Associations between promoter usage and alternative splicing of the glucocorticoid receptor gene

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Abstract

The glucocorticoid receptor (GR) is widely expressed in various tissues throughout the human body. At least three different 3'-splice variants of the GR have been reported: GR- α , which is functionally active; GR- β , which is a dominant negative inhibitor of GR- α function; and GR-P, which is thought to activate the function of GR- α . At least seven different variants for exon 1 exist, 1A–1F and 1H, each with its own promoter. In this study, we explored if tissue-specific splicing of the 3'-end variants of the GR is influenced by alternative promoter usage. cDNAs of different tissues and cell lines were used to investigate which part of transcripts carrying each of the three major variants for exons 1, 1A, 1B, or 1C, encodes for the splice variants GR- α , GR- β , and GR-P. Our data demonstrate that the expression of GR- α is preferentially regulated by promoter 1C and that for the expression of GR-P promoter 1B is predominantly used. This indicates that regulation of GR splice variants could partly occur through selective use of the multiple promoters, and that this is another way to sensitize cells and tissues to the different activities of the GR isoforms.

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Introduction

Glucocorticoids (GCs, cortisol, and its synthetic analogs) can influence gene expression via the glucocorticoid receptor (GR). Unliganded GR resides in the cytoplasm but translocates to the nucleus upon GC binding, where it acts as a factor regulating gene transcription (Buckbinder & Robinson 2002). The GR-encoding gene (*NR3C1*) is located on chromosome 5q31.3 and has nine exons (Hollenberg *et al.* 1985). Transcription of the gene results in a broad range of mRNA transcripts that can differ in their 5'- and 3'-ends (Fig. 1; Lu & Cidlowski 2004, Turner & Muller 2005).

Alternative splicing generates three different 3'-end mRNA transcripts, encoding the GR- α , GR- β , and GR-P isoforms respectively. The GR- α isoform (Fig. 1) consists of 777 amino acids and is the functionally active isoform that mediates GC action. The GR- β transcript is generated by linking exons 8 to 9 β instead of exon 9 α (Fig. 1), resulting in a 742 amino acid protein (Hollenberg *et al.* 1985). This GR- β protein is not able to bind ligand and is reported to be a dominant-negative inhibitor of GR- α action (Bamberger *et al.* 1995, Oakley *et al.* 1996). GR- β is expressed in almost every tissue and in many cell lines, albeit at a very low level (0–2%) compared with GR- α (Oakley *et al.* 1996, Pujols *et al.* 2002). Despite its wide tissue expression pattern, controversy remains about its physiological

relevance (Carlstedt-Duke 1999, Vottero & Chrousos 1999). A third 3'-end mRNA variant only contains exons 2–7 and continues into intron G. The corresponding truncated GR protein of 676 amino acids (Fig. 1) lacks a large portion of the ligand-binding domain, and is therefore also unable to bind ligand (Krett *et al.* 1995). GR-P is widely expressed in various tissues and cells, up to 55% of that of GR- α in several hematological malignancies (de Lange *et al.* 2001, Hagendorf *et al.* 2005). The function of GR-P is also still not fully understood, but in contrast to GR- β it has been reported to enhance GR- α -mediated gene transcription (de Lange *et al.* 2001).

In contrast to alternative splicing at the 3'-end of the GR mRNA, alternative splicing at the 5'-end does not affect the GR protein structure. The differences are restricted to the 5'-untranslated region (UTR), which is composed of exon 1 and the first 13 nucleotides of exon 2. Initially, three variant exons 1 (1A, 1B, and 1C; Breslin *et al.* 2001) were described. These alternative exons 1 are each preceded by their own promoter (Fig. 1; Nobukuni *et al.* 1995, Breslin *et al.* 2001). Moreover, exon 1A was reported to contain three alternative splice sites, resulting in mRNA transcripts containing exons 1A1, 1A2, or 1A3, which means that at least five GR transcripts could be expressed from three separate promoters (Breslin *et al.* 2001), possibly resulting in cell-type-specific GR expression patterns. Transcripts containing

