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Characterization of Conserved Tandem Donor Sites and Intronic Motifs Required for Alternative Splicing in Corticosteroid Receptor Genes

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Alternative splicing events from tandem donor sites result in mRNA variants coding for additional amino acids in the DNA binding domain of both the glucocorticoid (GR) and mineralocorticoid (MR) receptors. We now show that expression of both splice variants is extensively conserved in mammalian species, providing strong evidence for their functional significance. An exception to the conservation of the MR tandem splice site (an A at position +5 of the MR+12 donor site in the mouse) was predicted to decrease U1 small nuclear RNA binding. In accord with this prediction, we were unable to detect the MR+12 variant in this species. The one exception to the conservation of the GR tandem splice site, an A at position +3 of the platypus GR donor site that was predicted to enhance binding of U1 snRNA, was unexpectedly associated with decreased expression of the variant from the endogenous gene as well as a minigene. An intronic pyrimidine motif present in both GR and MR genes was found to be critical for usage of the downstream donor site, and overexpression of TIA1/TIAL1 RNA binding proteins, which are known to bind such motifs, led to a marked increase in the proportion of GR+12 and MR+12. These results provide striking evidence for conservation of a complex splicing mechanism that involves processes other than stochastic spliceosome binding and identify a mechanism that would allow regulation of variant expression. (Endocrinology 150: 4958–4967, 2009)

The glucocorticoid (GR) and mineralocorticoid (MR) receptors are thought to be descended from an ancestral fish corticosteroid receptor that underwent gene duplication some 450 million years ago. Elegant studies (1) suggest that the duplicated genes were originally capable of binding both glucocorticoids and mineralocorticoids, but with time the GR evolved to become specific for glucocorticoids. The common evolutionary history of corticosteroid receptors is apparent in their conserved structure which, like other nuclear receptors, incorporates specific domains allocated to functions such as ligand binding, activation of transcription, and DNA binding (2). The DNA binding domain, which is particularly highly conserved in all nuclear receptors, consists of two zinc fingers separated by a short loop. Each zinc finger, together with part of the intervening loop, is encoded by a separate exon (exons 3 and 4, separated by intron C). Two closely related genes, probably derived from a genome duplication that occurred at some time after the divergence of fish and tetrapod lineages, code for the GR in fish (3). Alternative splicing of one of these genes produces an isoform, first identified in rainbow trout (4, 5) and later reported in cichlid fish (6), with an additional nine amino acids encoded by 27 bases inserted between exons 3 and 4 (Fig. 1). The source of this variant, which has since been detected almost universally in fish species (7), is alternative splicing of an additional short exon located within intron C (8). Alternative splicing at the corresponding exon 3/exon 4 junction, but produced by a different mechanism, has

Abbreviations: GR, Glucocorticoid receptor; HA, hemagglutinin; MR, mineralocorticoid receptor; siRNA, small interfering RNA; snRNA, small nuclear RNA; S&S, Shapiro and Senapathy; WT1, Wilms’ Tumor 1.
been detected in the GR and MR of tetrapods. When first reported, the GR sequence of a New World monkey (Sagui- nus Oedipus) included an additional three bases located in the DNA binding domain (9). The same sequence was sub- sequently reported in mouse (10) and human (11) tumors. We found the identical variant in normal human tissues and consequently reported in mouse (10) and human (11) tumors. For transcriptional reg- ulation of some genes, GRGr genes, the variant proves to be markedly more active than GRGr. A similar splicing variant (MR + 12) of the MR has been reported in human (15, 16), rat (15), and xeno- pus (17). Again, an alternative splice site is located in the intron separating exons 3 and 4 of the MR gene, but in this case, 12 bases of the intron are retained, coding for an additional four amino acids, KCSW in humans and rats (Fig. 1), KCSR in xeno- pus. The results presented here show that both GR and MR variants are widely conserved in mammals and provide strong additional evidence in support of a functional role for both variants.

