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Type 1 and Type 2

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Alternative splicing is a hallmark of glycoprotein hormone receptor gene regulation, but its molecular mechanism is unknown. The LH receptor (LHR) gene possesses 11 exons, but exon 10 is constitutively skipped in the New World monkey lineage (LHR type 2), whereas it is constitutively spliced in the human (LHR type 1). This study identifies the regulatory elements of exon 10 usage. Sequencing of genomic marmoset DNA revealed that the cryptic LHR exon 10 is highly homologous to exon 10 from other species and displays intact splice sites. Functional studies using a minigene approach excluded the contribution of intronic, marmoset-specific long interspersed nucleotide-1 elements to exon 10 skipping. Sequencing of the genomic regions surrounding exon 10 from several primate lineages, sequence comparisons including the human and mouse LHR gene, revealed the presence of unique nucleotides at 3′-intronic position −19 and −10 and at position +26 within exon 10 of the marmoset LHR. Exon trap experiments and in vitro mutagenesis of these nucleotides resulted in the identification of a composite regulatory element of splicing consisting of cis-acting elements represented by two polypyrimidine tracts and a trans-acting element within exon 10, which affect the secondary RNA structure. Changes within this complex resulted either in constitutive exon inclusion, constitutive skipping, or alternative splicing of exon 10. This work delineates the molecular pathway leading to intronization of exon 10 in the LHR type 2 and reveals, for the first time, the essential function of regulatory and structural elements involved in glycoprotein hormone receptor splicing. (Molecular Endocrinology 21: 1984–1996, 2007)

H AND CHORIONIC GONADOTROPIN (CG) play an essential role in gonadal function, pregnancy, and male sexual development. The action of both LH and CG is mediated by one receptor, the LH receptor (LHR), a G protein-coupled receptor expressed in the Leydig cells of the testis and granulosa and theca cells of the ovary. The gene consists of 11 exons, 10 of which code for the extracellular domain, whereas exon 11 encodes the C-terminal part of the extracellular domain, the seven-transmembrane domain, and the intracellular domain (1, 2).

A hallmark of the LHR gene and the closely related FSH receptor (FSHR) and TSH receptor (TSHR) genes is a marked alternative splicing of the primary transcript (3). Several alternative splicing mechanisms have been identified, the most common type being exon skipping (removal of one or several complete exons), followed by exon inclusion, alternative acceptor, or donor splice sites (4). Whereas skipping of one or several exons results in in-frame deletion of amino acids without changing the open reading frame, exon inclusion or alternative acceptor or donor splice sites results in truncated receptors due to the presence of premature termination codons (5, 6).

Studies in sheep and rats, as well as other species, have shown that splicing of the LHR is regulated in a temporal and spatial manner during follicular or testicular development and that the abundance of the different variants varies significantly (7–9). Functional studies demonstrated that some of them are translated and, in principle, are capable of hormone binding. However, nearly all of them do not reach the membrane and remain trapped within the cell (10, 11). Recently, a LHR splicing variant lacking exon 9 was shown to interact with the full-length LHR when expressed in vitro and to modulate the expression of the wild-type LHR (12). Common to all studies is the lack of any information concerning the mechanism underlying splicing in the LHR gene. This also holds true for the TSHR and FSHR gene.

After the original observation that the wild-type LHR mRNA lacks exon 10 in the New World monkey (NWM) Callithrix jacchus (13), we proposed that this receptor belongs to a new class of LHR, which we named type 2. LHR type 2 represents the wild-type LHR in the whole NWM lineage (14). Interestingly, exon 10 is only missing at the mRNA and at the protein level because Southern blot experiments indicated the presence of a putative exon 10 at the genomic level (our unpublished data and
Ref. 13). Additional studies in several primate lineages indicated that exon 10 is completely lacking in the LHR cDNA of all NWMs, whereas in humans and other primates exon 10 is constitutively included in the mRNA (14, 15). This indicates tight functional constraints for constitutive splicing of exon 10, suggesting its essential role for LHR function in these species. Exon 10 encodes a part of the hinge region, linking the extracellular to the transmembrane domain. Removal of exon 10 from the human LHR results in different responsiveness to human CG and LH, with a significantly enhanced preference toward human CG (16, 17). Therefore, the part of the LHR protein encoded by exon 10 might be instrumental for receptor activation in species possessing CG.

Alternative splicing is regulated by a tightly regulated balance of trans- and cis-acting factors. Detailed studies on alternative splicing of the CFTR and SMN2 genes that are involved in human diseases indicate that single exonic or intronic point mutations can cause exon skipping (18). The fact that exon 10 is completely absent in LHR type 2 could therefore be due to mutations disrupting splice sites or affecting binding of splicing factors or, possibly, to species-specific splicing factors. Thus, LHR type 2, aside from its unique biochemical properties, represents an interesting model to investigate the molecular mechanisms underlying splicing in glycoprotein hormone receptors. In this study, we delineate, for the first time, the molecular mechanism underlying constitutive exon 10 inclusion in the human and exon 10 skipping in the marmoset LHR.