Figure 1 Organization and expression of the *GR* gene. The expression of the *GR* gene is controlled by multiple promoters yielding mRNAs containing various exons 1. mRNAs containing exons 1B and 1C are widely expressed and thought to play a role in the basal tissue-unspecific expression of the GR, while the expression of exons 1A, 1D–1F, and 1H appears to follow a tissue-specific expression pattern. Exons 1A and 1C are subject to (internal) differential splicing resulting in exons 1A1, 1A2, 1A3, and 1C1, 1C2, 1C3 respectively. The various 5'-end untranslated exons are spliced to the same splice acceptor site in exon 2. 3'-End alternative splicing results in mRNAs encoding three variants of the *GR* gene: GR-α, the functionally active protein; GR-β, a dominant negative inhibitor of GR-α function, unable to bind ligand; and GR-P, a truncated isoform, also unable to bind ligand but thought to increase the activity of GR-α.

exons 1B and 1C are ubiquitously expressed and are probably responsible for the basal GR expression (Nunez & Vedeckis 2002). Transcripts containing 1A1 and 1A2 are marginally expressed, even less than GR-β, while mRNA transcripts containing exon 1A3 are expressed in cells of the hematopoietic lineage (Pedersen & Vedeckis 2003, Pedersen *et al.* 2004). Footprint and functional analysis of the promoter regions reveal unique binding sites for several transcription factors, but only promoter 1A harbors a sequence resembling a glucocorticoid receptor element (GRE) and could probably be autoregulated by the GR (Pedersen *et al.* 2004, Purton *et al.* 2004). In the rat, a more complicated organization of the 5'-part of the *GR* gene was found (McCormick *et al.* 2000). Here, at least 11 potential alternatives for exon 1 have been identified that showed a tissue-specific expression pattern. Recently, it was shown (Turner & Muller 2005) that in

the human *GR* gene five additional exon 1 variants were present, based upon sequence homology between human and rat. The authors could detect expression of four of these five exons (D–F and H), and they also found that exon 1C is subject to (internal) differential splicing (Turner & Muller 2005; Fig. 1). As in the rat, the expression of these exon 1 variants appears to follow a tissue-specific pattern.

Heterogeneity in the 5'-ends of mRNAs generated by alternative promoter usage seems to be a common feature among the members of the steroid hormone receptor family (Flouriot *et al.* 1998, Auboeuf *et al.* 2005). Furthermore, recent studies reported that promoter structure could modulate alternative splicing and suggest a physical and functional coupling between transcription and splicing (Auboeuf *et al.* 2002, 2004, Kornblihtt 2005). To determine whether splicing of the 3'-end variants of the GR is influenced by alternative promoter usage,

we explored whether correlations exist between the most abundantly expressed exon 1 variants (1A, 1B, and 1C) in the mRNA and 3'-end splicing (α , β , or P).

Materials and methods

Cell culture

Epstein-Barr virus (EBV)-transformed lymphoblast cell lines were established from PBMLs as described previously (Chrousos *et al.* 1986, Tomita *et al.* 1986). Cells were grown in Roswell Park Memorial Institute (RPMI)-1640 medium supplemented with 15% FCS, 100 μ g/ml penicillin and streptomycin under standard culture conditions.

cDNA

cDNAs were prepared as described previously (Dalm *et al.* 2004). Normal tissue samples were kindly provided by the Erasmus MC Tissue Bank, Rotterdam, The Netherlands. Thymic tissue samples were collected during operations. The tissue samples, taken during postmortem or operation, were directly frozen and stored at -80°C until use. The protocols were in accordance with the Helsinki Doctrine on Human Experimentation. Informed consent was obtained. cDNAs prepared from tissues taken during postmortem or operations were: lung ($n=2$), ileum ($n=3$), colon ($n=2$), rectum ($n=2$), thyroid ($n=2$), testes ($n=2$), adrenal ($n=4$), kidney ($n=2$), jejunum ($n=2$), pancreas ($n=2$), stomach ($n=1$) according to protocols as previously described (Dalm *et al.* 2004). cDNAs