Alternative splicing of pre-mRNA increases protein diversity, enhancing proteome size considerably and providing a significant source for the increased complexity of regulat- ory networks in higher organisms (18). It is unclear, how- ever, to what extent the production of splice variants might result from errors in the splicing process. The class of alternative splicing that involves tandem 3’ and 5’ splice sites has been shown to be used extensively (19), but it has been sug- gested that such alternative splicing could simply represent noise caused by slipping of the spliceosome between the two sites in a stochastic process (20). The present study provides convincing evidence to refute this argument by demonstrating the essential role played by conserved intronic sequences. In addition, the TIA1 and TIAL1 proteins, which are known to facilitate recognition of 5’ donor sites, are shown to be capable of regulating the relative usage of the tandem donor sites.

Materials and Methods

Detection of splice variants

Using RT-PCR, a region spanning the exon 3/4 splice site was amplified from hepatic RNA obtained from each species. Total
RNA was extracted from tissues preserved in RNAlater (Am- bion, Abingdon, UK) using the RNeasy minikit (QIAGEN, Crawley, UK) and reverse transcribed using the cloned AMV first-strand cDNA synthesis kit (Invitrogen, Paisley, UK). All oligonucleotides used are given in supplemental Fig. S1, published as supplemental data on The Endocrine Society’s Journals Online web site at http://endo.endojournals.org.

Amplified PCR products were cloned using the TA cloning kit (Invitrogen). The retained three bases in the human GRγ sequence create an Accl restriction site, and the same insertion would generate this restriction site in the other mammalian species we investigated. Putative GRγ clones were therefore identified using restriction analysis before sequencing. The platypus MR+12 variant clones were identified by excising the cloned PCR products and separation by gel electrophoresis. All sequencing was performed by Geneservice (Cambridge, UK).

Quantitation of variant mRNA expression

Expression of GRγ in mouse tissues was measured by Accl digestion as described previously (12) using the MTC panel (BD Biosciences, CLONTECH, Palo Alto, CA). Fragments were stained with Vistra Green (Amersham Biosciences, Buckinghamshire, UK) and fluorescence quantitated with a Molecular Dynamics Storm phosphorimager (Amersham Biosciences). Quantitative PCR was performed using the ABI 7500 real-time PCR sequence detection system (Applied Biosystems, Warrington, UK) with the SYBR Green PCR master mix (Applied Biosystems). Primers used for GR quantitation were based on those described by Beger et al. (21). Representative standard curves for quantitative PCR assays are shown in supplemental Figs. 2 and 3. For rat tissues, the MTC rat panel (CLONTECH) was used.

Construction of minigenes

The MR minigene was constructed by two rounds of PCR amplification of the MR gene from rat genomic DNA (Bioline, Randolph, MA) using primers MR1-4 and proofreading DNA polymerase (PhuUltra; Stratagene, La Jolla, CA). Two separate regions of the MR gene were amplified, exon 3 with 300 bp downstream intronic sequence (primers MR1 and MR2) and 300 bp intronic sequence upstream of exon 4 together with exon 4 (primers MR3 and MR4). Primers MR2 and MR3 had 3′ sequence complementarity, allowing a second round of PCR amplification of both exons and intronic sequences before cloning into the pcDNA3 vector (Invitrogen). The GR minigene was constructed in the same manner, using primers GR1-4 to amplify regions of the glucocorticoid receptor from human genomic DNA.

Single-base modifications were introduced using QuikChange II site-directed mutagenesis (Stratagene). Deletion plasmids were constructed by two rounds of PCR amplification from the complete GR minigene and recloning into the pcDNA3 vector using appropriate pairs of primers. The intronic mutations in the MR minigene plasmids (CTTT to GGAG) and GR minigene plasmids (TTTT to CAGA) were constructed in the same way.

Transfection and Western blotting

COS-1 and A549 cells (both obtained from ECACC, Salisbury, UK) were cultured in DMEM supplemented with 10% fetal bovine serum and 2 mM L-glutamine. Cells (175,000 cells/well in six well plates) were seeded 24 h before transfection, and each well was transfected with 0.8 µg plasmid combined with 4 µl Lipofectamine-2000 (Invitrogen). The cells were harvested 24 h after transfection, and RNA was extracted using a RNeasy minikit (QIAGEN). Expression vectors for hemagglutinin (HA)-tagged short isoforms of human TIA1 and TIAL1 subcloned into pMT2 were generously supplied by H. Lou (Department of Genetics, Case Western Reserve University, Cleveland, OH) and N. Kedersha (Division of Rheumatology, Immunology, and Allergy, Brigham and Women’s Hospital, Boston, MA). For small interfering RNA (siRNA) knockdown of TIA1 and TIAL1, ON-TARGET SMARTpools L-013042-00-0005 and L-011405-00-0005 (Dharmacon, Lafayette, CO) were used. For Western blotting, cells were lysed as described (22). TIA1 and TIAL1 were detected using Ab H-120 (Santa Cruz Biotechnology, Santa Cruz, CA), and HA tag was detected using Ab 16B12 (Cambridge BioScience, Cambridge, UK).