RESULTS

Identification and Characterization of a Cryptic Exon 10 Sequence and Its Flanking Regions in the Marmoset Gene

Previous studies by Zhang et al. (13) and by us (14) reported the existence of a putative exon 10 sequence within the marmoset LHR type 2 gene. However, because of the nature of the experiments, mainly consisting of Southern blots, sequence information was lacking. To obtain the nucleotide sequence of the putative exon 10, we amplified the genomic region between exon 9 and 11 of the marmoset LHR gene. Using a long-template PCR approach, we cloned and sequenced an amplicon of approximately 15 kbp. This fragment contains a putative exon 10 sequence consisting of 81 bp together with an intronic region 9 (between exon 9 and 10) of 7053 bp and an intronic region 10 (between exon 10 and 11) of 7950 bp, respectively (Fig. 1) (EMBL accession no. AM181324).

Compared with the corresponding regions of the LHR gene in human (Gene identification no. 3973) and mouse (Gene identification no. 16867), a remarkable expansion of approximately 5 kbp shared in both introns was observed. Using the Repeatmasker software, a drastic increase of the percentage of repetitive sequences was detected therein: 67.9% in the marmoset compared with 38.9% and 34.7% in the corresponding human and mouse regions, respectively. This increase is mainly due to the presence of LINE-1 elements (long interspersed nucleotide elements) in both introns 9 and 10, not found in the human and in the mouse gene. The number and location of other repetitive elements such as ALU elements and mammalian-wide interspersed repeats were not significantly different among the three species in this region of the genome (data not shown).

The putative marmoset exon 10 consists of 81 bp and displays 86% nucleotide identity to exon 10 of the human and mouse LHR (Fig. 2). Interestingly the splice sites are conserved, with AG at the 3' and GT at the 5' end (Fig. 2). A splice site score calculator program revealed high scores for a normal splicing mechanism of the putative exon 10, which perfectly maintains splicing phase 2 with CT at the 3’-end encoding for leucine (CTN), as in all other exons of the LHR including exon 10 from other species (14). The deduced amino acid sequence of the putative marmoset exon

![Fig. 1. Schematic Representation of the Genomic Region Covering Exons 9–11 of the Human, Marmoset, and Mouse LHR Gene](image-url)
region upstream of the 3' splice site

<table>
<thead>
<tr>
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<th>Nucleotide Comparison of Exon 10 and the Adjacent 45-bp Intronic Sequence</th>
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<tr>
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</tr>
<tr>
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<td>Bulemur coronatus</td>
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<td>Rodents</td>
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Nucleotide Comparison of Exon 10 and the Adjacent 45-bp Intronic Sequence

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<tr>
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<tr>
<td>L. lagotricha</td>
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<tr>
<td>E. coronatus</td>
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region downstream of the 5' splice site

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<td>E. coronatus</td>
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deduced putative exon 10 amino acid sequence

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<tr>
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<tr>
<td>C. jacchus</td>
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</tr>
<tr>
<td>M. musculus</td>
<td>QNFSHSLSEFNSKQCESTVRKVNNTL 27</td>
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</table>

Fig. 2. Nucleotide Comparison of Exon 10 and the Adjacent 45-bp Intron Sequence

The genomic sequences corresponding to this region of the human, chimpanzee (Pan paniscus), cynomologus (M. fascicularis), marmoset (C. jacchus), four further NWMs (Saimiri sciureus, Saginus fusciollis, Lagothrix lagothricha, Pithecia pitheria), lemur (Eulemur coronatus), and mouse LHR genes were aligned. The nucleotides mainly specific to the group of NWMs are boxed. Identical nucleotides are marked by an asterisk. The two polypyrimidine stretches in the 3' upstream region and the SC35 signature in exon 10 are underlined. The EMBL accession no. for the complete cynomologus LHR cDNA cloned by the authors is given AM231185. Lower panel, Amino acid sequence comparison of exon 10. The amino acid specific to NWM is boxed.

10 revealed 77% identity compared with human and mouse exon 10. This homology is similar to the overall homology between the marmoset and the human LHR (13). Thus, no obvious sequence alterations were identified that could explain the absence of exon 10 usage in the marmoset.

LINE-1 Elements Are Not Involved in Exon 10 Skipping

To understand why exon 10 is constitutively skipped in the marmoset but constitutively spliced in the human LHR, we first used a minigene approach. The
minigenes consisted of exons 9 and 11, which are part of transcripts in all species analyzed, and exon 10, which is cryptic in the marmoset and functional in the human, as well as the intervening intronic sequences. As expected, in COS-7 cells transfected with the human minigene we obtained an amplicon of size corresponding to an mRNA including exon 10 (Fig. 3). For the marmoset minigene only a smaller amplicon lacking exon 10 could be detected. No alternative splicing was observed for any minigene. This corroborates original findings in ovaries and testes (14, 15) showing no alternative splicing of exon 10 and strengthens the view of a very tightly regulated mechanism controlling exon 10 processing.