prepared from cells of the hematopoietic lineage: T cells ($n=2$), B cells ($n=2$), macrophages ($n=4$), monocytes ($n=2$), dendritic cells ($n=2$), and thymocytes ($n=2$). cDNAs prepared from several untreated cell lines: B lymphoblasts ($n=2$), Jurkat ($n=1$), SaOs-1 ($n=2$), Mg63-1 ($n=2$), simian virus40 transformed human fetal osteoblast (SV-HFO) ($n=2$), PancI ($n=1$), Miapaca ($n=1$), Bon ($n=1$), and BxPc3 ($n=1$). cDNAs prepared from tumor tissue were: liver tumor ($n=1$), pituitary tumor ($n=1$), lymph tumor ($n=1$), pheochromocytoma ($n=1$), and renal tumor ($n=1$).

RT-PCR

To investigate if GR- α -, GR- β -, and GR-P-encoding transcripts are transcribed from all three different promoters, 1A, 1B, and 1C, RT-PCR was performed by using 5'-end (exon 1 specific) and 3'-end (3'-splice variant specific) primer combinations as indicated. These primers were also used for the quantitative real-time RT-PCR experiments and are shown in Table 1. PCR conditions, using the RT-PCR One Step Titan One Tube RT-PCR System (Roche), were as follows. A total of 250 ng RNA was incubated with a reaction mix containing 0.4 μ l enzyme mix, 0.2 mM dNTPs, 0.25 μ M forward (FW) primer, 0.25 μ M backward (BW) primer, 5 mM DDT, 1 U Protector RNase, and water in a total volume of 20 μ l. Cycle conditions were: 30 min 50°C ; then 10 cycles: 2 min 94°C , 10 s 94°C , 10 s 60°C , 2.5 min 68°C ; then 25 cycles 10 s 94°C , 10 s 60°C , 2.5 min 68°C , with an additional 5 s extension to the elongation time for each cycle. A PE-Applied Biosystem Gene Amp 9700 PCR-system (Nieuwerkerk aan de IJssel,

Table 1 Primers used for Quantitative real-time RT-PCR analysis

	Sequence	Q-RT-PCR of
Primer		
GR exon 6 (FW)	5'-TGTTTTGCTCCTGATCTGA-3'	GR- α mRNA
GR- α (BW)	5'-TCGGGGAATTCAATACTCA-3'	
Probe	FAM-TGACTCTACCCTGCATGTACGAC-TAMRA	
GR exon 6 (FW)	5'-TGTTTTGCTCCTGATCTGA-3'	GR- β mRNA
GR- β (BW)	5'-TGAGCGCCAAGATTGT-3'	
Probe	FAM-TGACTCTACCCTGCATGTACGAC-TAMRA	
GR exon 6 (FW)	5'-TGTTTTGCTCCTGATCTGA-3'	GR-P mRNA
GR-P (BW)	5'-CCTTTGTTTCTAGGCCTTC-3'	
Probe	FAM-TGACTCTACCCTGCATGTACGAC-TAMRA	
GR-1A3 (FW)	5'-CCCTCCTCGGGCTTTAGATTGT-3'	GR-1A mRNA
GR exon 2 (BW)	5'-GGGTGAAGACGCAGAAACCTT-3'	
Probe	FAM-CCCCAGCAGTGTGCTTGCTCAGGA-TAMRA	
GR-1B (FW)	5'-CCGGCCCCAAATTGATATTC-3'	GR-1B mRNA
GR exon 2 (BW)	5'-GGGTGAAGACGCAGAAACCTT-3'	
Probe	FAM-CCCCAGCAGTGTGCTTGCTCAGGA-TAMRA	
GR-1C (FW)	5'-GGCTCCTCTGCCAGAGTTGA-3'	GR-1C mRNA
GR exon 2 (BW)	5'-GGGTGAAGACGCAGAAACCTT-3'	
Probe	FAM-CCCCAGCAGTGTGCTTGCTCAGGA-TAMRA	

FW, forward; BW, backward.

