is triggered by recognition of the 5′ splice site by the U1 small nuclear ribonucleoprotein and of the 3′ splice site by U2AF followed by the U2 small nuclear ribonucleoprotein recognition of the branch point (2).

The poly(pyrimidine) tract is one of the important cis-acting elements that may affect the splicing efficiency of apoA-II exon 3. This tract deviates significantly from the consensus (3). This characteristic GT tract is also found within the intron 8/exon 9 junction of the human CFTR gene, but in this context, it is followed by a polymorphic poly(T) tract (7–9).

This apparent sequence similarity concerning the GU tract is contrasted by the different splicing patterns exhibited by the two genes. In fact, CPTR exon 9 undergoes alternative splicing, and its inclusion is inversely correlated with the length of the GU tract and directly proportional to the length of the poly(T) tract, whereas apparently apoA-II exon 3 is constitutively spliced, and its inclusion is dependent on the presence of the GU tract (11). In other words, in the CPTR intron 8/exon 9 context, the stretch of pyrimidines alternated with purines alone is not equivalent to a functional continuous poly(pyrimidine) tract, in contrast to what has been observed for the apoA-II gene (11). Altogether these observations prompted us to investigate the mechanisms underlying the constitutive splicing of apoA-II exon 3 and, in particular, to characterize the cis-acting elements and the trans-acting factors involved in apoA-II exon 3 definition.

We show here that the third exon of apoA-II contains an exonic splicing enhancer (ESE), which is essential for the inclusion of exon 3 in mRNA and which possibly balances the presence of the noncanonical 3′ splice site. The trans-acting factors that target the apoA-II exon 3 ESE have been identified as ASFP2 and SC35.

**EXPERIMENTAL PROCEDURES**

Constructs—To generate the pApo gene system, the full sequence of the gene was obtained by overlapping the PCRs of different fragments from the apoA-II gene (GenBank™ accession number X04898.1) and then cloned XhoI–SacII in pBluescript SK. Each fragment of the complete apoA-II gene was amplified by PCR (94 °C for 30 s, 60 °C for 30 s, 72 °C for 30 s, 30 cycles) using -200–400 ng of genomic DNA as the template. This work was supported by Grant GGP02453 from the Telethon Onlus Foundation—Italy (to F. E. B.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) X04898.1. § These authors contributed equally to this paper.

1 The abbreviations used are: apoA-II, apolipoprotein A-II; ESE, exonic splicing enhancer; EMSA, electrophoretic mobility shift assay; RT, reverse transcription; CFTR, cystic fibrosis transmembrane regulator; SR, serine/arginine-rich; ASFP2, alternative splicing factor/splicing factor 2; EDA, extra domain A of human fibronectin.

2 The paper is available on line at http://www.jbc.org

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**ESE Offsets Atypical 3’ Splice Site of ApoA-II Exon 3**

**Consensus**

Fig. 1. Comparison of splice sites of the human apoA-II exon 3 with human CFTR exon 9. Aligned acceptor and donor splice sites of human apoA-II exon 3 (hAII) and human CFTR exon 9 (hCFTR) are compared with consensus sequences (bold lower line). (Y), pyrimidine; (R), purine. **Uppercase letters, exons; lowercase letters, introns.** The scores for the authentic splice sites (under the junctions (i)) were calculated by the SPPN program (www.fruitfly.org/seq_tools/splice.html).

**Template**

The primer sequences at the 5’ and 3’ extremes of the apoA-II gene were: hapoA-II-1173 XhoI 5’–ctctggacactgcggccg–3’ and hapoA-II poly(A) SacII 5’, 5’–cacaacagagtgtgtgtgtgtgtgtgtgtgtgtgcagacattgat–3’. After amplification, the PCR products were purified through a MicroSpin S-400HR column (Amersham Biosciences AB, Uppsala, Sweden). At position 1549 (intron 2) and 2017 (intron 3), the Sall and EcoRI sites were introduced by PCR-mediated site-directed mutagenesis, respectively, to facilitate subsequent cloning procedures. Also a target sequence was included in the apoA-II expression system at the end of exon 4 to allow the specific amplification and analysis of transfected apoA-II RNA with specific primers.