Scoring of 5′ splice sites

Shapiro and Senapathy (S&S) and Maxent 5′ splice site scores were computed using online tools (http://ast.bioinfo.tau.ac.il/splicesiteframe.htm and http://genes.mit.edu/burgelab/maxent/xmaxentscan_scoreseq.html).

Results

Evolutionary conservation of 5′ splice sites at the exon 3/exon 4 junction of the GR gene

The consensus sequence for 5′ splice sites is CAG/GT/GRAGT (where / denotes the exon/intron junction and R represents purine) (23). The bases, which are conventionally designated −3 to −1 for the last three exonic bases and +1 to +6 for the first six intronic bases, allow for exact complementarity with the consensus sequence of the U1 small nuclear (snRNA) 5′ terminus, although individual splice sites may differ substantially from the consensus sequence. To assess the degree of evolutionary conservation of the GRγ variant, we first examined genomic sequences at the exon 3/intron C boundary of the GR gene in various species (Fig. 1B). In all mammalian species examined, a potential additional splice site with the consensus GT dinucleotide is present at positions +4 and +5 of the GRα donor site. This base pair is absent in chicken, xenopus, and fish sequences, however. Various methods have been used to assess the strength of putative splice sites.

To provide an initial estimate of the likelihood that tandem donor sites would direct splicing of both GRα and GRγ variants, we used a method based on nucleotide frequency matrices (S&S score) derived by Shapiro and Senapathy (24), which reflects the degree of conservation of individual nucleotides relative to the consensus sequence. With the exception of platypus, in all other mammalian species examined, the 5′ splice sites that would give rise to GRα and GRγ have scores of 72 and 62%, respectively (the maximum score for a consensus sequence being 100%). These figures suggest that expression of the two
variants is maintained by use of a relatively weak α-site together with a slightly weaker γ-site. The one exception is the platypus sequence, in which the presence of an A instead of G at position +3 of the putative GRγ donor site, which should strengthen the site (score 66%). The presence of this sequence in platypus suggests that GRγ arose at a very early stage in the evolution of mammals; two recent estimates for the time elapsed since human and platypus lineages diverged have been 166 (25) and more than 200 (26) million years ago.

There is also a particularly striking conservation that extends further to include a T-rich sequence (53% of the 30 bases downstream from the GRγ donor site). Previous studies have shown increased conservation of intronic sequences linked to alternatively spliced exons (27, 28), suggesting that these sequences might be important for alternative splicing. More recently Aznarez et al. (29) have identified U-rich sequences in precursor mRNA as important determinants of 5′ donor site choice, suggesting that the T-rich intronic sequence might play a role in choice of splice site. Another putative regulatory sequence, TATGCA, that has been shown to function as an exon splicing regulatory sequence (30) is located 77 bases downstream from the GRα splice site of the human GR.

**Evolutionary conservation of 5′ splice sites at the exon 3/exon 4 junction of the MR gene**

Examination of corresponding intron sequences for the MR gene also indicates conservation of the alternative MR+12 splice site in mammals (Fig. 1C). Predicted S&CS scores for MR and MR+12 in most mammals are 87.9 and 72.4%, respectively, with the alternative MR+12 splice site again being the weaker of the two sites. One interesting exception is the mouse sequence, in which there is a high degree of conservation, except that one base (A at +5 of a possible MR+12 splice donor site) replaces the G found in other mammals. As a result, the score for this alternative site is decreased to 59.8%. In Xenopus, despite some differences relative to mammalian genes, the score for the MR+12 donor site (73.0%) is similar to that in mammals. There is no evidence of an alternative splice site in the chicken.