The Netherlands) was used. PCR products were examined on a u.v. light source after electrophoresis on a 0.7% agarose gel containing ethidium bromide.

Quantification of the mRNA variants of the GR

Real-time quantitative (qPCR) analysis of mRNA was performed to investigate the amount of transcripts containing exons 1A, 1B, or 1C and the amount encoding GR- α , GR- β , or GR-P. The primers and probes used are shown in Table 1. For each splice variant, 2.5 μ l cDNA, corresponding to 50 ng total RNA, were amplified in separate reactions in a total volume of 25 μ l, containing 7.5 pmol of each primer and 5 pmol probe using the Universal Master Mix (Applied Biosystems, Nieuwerkerk a/d IJssel, The Netherlands) for PCRs amplifying the 5'-UTR and the qPCR Core-Kit (Eurogentec, Maastricht, The Netherlands) for PCRs amplifying the 3'-splice variants. Correction for assay variability between the separate reactions for which the same cDNA was used was performed by the house-keeping gene hypoxanthine phosphoribosyltransferase (HPRT) as described previously (Russcher *et al.* 2005), and calculation of the amounts of mRNA was carried

out using the comparative threshold cycle method (Livak & Schmittgen 2001).

Statistical analysis

Pearson correlation analysis was performed on data shown in Fig. 2 by using InStat software version 2.01 (GraphPad Software, Inc., San Diego, CA, USA). Data points were fitted with regression lines using the least-squares method. Statistical significance was set at $P < 0.05$.

Results

Tissue distribution of GR exons 1A, 1B, and 1C and 3'-end splice variants

Human cDNAs, reverse transcribed from RNA isolated from different organ tissue biopsy samples and different cells of the hematopoietic lineage, were used to quantify expression levels of exons 1A, 1B, and 1C, and of GR- α , GR- β , and GR-P mRNA. Figure 3 shows that the different exons 1 and the 3'-end splice variants were differentially expressed in these tissues. The major GR mRNA encoded the GR- α isoform. Expression of GR-P was

Figure 2 Positive significant correlations between contributions of exons 1 to the total amount of GR mRNA and mRNA encoding 3'-end splice variants. Pearson correlations between the contribution of exon 1C and GR- α and exon 1B and GR-P to the total amount of mRNA expressed in the 17 tissues described in Fig. 3, and (B) in these 17 tissues combined with 13 additional tissues and cell lines to increase statistical power.

Figure 3 Quantification of mRNA molecules encoding the GR- α , GR- β , GR-P isoforms and carrying exons 1A, 1B, or 1C in different tissues. (A) The number of copies of GR- α mRNA/50 ng total RNA in descending order. (B)–(F) GR- β , GR-P, exon 1A3, exon 1B, and exon 1C mRNA expressions respectively, in the same tissues.

about 4–20% of the expression level of GR- α and GR- β mRNA represented <1%, if at all detectable. Approximately, equal expression levels of exons 1B and 1C were found. mRNA containing exon 1A (1A3) was only detectable in adrenals and in cells of the hematopoietic lineage (T cells, B cells, dendritic cells, macrophages, thymocytes, and monocytes). The expression of exons 1A1 and 1A2 was very low, even less than GR- β , and it could not be further analyzed. The total amount of different exons 1 and the total amount of 3'-end splice variants are significantly correlated (Pearson $r=0.81$; $P<0.001$), indicating that both represent the amount of total GR present in the different tissues.