The CF/apoA-II hybrids were generated by exchanging the Sall-EcoRI cassettes or through a two-step PCR overlap extension. The three constructs with overlapping 21-bp deletions within the exon 3 were generated by exchanging the Sall-EcoRI cassettes in the pApo gene system.

The introduction of artificial point mutations within the ESE in apoA-II exon 3 were carried out by PCR-mediated site-directed mutagenesis. The amplified fragments were digested by Sall-EcoRI and substituted with the appropriate Sall-EcoRI cassettes created in the previously described pApo gene system.

Before expression, the identity of all constructs was checked by a CEQ20000 sequencer (Beckman Coulter, Fullerton, CA) according to the manufacturer’s instructions. The wild type poly(A) tract in the DNA template in the exon 3 was the 5'-globin/keratin reporter system (12) was replaced by (GT)_{16} repeats. Sense and antisense oligos carrying a (GT)_16 were designed such that they annealed at the flanking sequences of the EDA template. A first PCR reaction (94°C for 30 s, 60°C for 30 s, 72°C for 30 s, for 30 cycles) was performed by using a sense oligo Ex-1S, 5’–ataacaagaatgtgacctgactga-3’ annealing at -1 EDA exon and the antisense oligo FN-AII rev, 5’–atacacttgctacaacctaccacagccacaccacacacacccctggtg-3’ carrying the (GT)_16 sequence. A second reaction (same PCR conditions) was carried out by using the sense oligo FN-AII dir, 5’–cacaacagagtgtgtgtgtgtgtgtgtgtgtgtgtgctcagacctgt-3’ carrying the (GT)_16 sequence. The primer annealing at the extremes Ex-1S and Ex-1S and as a template, 1 μl each of reactions 1 and 2. The final PCR product was visualized in a 1% agarose gel, purified, cloned, and the plasmid transfected in Hep3B cells.

**Transfections**—The DNA used for transfections was prepared with JetStar purification kit (Genomed, GmbH, Löhne, Germany) following the manufacturer’s instructions. Liposome-mediated transfections of 3 × 10^5 human hepatocarcinoma Hep3B cells were performed using DOTAP liposomal transfection reagent (Alexis Corporation, Lausanne, Switzerland). 5 μg of construct DNA were mixed with 5 μg of DOTAP reagent for each transfection, and the mixture was incubated at room temperature for 15 min to allow the formation of DNA-liposome complexes. The mixture was added to the cells in 3 ml of serum-free culture medium and incubated at 37°C. After 12 h, the medium was replaced with fresh medium, and 24 h later the cells were harvested. Total RNA was extracted using RNAz reagent (Ambion, Austin, TX) and retrotranscribed with poly(dT) primer. To amplify only the messenger de-
RESULTS

Analysis of the ApoA-II Exon 3—As a first approach, we studied the association between the splice site strength and the different splicing behaviors of human apoA-II exon 3 and human CFTR exon 9. This was done by computer in a comparative sequence analysis of the 5'H11032 and 3'H11032 splice sites of the human apoA-II exon 3 and human CFTR exon 9.

The strength of these splice sites was calculated according to the Splice Site Prediction by Neural Network program (SSPNN, www.fruitfly.org/seq_tools/splice.html). In general, sequences that have a high score are considered to be strong (the maximum is 1.0 and corresponds to the consensus sequence). The program found the authentic 5'H11032 and 3'H11032 splice sites of human CFTR exon 9 (T7 version of the polypyrrimidine tract) in which the scores were 0.83 and 0.91, respectively (Fig. 1).

The calculated scores for the human apoA-II exon 3 demonstrated that the authentic donor splice site is ranked as the best possible 5'H11032 splice site (score = 1.0) within the 600 bp encompassing the genomic region between apoA-II exon 2 and exon 3. On the other hand, the authentic 3'H11032 splice site of apoA-II exon 3 is not ranked among the possible 3'H11032 splice sites within the same genomic region (at least with both donor and acceptor cut-off at 0.1). Five cryptic 3'H11032 splice sites (in which scores ranged from 0.11 up to 0.99) were also identified, (not shown). Overall, these observations suggest that the authentic acceptor splice site of human apoA-II exon 3 might be weak because of the presence of the noncanonical (GU) dinucleotide repeats within the 3'H11032 site.