To elucidate the role of the LINE-1 elements in exon skipping, we performed additional experiments with truncated intronic regions. After restriction enzyme digestion of the marmoset constructs using AccI or XcmI to remove large portions including the LINE-1 elements, transfection of the truncated minigenes resulted in the same splicing pattern, showing that shortening the introns flanking exon 10 does not activate its usage (Fig. 3, m2–m4). This strongly suggests that no repressing factor/activity was binding to the marmoset-specific intronic sequences.

Exon 10 Usage Requires Both Intronic and Exonic Sequences

To circumscribe and to identify the intronic sequences adjacent to exon 10 responsible for its usage, genomic sequences encompassing exon 10 from human or marmoset DNA and including 450, 300, 150, or 45 bp 5' and 3' of exon 10, respectively, were cloned into the exon trap vector pSPL3, followed by transient transfection of COS-7 cells and RT-PCR. The typical pattern of exon 10 usage of the human and marmoset construct, respectively, was completely maintained when using the 450-bp or the 300-bp construct (data not shown) as well as when using the 150-bp or the 45-bp construct (Fig. 4, upper and middle panel). This indicates that sequences responsible for exon 10 usage must be located within the region encompassing exon 10 and 45 bp upstream and downstream of it.

Next we generated chimeric constructs of 171 bp in which the human exon 10 was flanked by marmoset intronic sequences and vice versa (Fig. 4). Transfection and RT-PCR of these chimeric, exon trap constructs showed alternative splicing. In particular, the marmoset-human-marmoset construct, consisting (from 5'H11032 to 3'H11032) of the 5'-flanking intron of the marmoset exon 10 (45 bp), the human exon 10 (81 bp), and the 3'-flanking

![Fig. 3. Splicing of Genomic Fragments Encompassing Human Exons 9–11 and Marmoset Exons 9–11 Cloned into the pTarget Vector](image-url)

Constructs were transiently transfected into COS-7 cells, and RNA was isolated 48 h later. RT-PCR were performed using primers specific for exon 9 and exon 11. The amplicon obtained corresponds to a partial LHR mRNA transcript containing exon 10 (human, lane h1) and lacking exon 10 (marmoset monkey, lane m1). By the use of the restriction sites AccI and BsmI, genomic fragments were removed to study the importance of these regions for splicing. The construct was restricted as indicated in the upper part of the figure. Each construct was transiently transfected into COS-7 cells, and RNA was isolated 48 h later. RT-PCR was performed using primers specific for exon 9 and exon 11. The partial LHR mRNA transcripts are always identical to the wt mRNA (lane m1) independently of a 5' (lane m2), 3' (lane m3) or 5', and 3' (lane m4) truncation. C, Control without reverse transcriptase. The fragments were sequenced and their identity confirmed. The integrity of the isolated RNA was confirmed by simultaneous amplification of β-actin (bottom panel of the photo). Experiments were performed in triplicate.
intronic (45 bp) sequences (m-h-m) and generated chimeric constructs in which the intronic part was exchanged yielding constructs in which the human exon 10 was conserved of expressed vector sequences plus exon 10 (81 bp). Experiments were performed with or without reverse transcriptase.

For the human constructs 150 bp and 45 bp, only one amplicon corresponding to a transcript including exon 10 was obtained, whereas for the marmoset using similar constructs, only one amplicon lacking exon 10 was observed. We then generated chimeric constructs in which the intronic part was exchanged yielding constructs in which the human exon 10 was flanked by marmoset intronic (45 bp) sequences (m-h-m) and vice versa (h-m-h). For both chimeric constructs alternative splicing was obtained, indicated by the presence of one amplicon lacking exon 10 (263 bp) and one containing exon 10 (344 bp). Lower panel, Transfection of the human granulosa cell line COV 434 using both chimeric constructs and the wild-type 45 bp constructs for human and marmoset exon 10. All experiments were performed in triplicate. β-Actin was used as an internal standard for RNA integrity and the fidelity of PCR (data not shown).

Fig. 4. Functional Analysis of the Role of Genomic Sequences Close to Exon 10

Upper panel, Constructs covering either 150 bp or 45 bp 5’ and 3’ of exon 10 from the human or the marmoset monkey LHR were cloned into the exon trap vector pSPL3. The constructs were transiently transfected into COS-7 cells, and RNA was isolated 48 h later. RT-PCR was performed using primers specific for expressed vector sequences (SA2 and SD6). An amplicon with 175 bp corresponds to vector mRNA (constitutive exons) without any additional expressed sequences. An amplicon of 256 bp corresponds to expressed vector sequences plus exon 10 (81 bp). Experiments were performed with or without reverse transcriptase.