Again, there is conservation in the 30 bases downstream from the 5′ splice site. Like the GR gene, this sequence is T-rich (53% of the 30 bases downstream from the human MR+12 donor site).

**Expression and quantitation of the GRγ splice variant in different species**

To confirm the predicted expression of GRγ mRNA, we tested for the presence of mRNA encoding GRγ in liver samples from various species. Sequences spanning the GR exon 3/exon 4 junction were amplified by PCR, cloned, and then screened by digestion with the AccI restriction enzyme. The presence of the additional GTA triplet in an AccI-positive clone from each species was confirmed by sequencing, and the species identity of each sequence was confirmed by comparison with published sequences. Previously we identified GRγ in various human tissues, including liver (12). We have now confirmed the expression of GRγ in a wide range of mammals including pig, rabbit, rat, mouse, and platypus (results not shown). As predicted from the intron sequences that lack appropriate alternative 5′ splice sites (Fig. 1C), we detected no GRγ mRNA in chicken or Xenopus.

GRγ expression was measured in mouse tissues using the AccI digestion assay (12). The results were similar to those found previously in human tissues, ranging between 6.1 and 8.4% of total GR mRNA, with a mean of 7.1 ± 1.0% (Table 1). We then established a quantitative PCR assay for rat and platypus GRγ mRNA. With this assay, we obtained similar, although slightly higher, levels of GRγ mRNA ranging from 7.4 to 10.6% in rat tissues (Table 1), with a mean of 9.0 ± 1.2%. Unexpectedly, despite the higher score for the putative downstream donor site, we found no GRγ mRNA in chicken or Xenopus.

**Quantitation of MR+12 mRNA variant expression in rat and mouse tissues**

The MR+12 splice variant has been described previously in rat and human (15) as well as Xenopus (17) tissues. Using quantitative PCR, we found expression of MR+12 mRNA to range between 2.0 and 4.0% of total receptor

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**TABLE 1.** Expression of GRγ and MR+12 mRNA in different species

<table>
<thead>
<tr>
<th>Gene</th>
<th>Mouse</th>
<th>Rat</th>
<th>Platypus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td>8.2 ± 1.6</td>
<td>8.6 ± 1.1</td>
<td>3.5 ± 0.6</td>
</tr>
<tr>
<td>Spleen</td>
<td>8.4 ± 1.4</td>
<td>9.1 ± 1.9</td>
<td></td>
</tr>
<tr>
<td>Skeletal muscle</td>
<td>7.5 ± 1.2</td>
<td>7.6 ± 0.1</td>
<td></td>
</tr>
<tr>
<td>Heart</td>
<td>6.1 ± 1.6</td>
<td>9.8 ± 1.0</td>
<td></td>
</tr>
<tr>
<td>Kidney</td>
<td>6.4 ± 0.7</td>
<td>7.4 ± 1.2</td>
<td></td>
</tr>
<tr>
<td>Lung</td>
<td>6.1 ± 0.7</td>
<td>9.8 ± 0.5</td>
<td></td>
</tr>
<tr>
<td>Brain</td>
<td>6.9 ± 0.8</td>
<td>10.6 ± 1.9</td>
<td></td>
</tr>
</tbody>
</table>

Results represent the mean and SD from at least three separate experiments. The GRγ variant was measured by AccI digestion in mouse tissues and quantitative PCR in rat tissues. The MR+12 variant was measured by quantitative PCR in mouse and rat tissues.
mRNA in rat tissues (mean 3.0 ± 0.7) (Table 1). We were interested to measure MR+12 expression in the mouse because the predicted strength of the donor site for this variant was markedly lower than in other mammals. In agreement with this prediction, we were unable to detect MR+12 in mouse tissues using quantitative PCR (Table 1). The presence of MR+12 in platypus liver was confirmed, however, by sequencing the region spanning the 5′ splice site in a clone selected on the basis of size (results not shown).