The apparent weakness of the human apoA-II exon 3 acceptor splice site (strength <0.1) in comparison with the human CFTR exon 9 acceptor splice site (strength = 0.91) is in striking contrast with the splicing behavior of the two exons. In fact, human apoA-II exon 3 is constitutively included in mRNA; whereas human CFTR exon 9 is alternatively spliced. Hence, it is plausible that novel accessory cis-acting elements might be involved in the apoA-II exon 3 definition to support its constitutive splicing.

In Vivo System for the Study of ApoA-II Exon 3 Splicing—To study the relevance of the splice site strength and map the possible cis-acting elements involved in the apoA-II exon 3 definition, we generated a eukaryotic gene expression system by cloning the whole 3.2-kbp apoA-II gene including the promoter region and its polyadenylation site (pApo-wt) (Fig. 2A).

In comparison with the minigene system widely used to study other splicing models (13–17), where only one exon and its flanking regions are cloned in a heterologous gene context, the apoA-II expression system that we generated contains most of the elements necessary for its transcription and RNA processing, which are also present in the endogenous apoA-II gene. Such a construct should allow the study of the cis-acting elements affecting apoA-II exon 3 splicing in a context as close as possible to the chromosomal background.

The transient transfection of the pApo-wt gene system in Hep3B cells was followed by reverse transcription (RT) using a primer specific for the pApo-wt construct or the endogenous gene. The splicing pattern was then determined by PCR amplification using primers that recognized slightly modified sequences that have a high score are considered to be strong (the maximum is 1.0 and corresponds to the consensus sequence). The program found the authentic 5'H11032 and 3'H11032 splice sites of human CFTR exon 9 (T7 version of the polypyrrimidine tract) in which the scores were 0.83 and 0.91, respectively (Fig. 1).

The calculated scores for the human apoA-II exon 3 demonstrated that the authentic donor splice site is ranked as the best possible 5'H11032 splice site (score = 1.0) within the 600 bp encompassing the genomic region between apoA-II exon 2 and exon 3. On the other hand, the authentic 3'H11032 splice site of apoA-II exon 3 is not ranked among the possible 3'H11032 splice sites within the same genomic region (at least with both donor and acceptor cut-off at 0.1). Five cryptic 3'H11032 splice sites (in which scores ranged from 0.11 up to 0.99) were also identified, (not shown). Overall, these observations suggest that the authentic acceptor splice site of human apoA-II exon 3 might be weak because of the presence of the noncanonical (GU) dinucleotide repeats within the 3'H11032 site.

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quences in the plasmid pApo-wt, which allowed us to discriminate its transcript from the endogenous one.

Fig. 2B shows that the splicing efficiency of the apoA-II construct in Hep3B cells was similar to that observed for the endogenous apoA-II gene, displaying about 90% of exon 3 inclusion. An identical experimental strategy was used in all of the following experiments using mutated versions of the pApo-wt gene construct.

In Vivo Effects of (GT)16 Tract Deletion and Replacement—We have shown previously that when the U tract is removed from human CFTR intron 8, total exon 9 exclusion occurs (10). Moreover, an increase of the number of (GU) dinucleotide repeats reduces the efficiency of exon 9 inclusion. This splicing pattern is in contrast with what is observed in the apoA-II context, where exon 3 inclusion reaches 90%. Therefore, in the context of apoA-II, the (GU) dinucleotide repeats seem to work as a functional polypyrimidine tract. To test this hypothesis, we deleted all the (GT) dinucleotide repeats from the apoA-II intron 2 (Fig. 3A, construct Δ(gu)). As a control, a sequence containing (CA)16 dinucleotide repeats was used to replace the (GT)16 tract maintaining the same length (Fig. 3A, construct (ca)x(gu)). The deletion and replacement of the (GU) tract resulted in almost 100% of exon 3 being excluded (Fig. 3B, lanes 2 and 3). This result supports previous studies showing that the removal of all the GU repeats in the apoA-II intron 2 caused ~90% of exon 3 to be skipped (11).