Middle panel, For the human constructs 150 bp and 45 bp, only one amplicon corresponding to a transcript including exon 10 was obtained, whereas for the marmoset using similar constructs, only one amplicon lacking exon 10 was observed. We then generated chimeric constructs in which the intronic part was exchanged yielding constructs in which the human exon 10 was flanked by marmoset intronic (45 bp) sequences (m-h-m) and vice versa (h-m-h). For both chimeric constructs alternative splicing was obtained, indicated by the presence of one amplicon lacking exon 10 (263 bp) and one containing exon 10 (344 bp). Lower panel, Transfection of the human granulosa cell line COV 434 using both chimeric constructs and the wild-type 45 bp constructs for human and marmoset exon 10. All experiments were performed in triplicate. β-Actin was used as an internal standard for RNA integrity and the fidelity of PCR (data not shown).

We previously showed that exon 10 is lacking in all NWMs whereas it is present in all other primate lineages (14). Assuming a common mechanism underlying exon 10 skipping in all NWMs, we reasoned that comparing the corresponding sequences from different primates could help to identify candidate nucleotides. We therefore cloned and sequenced the 171-bp genomic region comprising exon 10 and the 45 bp upstream and downstream of it from four additional NWMs (Saimiri sciureus, Saguinus fuscicollis, Lagothrix lagotricha, Pithecia pitheca) as well as from the Old World monkey Macaca fascicularis, the lemur Eulemur coronatus, and the chimpanzee (Pan paniscus). Primers were designed based on sequence homology of human, marmoset, and mouse LHR. Inspection of the sequences obtained revealed the presence of only a few nucleotide changes specifically present in the NWM (Fig. 2, gray boxes). We pinpointed unique nucleotide substitutions at position -19 within the 3’-intrinsic sequence upstream of exon 10, in which a T present in all other species is replaced by an A in M. fascicularis and in the NWM, and at position +26 within the coding region of exon 10 where a G is substituted by an A. In addition, a highly conserved T at position -10 is replaced by a C only in C. jacchus.

Sequence analysis using the ESE (exon splicing enhancer) software program yielded several important splicing motifs. First, two polypyrimidine (Y = C or T)
stretches (n = 7 and n = 12), important cis-acting sequence elements directing intron removal in pre-mRNA splicing, are directly adjacent to the 3'-splice site of human exon 10 (Fig. 2, underlined). In the marmoset the first poly-Y stretch is disrupted by the purine A at position −19 (Fig. 2, gray box) and in the second stretch the pyrimidine T at position −10 is replaced by the pyrimidine C (gray square). Second, within the coding sequence of human exon 10 a sequence stretch displayed some homology to an SC35 binding site signature (consensus sequence GRYYc-SYR; human sequence CATTTCCTG), which was lacking in the marmoset and in all other NWMs due to the A substitution at position +26. Other than these differences, binding sites for other splicing factors such as SRp40, SF2/AF, and SRp55 were identical both in human and marmoset exon 10 (data not shown).

Three Nucleotides within a 45-bp Stretch at the Intron 9-Exon 10 Boundary Specifically Regulate Exon 10 Usage

Through oligonucleotide site-directed mutagenesis, the nucleotides at positions −19 and +26 in the marmoset sequence were changed into the corresponding human nucleotides and vice versa (Fig. 5, upper part). When the intronic marmoset −19 A was mutated into human T, a small increase in exon 10 inclusion (11% compared with < 1%) was observed, whereas no change in splicing was observed in the corresponding human construct (Fig. 5, m −19 and h −19). However, when the human sequence was restored in the marmoset by mutating the nucleotide A at position +26 into a G, significantly increased splicing (60%) could be observed (Fig. 5, lane m 26). Conversely, by replacing the human G at position +26 by an A, only a slight decrease in exon 10 splicing efficiency could be detected (93%; Fig. 5, lane m 26 and h 26). Surprisingly, combining both exonic and intronic changes at position −19 and +26 in both species resulted in a slightly more pronounced exon 10 usage, but neither complete exon 10 skipping in the human nor complete splicing in the marmoset LHR (data not shown) was achieved. We therefore considered the unique exchange at position −10 present only in the marmoset sequence (Fig. 2). Replacing the C at position −10 of the marmoset by a T resulted in a drastic effect on exon 10 inclusion in the marmoset sequence (56%), whereas in the corresponding human construct a drastic reduction of exon 10 splicing (61%) was observed (Fig. 5, lane m −10 and h −10). When both intronic nucleotide changes at position −19 and −10 were introduced, exon 10 inclusion further increased to 91% for the marmoset, whereas in the human exon 10 splicing was lowered to 60% (Fig. 5, lane m −19/−10 and h −19/−10). A similar high inclusion rate was obtained for marmoset exon 10 by changing the two nucleotides at position −19 and +26 (80%), which reached 100% inclusion when the nucleotides at position −10 and +26 were changed (Fig. 5, lane m −19/26 and m −10/26). In the final experiments all three nucleotide changes at position −19, −10, and +26 were combined within one construct. This almost completely reversed the wild-type pattern of human exon 10 splicing (only 5% residual inclusion) (Fig. 5, lane h −19/−10/26) and of marmoset exon 10 skipping (100% inclusion) (Fig. 5, lane m −19/−10/26). Thus, the nucleotides at position −10 and +26 and, to a lesser extent at position −19, represent key sites in regulating exon 10 skipping or splicing of the LHR. To study further whether SC35 interacts with the identified putative binding site at position +26 we performed RNA interference (RNAi) experiments. Although SC35 protein expression, as revealed by Western blots, could be significantly reduced in COS-7 cells transiently transfected with RNAi specific for SC35 by at least two of the three RNAis tested (356 and 589; Fig. 6A), an effect on exon 10 usage could be detected neither for the wild-type human LHR nor for the marmoset LHR mutated at the three positions −19, −10, and +26 (Fig. 6B). Thus, although the nucleotide sequence displays a putative SC35 binding site signature, this is not involved in the regulation of exon 10 usage.