Alternative splicing in minigenes with point mutations in donor sites

Minigenes to allow further analysis of GR and MR alternative splicing were constructed that consisted of exons 3 and 4 of each gene together with about 300 bases from the 5′ and 3′ ends of the intron. To investigate the importance of individual bases in the tandem 5′ donor sites of the GR gene, mutations were introduced at sites +3 to +6 of the GR δ donor (Fig. 2A), and the effect of these changes on the ratio of α- and γ-splice variants was determined in COS-1 cells. With the wild-type minigene, the GR γ splice variant was produced at a consistently lower level than GR δ, ranging between 17 and 37% of total transcripts in individual experiments. None of the mutations had any significant effect on production of GR δ (results not shown), but mutations at +5 and +6 resulted in a marked decrease in splicing to the GR γ variant (Fig. 2B), as predicted by lower S&S scores for these mutations (50 and 56%, compared with 62% for the wild type sequence). A higher S&S score (72%) for the mutation at +4 (T to A) also appeared to result in increased formation of the GR γ variant, although the difference was not statistically significant. Importantly, the effect of the +3 G-to-A mutation confirms the anomalous finding with the platypus receptor, i.e. an A at position +3 would be expected to increase base pairing to U1 snRNA, as reflected in the S&S score but in practice decreases usage of this donor site.

With the MR minigene, 2.8% of total transcript consisted of MR+12 variant. In agreement with the absence of MR+12 in mouse tissue, mutation of the rat MR δ+12 donor site (Fig. 2C), as found in the mouse genome, drastically reduced the proportion of MR+12 variant to just 19% of that produced by the wild-type minigene (Fig. 2D).

Alternative splicing in minigenes with deleted intronic sequences

Progressive deletions of the 5′ intron sequence (Fig. 3A) were then introduced into the GR wild-type minigene. The Δ86 minigene still included the TATGCA hexamer putative regulatory sequence (at +77), but the hexamer was deleted from the Δ77 minigene. The Δ10 minigene retained only 10 bases (i.e. the nine bases encompassing the GR δ and GR γ splice sites, plus one additional base from the downstream intron). The Δ13 and Δ16 mini-
gene sequences contained the GR\(\alpha\) and GR\(\gamma\) splice sites plus four and seven bases of the downstream intron, respectively.

Deletion of intronic sequences to create the \(\Delta 86\) and \(\Delta 77\) minigenes resulted in an increase \((P < 0.05)\) in the number of both GR\(\alpha\) and GR\(\gamma\) transcripts (Fig. 3B), suggesting that these sequences, and particularly the TATGCA hexanucleotide, exert repressor functions. Further deletions to create \(\Delta 16, \Delta 13\) and \(\Delta 10\) minigenes resulted in a decrease \((P < 0.05)\) in both products, a finding that is compatible with previous studies showing that T-rich intronic sequences enhance usage of adjacent upstream 5′ donor sites (29).

The most striking effect of intronic deletions was on the proportion of GR\(\gamma\) variant produced (Fig. 3B). There was no significant change in the relative amount of GR\(\gamma\) produced by the \(\Delta 86\) and \(\Delta 77\) minigenes \((i.e.\ up\ to\ and\ including\ the\ TATGCA\ sequence)\), but deletion of the T-rich intronic sequence caused a drastic reduction in the proportion of GR\(\gamma\). A small amount \((10%\ of\ total)\) of GR\(\gamma\) was produced by the \(\Delta 16\) minigene, which still included the TTTT sequence just downstream from the GR\(\gamma\) donor site, but further deletion resulted in almost complete loss of the GR\(\gamma\) variant \((to\ 0.9\ and\ 1.9%,\ respectively,\ in\ the\ \Delta 10\ and\ \Delta 13\ minigenes)\). Thus the T-rich intronic sequence not only enhances usage of 5′ donor sites but may be essential for usage of weaker downstream donors at tandem sites.

**Alternative splicing in minigenes with point mutations in an intronic pyrimidine motif**

The experiments with GR deletion mutants showed that the extended T-rich intronic sequence plays a major role in directing the spliceosome to the downstream donor. Because these experiments also suggested that the first pyrimidine tetranucleotide motif located immediately downstream from the donors might be particularly important, we decided to test the effect of mutating this motif in the GR and MR minigenes. In both human genes, the motif is TTTT (Fig. 3C), but in the wild-type rat MR minigene, it is TTTT (Fig. 3E). Mutation of these motifs resulted in almost complete suppression of both human GR\(\gamma\) (Fig. 3D) and rat MR+12 (Fig. 3F) variants, without significantly affecting expression of the variant lacking an insert.
TIA1 and TIAL1 regulate alternative splicing of endogenous corticosteroid receptor genes