To establish if the GU tract is functional or detrimental in systems different from the exons under study (apoA-II exon 3 and CFTR exon 9), we have tested the effect of replacing a canonical polypyrimidine tract with GU repeats. Using a minigene system for the mouse fibronectin EDA exon (12), we found that the substitution of the EDA exon polypyrimidine tract with the apoA-II (GT)16 tract resulted in 95% of the EDA exon being excluded (Fig. 4).

Altogether these results indicate that the (GU)16 tract functions as a polypyrimidine tract, which depends on its context and location in the nucleotide sequence. Moreover, these data suggest that the 3’ splice site definition of the apoA-II exon 3 might be supported by strong and specific cis-acting elements that counteract the effect of the noncanonical polypyrimidine tract.

Mapping of Regulatory Elements within ApoA-II Exon 3—To identify regulatory elements placed within the apoA-II exon 3, we generated hybrid constructs between apoA-II exon 3 and
CFTR exon 9 in the same manner as the splicing regulatory elements of CFTR exon 9 had been previously characterized (18).

A set of five CFTR/apoA-II hybrids was generated in which the exon 3 was progressively replaced by CFTR exon 9 and its 3′ splice site replaced with the allelic configuration (GT)11(T)5. The constructs were named by indicating the number of apoA-II exon 3 nucleotides replaced by CFTR exon 9 (i.e., apoA-II exon 3, which is observed as a faint band in apoA-II pApo-wt). This cryptic splice site has been characterized previously (10). The transfection of the pCF/Apo113 construct showed 80% exclusion of exon 3 (Fig. 5B, lane 3). On the other hand, the pCF/Apo60 constructs showed a new cryptic 3′ splice site introduced with the extension of the CFTR sequence from nucleotides 39 to 60 (Fig. 5B, lane 4). This cryptic splice site has been characterized previously (10). The transfection of the pCF/Apo87 construct showed 100% inclusion of exon 3, whereas the pCF/Apo113 construct showed 80% exclusion of exon 3 (Fig. 5B, lanes 5 and 6). In principle, the strikingly different splicing patterns of pCF/Apo87 and pCF/Apo113 constructs might be due to the removal of an enhancer element placed within the 26 bp of the apoA-II exon or to the addition of a silencer element introduced within the 26 bp of the CFTR exon 9. However, considering that previous mapping studies did not highlight the presence of any exonic CFTR regulatory element in this 26-bp range (18), we focused attention on the enhancer-like sequence possibly present in the removed sequences of apoA-II exon 3. To identify the nucleotides within this 26-bp region that contributed to efficient exon 3 splicing, we constructed three 21-bp overlapping deletions encompassing the 26-bp sequence (Fig. 6A). The three constructs (Δ1843–63, Δ1854–74, Δ1866–86) were transiently expressed in Hep3B, and the RNA was analyzed by RT-PCR. The Δ1843–63 deletion caused 80% of exon 3 exclusion, whereas both the Δ1854–74 and the Δ1866–86 deletions resulted in 55% of exon 3 exclusion (Fig. 6B). Therefore, the region spanning from nucleotide 1843 to 1886 seems to affect positively apoA-II exon 3 definition. In previous studies, a broad spectrum of enhancer-like sequences capable of promoting exon inclusion have been identified (19). Interestingly, the motif GGAGA (Class I of purine-rich enhancers) is also present in the 1852–1860 nucleotide region of apoA-II exon 3. Moreover, the 1852–1856 region is compatible with the ASF/SF2 motif found through functional systematic evolution of ligands by exponential enrichment (SELEX) (20). Therefore, we focused attention on the sequence ranging from nucleotide 1852 to 1860 (corresponding to nucleotides 91–99 of exon 3) to be analyzed as a possible regulatory element (Fig. 7A, boxed). To map finely which base(s) in such a region was directly affecting exon inclusion have been identified (19). Interestingly, the motif GGAGA (Class I of purine-rich enhancers) is also present in the 1852–1860 nucleotide region of apoA-II exon 3. Moreover, the 1852–1856 region is compatible with the ASF/SF2 motif found through functional systematic evolution of ligands by exponential enrichment (SELEX) (20). Therefore, we focused attention on the sequence ranging from nucleotide 1852 to 1860 (corresponding to nucleotides 91–99 of exon 3) to be analyzed as a possible regulatory element (Fig. 7A, boxed). To map finely which base(s) in such a region was directly affecting exon 3 definition, point mutations were introduced within this 9-bp region. Each base, one at a time, was replaced by its complementary one, generating nine different constructs that were used to transfect the Hep3B cell line (Fig. 7B). Although most of the substitutions did not have a significant effect on exon 3 processing in Hep3B cells, G92C, A99T, and more noticeably, A97T caused 20, 60, and 85% of exon 3 skipping, respectively (Fig. 7C, lanes 3, 8, and 10).