The Nucleotide Changes at Position −19, −10, and +26 Modify the Secondary RNA Structure

Based on the experimental data that all three nucleotide changes contribute to exon usage or skipping, we examined whether the secondary RNA structure might be involved in the observed effects. We applied the MFOLD program (19), which analyses RNA sequences and predicts folding of RNA resulting in stem/loop formation. Using the nucleotide sequences investigated in the exon trap experiments, i.e., exon 10 and both its 45-bp flanking sequences, we first analyzed the secondary structure of the wild-type human and marmoset sequences and then the impact of the mutations at position −19, −10, and +26. As depicted in Fig. 7, the secondary RNA structure of human (H) and marmoset (M) wild-type exon 10 and its adjacent intronic sequences are markedly divergent. Whereas for the human exon 10 the 3'-acceptor splice site (uucAG) is located in a short stem (see inset H), in the marmoset the acceptor site is positioned at the edge of a loop (inset M). In the human RNA the putative SC35 sequence stretch (ranging from nucleotides 23–28; Fig. 2) is the RNA stretch. Thus, both the 3'-intron sequence and part of exon 10 form a short stem, obviously necessary for the recognition of the 3'-splice site. In the marmoset, by contrast, only a loop with no complementary sequences can be observed. In addition, the overall secondary structure and the number of stems and loops are different between the two species. Introducing sequentially point mutations at position −19, −10, and +26 or combinations thereof significantly changes the secondary structure predictions [supplemental Fig. 1, a–h, for human exon 10 and...
supplemental Fig. 2, a–h (published as supplemental data on The Endocrine Society’s Journals Online web site at http://mend.endojournals.org). For the human exon 10 this results in transformation of the 3′-acceptor site to a loop structure (H and inset), whereas a stem transformation is found in the marmoset (M and inset) (Fig. 7). Interestingly, the stem formed resembles exactly the human exon 10 wild-type conformation with a complementary strand consisting of nucleotide position 23–28 (see inset) of the putative SC35 binding motif. Moreover the overall secondary structure including number of loops and stems is very similar to that of the human RNA (compare H and M′). Single mutations (−19, −10, and +26) resulted in an intermediate configuration between H and H′ and between M and M′ (supplemental Figs. 1 and 2); however, no association between free energy values and extent of exon splicing was found. Thus, the formation of stems covering the 3′-acceptor splice site of exon 10 is associated with exon 10 usage.

Fig. 5. Oligonucleotide Site-Directed Mutagenesis to Delineate Nucleotides Essential for Exon 10 Inclusion or Skipping

The pSpl 3 vector containing either the human (h) or marmoset (m) 45 bp-exon 10–45 bp was altered using site-directed mutagenesis. Nucleotides at position −19, −10, and +26 were changed into the corresponding nucleotide present in the human or marmoset exon 10 sequence or adjacent intronic sequences (upper panel). The different constructs were transfected into COS-7 cells and RT-PCR was performed. The amplicons were subjected to agarose-gel electrophoresis (middle panel), and the intensity of the bands was determined by densitometric measurements and is given in the corresponding diagram by percentage of exon 10 inclusion (lower panel). The gel photo is representative of three independent experiments for which means and SD were calculated. The position of the changed nucleotide and the species (h for human and m for marmoset) is given, the transcription of the empty pSpl 3 vector served as a negative control. Black bars correspond to marmoset constructs, whereas gray bars indicate human constructs.
Previously reported right shift of the LH dose-response curve with the LHR lacking exon 10 (17) cannot be appreciated in this experiment.