Correlation between the different exons 1A, 1B, and 1C and the 3'-end splice variants

Transcripts encoding GR- α , GR- β , and GR-P could theoretically be formed from each of the three different promoters that we investigated: 1A, 1B, and 1C. Due to the long coding sequence (>2000 nucleotides) between exons 1 and 9, no quantitative real-time RT-PCR could be performed to investigate how the different promoters control expression of these 3'-end splice variants. Therefore, we used semi-quantitative PCR analysis using RNA from a B lymphoblast cell line. Figure 4 shows that GR- α and GR-P mRNA could be expressed from all three promoters 1A, 1B, and 1C. However, no combinations could be made with the GR- β -specific primer, possibly due to low GR- β mRNA levels. Band intensities of the reaction products vary but could be due to varying reaction efficiencies. Different

primer combinations were used to amplify the mRNAs and, therefore, further conclusions based on the different signal intensities cannot be drawn. Furthermore, by calculating the percentage of each variant

Figure 4 Expression of different GR mRNA isoforms in B lymphoblasts. PCR analysis was performed in B lymphoblasts to investigate the expression of the indicated mRNA molecules. The specific amplification of these mRNA molecules was achieved by using the underlined primers shown in Table 1. In these B lymphoblasts, GR- α as well as GR-P expression is driven from promoters 1A, 1B, and 1C. No amplification of GR- β encoding mRNA was detected.

contributing to the total amount of GR mRNA presented in Fig. 3, correlations were made between the different exon 1 and the 3'-end splice variants. Positive significant correlations were found between the contributions of exon 1B and the GR-P splice variant (Pearson $r=0.66$, $P=0.004$) and between exon 1C and the GR- α splice variant (Pearson $r=0.6$, $P=0.012$; Fig. 2A). No positive correlations were found between exon 1A and the 3'-end splice variants and between GR- β mRNA expression and the various exons 1. The correlations presented in Fig. 2B are based on 17 tissue samples and 13 additional cDNAs, which were obtained from different cell lines and tumor tissues to increase statistical power. These samples were obtained from various other studies and not included in Fig. 3, because cDNA was prepared with non-comparable amounts of RNA. In these additional tissues, similar correlations were found between exons 1B and GR-P (Pearson $r=0.67$, $P<0.0001$) and between exons 1C and GR- α (Pearson $r=0.61$, $P=0.0004$; Fig. 2B). The proportion of 1B usage strongly influences the GR-P splicing route, whereas the proportion of 1C usage favors the GR- α splicing route.

Discussion

In 60% of the human genes, processing of pre-mRNA by specific splice factors results in multiple mRNAs and, hence, multiple proteins (Auboeuf *et al.* 2002). The factors that influence the selection of splice sites are not understood very well. However, recent evidence reveals that transcription regulation and alternative splicing can be coupled events (Cramer *et al.* 1997, 1999). The promoter may affect alternative splicing through recruitment of factors with dual functions in transcription and splicing (i.e. factors that contain functional domains for both processes and hence link them). Studies in which

promoter structure was changed led to differences in alternative splicing of the transcript, which could not be explained by differences in promoter strength (Cramer *et al.* 1997). Furthermore, a decreased processing rate of the RNA polymerase II or internal pauses could favor the inclusion of alternative exons, whereas a highly processive polymerase would favor exclusion of these exons. Elongation speed is regulated by the promoter and depends not only on the composition of the recruited transcription complex (de la Mata *et al.* 2003, Rosonina *et al.* 2003), but also on the secondary structure of the pre-mRNA (Eperon *et al.* 1988). When multiple promoters are involved in the transcription of a particular gene, occupation by promoter-specific transcription factors might differentially influence the transcription/splicing coupling mechanism, leading to alternative splicing (Nogues *et al.* 2002, Auboeuf *et al.* 2004).

The GR mRNA is expressed from multiple promoters. Initially, three exon 1 variants (1A–1C) were described (Breslin *et al.* 2001), each with its own promoter, and recently four additional exon 1 variants (1D–1F and 1H) were discovered, based upon sequence homologies between the rat and the human GR gene (McCormick *et al.* 2000, Turner & Muller 2005). Promoter 1A is mainly active in cells of hematopoietic origin and contains binding sites for nuclear factor- κ B, interferon regulatory factor, and GR (Breslin *et al.* 2001). Promoters 1B and 1C, however, are thought to play a role in the constitutive expression of GR. Constitutive or ‘housekeeping’ genes typically lack a TATA or CAAT box, but contain multiple GC regions that are potential sites for ubiquitous transcription factors such as SP-1. Four SP-1-binding sites have been identified in promoter 1B, whereas five were identified in promoter 1C (Nobukuni *et al.* 1995, Breslin & Vedeckis 1998). Yin-Yang 1 (YY-1)-binding sites are identified in promoter 1B (three sites) and promoter 1C (one site). YY-1 is a transcription factor that can act