Interaction of SR Proteins with apoA-II 9-Nucleotide ESE—To identify the trans-acting factors able to bind the
9-nucleotide enhancer-like sequence within apoA-II exon 3, both EMSA and UV cross-linking assays were performed. For the EMSA experiment, we used in vitro transcribed RNAs including the 9-nucleotide wild type core region of the apoA-II exon 3 (ESEwt) or this same sequence point-mutated (ESEA97T) or deleted (ESE-9) (Fig. 8A). The RNAs were incubated with HeLa nuclear extract in the presence of the nonspecific competitor heparin (see "Experimental Procedures"). As shown in Fig. 8B, ESEwt RNA showed a broad band of shifted material, which is slightly weaker with the ESEA97T RNA and almost nonexistent with the ESE-9 RNA (lanes 4–6). Nevertheless, it should be noted that the region used as the EMSA probe spans the deletion of Δ1843–63 = 80%; Δ1854–74 = 55%; Δ1866–86 = 55%. Standard deviations were <10%.

The nature of the protein(s) that bind(s) to the wild type apoA-II exon 3 across the 9-nucleotide sequence was then investigated by UV cross-linking of RNA-protein complexes. 32P-labeled ESEwt, A97T, and ΔESE-9 RNA probes were incubated with HeLa nuclear extract and were cross-linked to proteins by exposure to UV light. The resulting 32P-labeled protein(s) was (were) separated by SDS-PAGE. The pattern of UV cross-linked proteins obtained with these three different constructs did not display any significant difference (data not shown).

To test the possible differential recruitment of SR proteins by the wild type, mutated, or deleted apoA-II 9-nucleotide ESE that may be obscured in the UV cross-linking assay, these were followed by immunoprecipitation with anti-SR protein monoclonal antibodies. The constructs used for these experiments carry ESEwt, ESEA97T, and ΔESE-9 (Fig. 9A).

The immunoprecipitation of the UV cross-linked material with monoclonal antibody 1H4 directed against SRp40, SRp55, and SRp75 did not produce any specific immunoprecipitated material (not shown). Instead, the monoclonal antibody 96 anti-SF2/ASF monoclonal antibody (Fig. 9B, left panel) and anti-SC35 monoclonal antibody (right panel) immunoprecipitated specific proteins (lanes 1) following UV cross-linking with nuclear extract of a labeled ESEwt RNA but only traces with the ESEA97T and ΔESE-9 control RNAs (lanes 2 and 3). Thus,
FIG. 7. Effect of point mutations within the apoA-II exon 3 putative ESE. A, scheme of apoA-II exon 3 and partial sequence containing the ESE sequence (box). B, scheme of the point mutations that were carried out. Briefly, each base at a time in the ESE was replaced by its complement. Changes are indicated in bold capital letters (diagonal). C, denaturing acrylamide gel of radioactive RT-PCRs of pre-mRNA splicing pattern of the point-mutated pApo constructs. Black arrows indicate the strongest effects of the point mutations on exon 3 skipping (left to right, G92C, A97T, and A99T). The relative amount of exon 3 skipping (\(\text{ex}3\)−) was quantified by phosphorimaging analysis of radioactive PCRs as described under “Experimental Procedures.” G92C = 20%; A97T = 85%; A99T = 80%. Standard deviations were <15%.