**DISCUSSION**

Alternative splicing of the primary transcript is a hallmark of the glycoprotein hormone receptor genes (3). However, aside from the description of splice variants and sparse functional studies, the underlying molecular mechanism remains completely unclear (12). Exon 10 skipping in the LHR gene type 2 could be considered as the extreme form of alternative splicing, and understanding the molecular events responsible could help explain the basic mechanism underlying splicing of the other exons, as well as the functional meaning of alternative splicing. In this study we exploited exon 10 usage in the LHR gene as a model to elucidate alternative splicing.

Inspection of the putative marmoset exon 10 sequence and its genomic organization revealed two interesting features. First, there is a significant expansion of the intronic regions 9 and 10 due to the presence of LINE-1 elements; second, exon 10 sequence is highly conserved when compared with other LHRs and the splice sites are intact. The LINE-1 elements, making up approximately 7500 bp of the total intronic regions 9 and 10, are considered a driving force in retrotransposition. Random integration of LINE-1 elements may cause gene or exon silencing or can generate additional exons and even new genes (21–23). However, most LINE-1 elements are transcriptionally silent. The LINE-1 elements found in the analyzed region of the marmoset LHR gene lack an open reading frame and are inactive. In addition, our experiments demonstrated that they do not influence exon inclusion (Fig. 3). Thus, it seems likely that the retrotransposition of LINE-1 elements was not the primary event causing loss of exon 10 inclusion. Rather, it could be a process secondary to the intronization of exon 10 in the LHR type 2.

**Mechanism of Exon 10 Intronization in the Marmoset LHR**

This study shows that in the marmoset and in the other NWMS a polypyrimidine stretch, important for positioning splicing factors such as U2AF, is interrupted at position −19 by the purine A replacing a T found in all other species investigated. Surprisingly, however, this nucleotide contributes only marginally to exon inclusion (Fig. 5), and only when the marmoset-specific C at position −10 is replaced by T, a significant increase in exon 10 inclusion is obtained. Correspondingly, a decrease in inclusion efficiency of the human exon 10 can be observed by replacing the human T at position −10 by the marmoset-specific C. This is peculiar because a nucleotide substitution of T by C does not
interrupt the polypyrimidinic nature of the stretch and because U2AF (or a related splicing factor) is thought to bind equally well to C and T (24). Therefore, the sterical properties of the region might be important for splicing as well. When both nucleotide substitutions at positions −19 and −10 are combined, inclusion of marmoset exon 10 is additionally enhanced to 91%, whereas the level of inclusion of the human exon 10 remains the same. From these experiments on the 3′-region of intron 9 we conclude that both nucleo-

![Secondary RNA Structure Prediction](image)

The nucleotide sequence covering 45 bp 5′ and 3′ of exon 10 and the complete exon 10 were analyzed using the MFOLD program. Regions covering the 3′-acceptor splice site (AG) are given in the inset at a higher magnification. The intronic sequences are given in *lowercase letters*, whereas exonic sequences and the splice sites AG and GT are indicated by *uppercase letters*. The intronic mutations −19 and −10 are shown in *capital letters* and the exonic mutation at position +26 in *lowercase*. The locations of the mutations are indicated by arrows. H, Human exon 10 wild-type; H′, human exon 10 plus mutations −19, −10, and +26; M, marmoset exon 10 wild type; M′, marmoset exon 10 plus mutations −19, −10, and +26. Free energy (∆G) calculations by the MFOLD program on the RNA secondary structures obtained did not result in any significant difference.
tides at position −19 and position −10 contribute significantly to exon 10 inclusion.

Another important nucleotide change is located within exon 10. The substitution of G by A at position +26 resulted in destruction of potential binding sequence with a SC35 signature (Refs. 25–27 and Fig. 2). However, our RNAi experiments indicated clearly that SC35 itself is not the regulatory splicing factor. Based on these experiments, we followed the hypothesis that the secondary RNA structure could be involved in exon 10 usage. Recent studies have underlined the important function of such structures for effective splicing. Changes within this structure seem to hinder the access of basic splicing factors to selected sequences, resulting in diminished intron processing and thereby promoting exon skipping (28). Such a scenario has been recently shown for the human TAU gene, the 3′-splice site of which is particularly sensitive to the presence of structured RNA (29).

The secondary RNA structure prediction using MFOLD corroborates the results of the in vitro mutagenesis experiments. For the human exon 10 the 3′-splice site is readily accessible for the splicing machinery, thereby resulting in constitutive exon 10 usage. For the marmoset exon 10, the experimental data and the structural prediction suggest that exon skipping is supported by the aberrant 3′-splice site structure conformation. Only when at least the two point mutations at positions −10 and +26 are introduced does the RNA assume a secondary structure highly reminiscent of the human exon 10, in which the 3′-splice site is forming a stem together with the SC35 sequence signature (supplemental Fig. 2g). The changes at position −10 and +26 of the human exon 10 significantly disrupt the secondary RNA structure, but it is only when all three nucleotide exchanges are operated that exon 10 splicing is maximally reduced (Fig. 5 and supplemental Fig. 1g). Thus, both intronic and exonic sequences have a substantial role in constitutive inclusion of exon 10. The partial exchange of these sequences immediately results in alternative splicing of exon 10, as revealed by experimental data in which only single point mutations have been studied (Fig. 5).