In view of their affinity for U-rich motifs and effect on choice of 5’ donors, TIA1 and TIAL1 proteins were expressed in A549 cells (Fig. 4A) and the proportion of variants expressed by endogenous genes was measured. There was a clear increase in the relative proportion of both GRγ (Fig. 4C) and MR+12 (Fig. 4D). Decreasing both TIA1 and TIAL1 by siRNA (Fig. 4B) resulted in a clear decrease in GRγ, whereas a small decrease in GRα was not significant (Fig. 4C). Knockdown of TIA1 and TIAL1 had no obvious effect on MR-12, whereas expression of MR+12 became too low to measure. These results clearly show that TIA1/TIAL1 proteins enhance usage of downstream splice sites in both genes. A small increase in GRα (P < 0.05) when these proteins were overexpressed suggests that they might also stabilize GR mRNA.

Discussion

Several instances of alternative splicing have been described in the GR and MR receptors that result in insertion of additional amino acids in the short loop joining the two zinc fingers of the DNA binding domain. We have expanded these observations to show a high degree of evolutionary conservation for both the GRγ and MR+12 splice variants, which extends at least to the platypus, a species belonging to a lineage that is thought to have diverged from other mammals 166 million yr ago or more (25). There was no evidence for the GRγ variant in reptiles or birds, but MR+12 has been reported in xenopus (17), as would be predicted by the presence of an alternative 5’ splice site in the xenopus MR intron sequence (Fig. 1C). One exception for the MR+12 variant is its almost complete absence in the mouse, which we predicted on the basis of a single base change in the alternative splice site of the mouse genomic sequence.

In an extensive study of the human genome, Hiller et al. (31) identified 85,500 tandem donor sites with the GYNGYN motif (where Y = C or T, N = A, C, G, or T). Of these, there was evidence of alternative splicing at 110 (1.3%) donors that used both GY splice sites. It has been suggested that such alternative splicing could simply represent noise caused by slipping of the spliceosome between the two sites in a stochastic process (20). The alternative splicing that occurs from tandem splice sites in the Wilms’ Tumor 1 (WT1) gene (32) provides a clear example of functional importance for tandem splice sites, however. Two isoforms that differ by the presence (+KTS) or absence (−KTS) of three amino acids, also located in a linker region between two zinc fingers, are produced by the WT1 gene (32, 33), and severe developmental defects result from changes in the ratio of these isoforms (34–36).

Complementarity to the U1 snRNA seems to be the dominant parameter in determining the extent of alternative splicing for two 5’ splice sites, which are predicted to have strong affinity for splicing factors (37). Although predicted binding for the alternative splice sites of the GR is less strong, the effects of point mutations in the donor sequence giving rise to GRγ generally confirm the importance of U1 binding. With the MR minigene, the G at position +5 in the MR+12 donor is particularly important, as shown by the apparent absence of MR+12 in the mouse and by the minigene experiment shown in Fig. 2D. Interestingly, the GRβ splice variant, which lacks the C-terminal domain (38), is also absent in the mouse (39). Evidence for the existence of determining factors other than simple strength of U1 binding in this system is provided by the decreased formation of GRγ when G is substituted by A at +3 in the 5’ donor of the GR minigene and
in the platypus, which was predicted to increase complementarity to U1. This unexpected finding is in accord with a number of studies showing that splice site choice cannot be predicted simply on the basis of complementarity to splicing factors.

In general, compared with introns flanking constitutively spliced exons, those surrounding alternatively spliced exons tend to be better conserved, with about 80% conservation for the first 30 bases of the downstream intron (27). In comparison, the first 30 bases of intron C of the glucocorticoid-receptor gene show a remarkable 97% conservation between human, rat, and mouse. A putative regulatory TATGCA sequence was identified 68 bases downstream from the tandem donor sites of the GR gene, and experiments with GR minigene deletion constructs provided some evidence for a suppressive effect of this hexanucleotide on overall splicing. The most striking result obtained with the minigene constructs related to deletion of the T-rich intronic sequence located between the tandem donor sites and the TATGCA motif. Several studies identified T-rich intronic sequences that regulate exon inclusion or exclusion (40–42), and a recent genome-wide study found widespread involvement of U-rich sequences in 5′ splice site recognition (29).