FIG. 8. Electrophoretic mobility shift assay with apoA-II exon 3 ESE RNA. A, scheme of the constructs carrying wild type (\(\text{ESE}_{\text{wt}}\)), point-mutated (\(\text{ESE}_{\text{A97T}}\)), and deleted (\(\Delta\text{ESE}_{\text{9}}\)) exonic splicing enhancer of apoA-II exon 3 used for the EMSA. B, radiolabeled RNAs were incubated with HeLa nuclear extract (ne) for 20 min at room temperature. Complexes were then fractionated on a 4% nondenaturing polyacrylamide gel. The position of bound (complex) or free RNA is indicated.
these results provide evidence that at least two SR proteins are able to interact with the nucleotides across the ESE sequence and are also consistent with the result obtained in the EMSA study, where a clear difference in the RNA-protein complex formation with a construct containing the ESEwt and \( \text{ESE-9} \) is observed.

**Effect of ASF/SF2 and SC35 Overexpression on Splicing of ApoA-II Exon 3**—To highlight the putative positive effect of ASF/SF2 and SC35, plasmids containing the open reading frame of these SR proteins were cotransfected along with pApo-wt construct. Thus, the possible positive effects of the overexpression of ASF/SF2 and/or SC35 should cause an increase of exon 3 inclusion, even though the percentage of basal exon 3 skipping is low (about 10%). As a control, the construct carrying the point mutation A97T within the ESE was used (pApo-A97T). Fig. 9C showed that the cotransfection of pApo-wt with ASF/SF2 (lane 2) and SC35 (lane 6) promoted exon 3 inclusion when compared with the cotransfection with the empty vector pCG (lanes 1 and 5). Conversely, the cotransfection of ASF/SF2 and SC35 with the construct carrying the A97T point mutation showed no significant variation in levels of exon 3 inclusion (Fig. 9C, lanes 4 and 8). Altogether these results confirm that the disruption of the ESE by the point mutation A97T prevents a functional binding of ASF/SF2 and SC35 to this regulatory sequence and inhibits the positive effect. Hence, both ASF/SF2 and SC35, in which specific interaction to the ESE had already been demonstrated by UV cross-linking/immunoprecipitation (Fig. 9B), have a functionally positive effect, increasing the apoA-II exon 3 inclusion.

**DISCUSSION**

Human apoA-II exon 3 and its flanking introns share only partially the canonical features of splicing sites. In fact, the 5′ splice site of intron 3 is well defined and resembles the consen-
The comparison of splice sites strength of human apo-A-II exon 3 and human CFTR exon 9 has delineated an apparent contradiction between the splicing behavior of the two exons and the strength of the splice sites. Taking advantage of the compact size of the human apo-A-II gene, we were able to create an in vitro expression system that includes the whole 1173-bp enhancer promoter region in which the 1226-bp coding region encompasses 4 exons and 3 introns and the 320-bp 3\'-UTR (where the polyadenylation signal is located). In this way, it was possible to study the exon 3 splicing within a context very similar to the chromosomal context. In addition, a liver-derived cell line (Hep3B) was used to provide a cellular context more similar to that of original tissue where the apoA-II gene is normally expressed. This represents a clear advantage in comparison to the chromosomal context. In this way, it is now clear that ESEs are functionally equivalent to cis elements that drive the selection of regulated exons even in the presence of a strong 5\' splice site.

A large body of evidence indicates that U2AF35 is required for constitutive splicing and also works as a mediator of enhancer-dependent splicing (31–33). In vitro protein-RNA interaction studies with pre-mRNAs containing either a constitutive or regulated splicing enhancer have shown that U2AF35 directly mediates interactions between U2AF65 and proteins bound to the enhancers (34). Thus, U2AF35 should recruit U2AF65 acting as a bridge between these proteins and the enhancer complex. In the case of apo-A-II exon 3, this recruitment should be mediated by the ESE sequence characterized in this paper and its interactions with AS/CF and SC35.

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