The fact that three nucleotide changes had to be combined for constitutive inclusion or exclusion of exon 10 indicates that splicing is an event tightly controlled by sequence constraints. The synergistic effect of a combination of nucleotides has been observed in the regulation of other genomic regions such as exon 9 of the CFTR gene and exon 7 of the SMN2 gene, where these sequence elements have been named “composite exonic regulatory element of splicing” (CERES) (18). We speculate that alternative splicing of other exons of the LHR and of the other glycoprotein hormone receptor genes might depend on similar mechanisms, residing in the gene sequence.

In conclusion, this study identifies the genomic checkpoints for exon 10 usage and shows, for the first time, the involvement of a CERES responsible for exon 10 inclusion in the LHR mRNA. Mutations in the CERES, which change the secondary RNA structure and the accessibility of the 3′-splice site, result in exon 10 alternative splicing or skipping in the LHR type 2.
We generated a human LHR constitutively skipping exon 10, and a marmoset LHR constitutively including exon 10. Understanding factors and structures involved in splicing of gonadotropin receptors provides the possibility to manipulate splicing, e.g., by knocking in a mutated LHR type 2 in transgenic mice to explore the consequences of altered splicing patterns in vivo.

MATERIALS AND METHODS

Genomic DNA Isolation

Genomic DNA was isolated from blood and tissue samples using the Nucleon DNA isolation kit as described previously (14).

Long-Template PCR

Long-template PCR was performed on 300–500 ng genomic DNA using the Expand Long Template PCR System (Roche, Penzberg, Germany) using the following primers: human exon 9 forward 5′-CAGAGGCTAATTGCCAGCTACCTC-3′ human exon 11 reverse 5′-AGTCCCAAGCACTCAGTTCACTTC-3′ marmoset exon 9 forward 5′-GCAATCTCTCTGATGCAGACGC-3′ marmoset exon 11 reverse 5′-CAGAGGACATCAGGGGTTGCTTG-3′ The amplicons obtained were cloned into the TOPO-XL vector (Invitrogen, Karlsruhe, Germany) and completely sequenced using the shotgun technique.

Minigene Construction

For analyzing exon splicing of the large genomic regions covering exons 9–11, we employed the minigene approach described by Stoss et al. (30). Consequently both DNA fragments were excised by the restriction enzymes XhoI and NotI and cloned into the pTarget (Promega, Mannheim, Germany) expression vector.

Preparation of Expression Vectors for Exon Trap Experiments

Analysis of the shorter genomic clones containing exon 10 and 450, 300, 150, and 45 bp upstream and downstream of it were performed using the exon trapping system (Gibco-BRL, Germany). Clones were generated by amplification of DNA fragments from human or marmoset genomic DNA using specific primers containing the restriction site XhoI within the forward primer or BamHI in the reverse primer. The amplicons were restricted by XhoI and BamHI and cloned into the exon trap vector pSPL3.

Generation of Chimeric Intron/Exon Constructs

To generate chimeric constructs, consisting of 3′- and 5′-intronic marmoset sequences joined to the human exon 10 and vice versa, we used the splicing by overlap extension by the PCR technique, first described by Horton et al. (31). In the present study, splicing by overlap extension by PCR involves five different PCRs to synthesize one chimeric construct. The intronic upstream element, representing 45 bp of the 3′-end of marmoset intron 9 was amplified using a forward primer (containing a XhoI restriction site) for the human LHR 5′-CCGCTCGAAGAGCTCAAAAGCTTTGATC-3′ or the marmoset LHR 45-bp intronic region 5′-CCGCTCGAAGAAAGCTCAGG-3′ and a chimeric reverse primer for the human exon 10 5′-CTTACATCGAAATACCTTTACTTACGGTATTGGTTTACCTAC-3′ (consisting of 3′-intronic and human exononic sequences) or a chimeric marmoset LHR 5′-CTTACATCGAATACTTACGGTGATTGGTTTACCTAC-3′ containing a BamHI restriction site.

The chimeric constructs obtained were gel purified, restricted by BamHI and XhoI, and cloned into the exon trap vector pSPL3.

Mutagenesis

Mutagenesis was performed using the oligonucleotide site-directed mutagenesis kit from Stratagene (Amsterdam, The Netherlands) according to the manufacturer’s protocol.