To identify the sequence responsible for this effect more precisely, the first pyrimidine tetramers downstream from the tandem donors in GR and MR minigenes were mutated. The consequence of these mutations was a drastic reduction in expression of both GRγ and MR+12 variants (Fig. 3, D and F). These experiments clearly show that choice of tandem donors is not simply a stochastic process determined by complementarity of the two splice donors to U1 snRNA and clearly refute the suggestion that alternative splicing at these tandem donor sites simply represents noise in the splicing process. Apart from the point mutations, the minigenes used for these experiments were identical with the wild-type minigenes, thus confirming that the decreased expression of GRγ in deletion mutants (Fig. 3B) was not simply a result of either reduced intrinsic sequence or proximity of the deletion site to the U1 binding sequences. Also, the conservation of intrinsic motifs required for expression of the GRγ and MR+12 variants provides additional evidence supporting their functional importance. Interestingly, while this manuscript was in preparation, a pyrimidine-rich intronic enhancer, which is required for production of the +KTS variant of WT1 was identified (43).

Cytotoxic granule-associated RNA binding protein (TIA1) and the related TIAL1 are multifunctional proteins involved in regulating splicing and mRNA stability. They bind U-rich sequences and have been shown to facilitate recognition of 5′ splice sites (29). In the present study, we show that this property extends to tandem donor sites, in which they markedly enhance usage of the downstream site (Fig. 4). The TIA1 and TIAL1 proteins are known to interact with other splicing regulators to determine tissue-specific expression of splicing variants (44), providing a mechanism whereby expression of the corticosteroid receptor variants could be regulated.

Comparing GRγ expression in a small number of rat and mouse tissues (Table 1), we found a relatively constant ratio of expression, with the variant constituting about 7–9% of total GR mRNA [similar to the level we found previously in human tissues (12)]. Other studies indicated a degree of tissue-specific regulation, however. In particular, there is evidence for an association between altered levels of GRγ expression and responsiveness to glucocorticoids in leukemic patients (21, 45, 46), a finding that could be explained by the regulatory factors that we have identified. With regard to the MR+12 variant, we found that it constituted between 2 and 4% of total MR receptor mRNA in the tissues studied, although Wickert et al. (47) detected variable expression of MR+12 in different regions of the brain.

Assuming that GRγ and MR+12 each play a distinct functional role in mammals, it is clearly important to identify the cellular processes that are controlled by each variant. Previous studies have shown that GRγ is less efficient than GRα at transactivation from a simple reporter gene containing glucocorticoid response elements (10, 11). Our more recent studies (paper to be submitted) show that with certain promoters GRγ may be more potent than GRα. These findings have now been confirmed by Meijsing et al. (14), who propose that the conformation of a short lever arm that is sensitive to differences in the sequence of DNA binding sites can mediate allosteric changes in receptor conformation and function, so allowing differential regulation of specific target genes. Importantly, the additional arginine in GRγ is located within this lever arm and is shown to alter its conformation. Distinct properties of the variants may also be linked to the dual function of the DNA binding domain, which mediates important protein/protein interactions (48–50) in addition to DNA binding. Insertion of arginine in GRγ could be of particular significance because this amino acid appears to have an important role in providing binding sites for protein/protein interactions (48–50) in addition to DNA binding. Insertion of arginine in GRγ could be of particular significance because this amino acid appears to have an important role in providing binding sites for protein/protein interactions, perhaps because it can form the basis for complex salt bridges linking more than two amino acids (51), and it is one of the three amino acids found most frequently in binding hot spots at protein-protein interfaces (52).

The results presented here show that the bases in the tandem 5′ donor sites of the GR and MR genes are crucial for determining the ratio of splice variants produced but not sufficient. These alternative splicing events have been
conserved in mammalian genomes over a considerable evolutionary period, possibly exceeding 200 million years, thus providing strong evidence in support of a functional role for the variants. Importantly, we have also identified conserved intronic sequences that play a crucial role in selection of GR and MR donor sites, and the ability of TIA1/TIAL1 proteins to modify the ratio of variant mRNA produced indicates the existence of a regulatory mechanism that may be of general relevance to splicing at tandem donor sites in other genes.

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