Transient Transfection and RT-PCR Using the Exon Trap Vector pSPL3

COS-7 cells or COV343 cells, a human granulosa cell line kindly provided by Dr. C. de Geyter [University Women’s Hospital, Basel, Switzerland (32)], were seeded on petri dishes and cultured until they reached 40–50% confluence. Plasmid DNA (12 μg per dish) was used for transfection by lipofectamine (Invitrogen, Amsterdam, The Netherlands) (12 μl/dish). After 6 h transfection was stopped by adding DMEM supplemented with 20% fetal calf serum. The cells were lysed 48 h later using Ultraspec (AMS, Germany) and RNA isolated according to the manufacturer’s protocol. Reverse transcription was performed using the vector-specific primers SA2 or β-actin primers. Subsequent PCR was performed with the primer combination SD6/SA2 and β-actin forward/reverse using the following program: 94°C for 50 sec, 60°C for 50 sec, 72°C for 90 sec for 35 cycles. The PCRs were subjected to 2% agarose gel electrophoresis and documented using the Multi-Image Light system (Biozym, Oldendorf, Germany).
Densitometric Analysis

Evaluation of splicing patterns using the exon trap vector were performed by densitometric analysis of the RT-PCR bands using the Chemilmager software (Alpha Innotech Corp., Biozym, Germany).

Generation of Chimeric Marmoset LHR Constructs

The marmoset LHR cDNA plasmid was kindly provided by Professor I. Huthaniemi (Imperial College, London, UK). Sequence and functional characteristics of the marmoset LHR cDNA have been previously reported (13). We generated marmoset LHR chimeric constructs in which either the putative marmoset exon 10 or the human exon 10 were inserted in the expected place. Constructs were produced by overlapping PCR according to Zhang et al. (13) and Müller et al. (17). The following primers were used for the introduction of marmoset putative exon 10:

- forward primer:
  5'-TCTGAACATGGTGGACAGACAGGAAGAACAAA-
  TAATGAAAACACTTATGCTCCTATTTGC-3' reverse primer

- forward primer:
  5'-TCACATGGTTGGAAGTCCTTTAGAAA-
  TGAAATTCTTGTCTTGTGACAGTTT-3'

For the introduction of exon 10 into the marmoset LHR the primers were

- forward primer:
  5'-TTCCAAACATTTGGAAGGCAAGATGAGAACAG-
  TGAATAAACAAAACAGCTTTATGCTCACATTTTG-3'

- reverse primer:
  5'-TCACACTTGTGGGAAGGTTCAGTTGTGTAACAGTTT-3'

The amplicons were gel purified and cloned into the pGEM-T easy vector (Promega, Heidelberg, Germany). By restriction site-directed ligation the fragments containing either human or marmoset exon 10 were cloned into marmoset wild-type LHR cDNA, which then was cloned in the pTarget expression vector (Promega).

RNAi Oligonucleotides

We have designed three different RNAis that, in principle, are capable of interfering with SC35 synthesis:

- RNAi 182 5'-GGGGAUGACCGUGCAUGGCTGTTT-3' RNAi 356 5'-GGGUAUGACCUCCUGGACAG-3' RNAi 589 5'-GGGGAUGACCGUGCAUGGCTGTTT-3'

Transfection experiments by lipofection were performed with varying doses of RNAi (5–10 nM) or plasmid DNA (100 ng to 1 µg plasmid DNA). Western blot were performed according to routine protocols, and for the detection of SC35 expression vector (Promega).

Functional Studies

Recombinant marmoset CG was produced and calibrated as described by Müller et al. (20). Highly purified human LH (Calbiochem, Germany) was calibrated against the WHO international standard for LH 80/522 as described by Müller et al. (17). COS7 cells were transiently transfected with constructs containing the wild-type marmoset LHR cDNA or the chimeric constructs into which the human or marmoset exon 10 had been inserted at the expected place as described above. Cells were stimulated in triplicate 24 h after transfection for 6 h at 37 °C with recombinant marmoset CG or human LH at concentrations of 0, 4, and 16 IU/liter (in terms of WHO LH 80/522) in 250 µl PBS solution, 1 mM glucose, pH 7.4, in the presence of 25 mM isobutylmethylxanthine (Sigma-Aldrich, Germany). After incubation the wells were frozen at −20 C and thawed, and the triplicates were pooled and boiled at 100 C for 2 min.

cAMP production of the COS7 cells was measured by ELISA (IHF, Hamburg, Germany) as described previously (20). The pooled samples of triplicate stimulations were measured in duplicate. Protein content of the samples was determined by Bio-Rad DC protein assay (Bio-Rad Laboratories, Inc., Hercules, CA) in duplicate. Results were statistically assessed by the Friedman test using GraphPad Prism software (GraphPad Software, Inc., San Diego, CA).

Sequence Analysis

Sequence alignments were performed using the ClustalW program (http://www.ebi.ac.uk/Tools/w/hmmer3). Exon splicing binding sites were identified using the ESE finder software (http://rulai.cshl.edu/tools/ESF/). Scoring of the splice sites was performed using Alex’s Splice Site Score Calculator (www.genet.sickkids.on.ca/~al/splicesitescore.html). Other searches were performed using the toolbox of the Alternative Splicing Database project (http://www.bioinfo.rpi.edu/applications/mfold/rna/form1.cgi).

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