Table 2 The abundance of GR exon 1 transcripts in various databases

Exon 1 variant	GenBank+EMBL+DDBJ ESTs (number of hits)	DBTSS (number of hits)	Turner & Muller (number of positive tissues, total=15)
1A1	0	0	n.d.
1A2	0	0	n.d.
1A3	15 (partial: 3'-end)	3	n.d.
1B	30	34	12
1C	30	13	14 (1C1+1C2+1C3)
1C1	0	0	n.d.
1C2	0	0	3
1C3	0	0	12
1D	0	0	1
1E	0	0	3
1F	3	3	3
1H	1	1	8

n.d., not done.

as an activator, a repressor, or an initiator of transcription, depending on the cellular context (Shrivastava & Calame 1994, Nunez & Vedeckis 2002). AP-1 and AP-2 sites are only identified in promoter 1C (Nunez & Vedeckis 2002). For the other variants of exon 1, no experimental information is available yet with regard to possible transcription factor binding sites (Turner *et al.* 2006). Several transcription factors are reported to contain functional domains to recruit splicing factors (Kornblihtt 2005), but whether SP-1, YY-1, AP-1, and AP-2 can directly interact with the splicing machinery is unknown.

The recent paper by Turner & Muller (2005) shows that exons 1B and 1C are the most widely expressed. This is confirmed by a BLAST search of the available human databases of expressed sequence tags (EST; Database of GenBank + EMBL + DDBJ sequences from EST division) and in the Database of Transcription Start Sites (DBTSS, <http://dbtss.hgc.jp/>, (Kimura *et al.* 2006)). Both searches yielded mainly entries for 1A, 1B, and 1C (see Table 2). Therefore, in this study, we focused on 1A, 1B, and 1C. We measured 1A, 1B, and 1C, but also GR- α , GR- β , and GR-P containing transcripts by quantitative real-time RT-PCR and found associations between promoter usage and alternative splicing of the GR gene. The proportion of 1B usage strongly influences the GR-P splicing route, whereas the proportion of 1C usage favors the GR- α splicing route (Fig. 2). It should be mentioned that our choice of primers for qPCR of exon 1C would detect 1C1, 1C2, and 1C3, similar to the 1C-total mentioned in Turner & Muller (2005), and from these data we cannot determine which of these exon 1C variants is actually present. However, a semi-quantitative PCR experiment in which 5'-specific primers for the different exons 1 was combined with specific primers for the 3'-splice variants showed that this is not an absolute effect, because PCRs with the possible primer combinations all resulted in amplification products (Fig. 4). Furthermore, associations were found neither with transcripts including exon 9 β , nor with those transcribed from promoter 1A, but this might be due to the low number of transcripts with these characteristics. Alternatively, GR- β splicing might be associated with one of the variants for exon 1 that we did not investigate here (1D–1F and 1H). However, in view of the similarity of the total amount of GR mRNA, whether estimated from the exon 1-specific qPCR or from the 3'-end-specific qPCR, suggests that the amount of GR- β mRNA incorporating one of these alternatives for exon 1 cannot be very large. The differences in amount of SP-1 and YY-1 transcription factor binding sites in promoters 1B and 1C, the presence of AP-1 and AP-2-binding sites in promoter 1C, or the abundance of transcription and splicing factors in the different tissues that are studied might

result in a different composition of transcription complexes that are recruited on these promoters, and through associations with splicing factors, influence the ratio in which GR- α and GR-P mRNA is expressed.

The cell-type-specific use of different promoters in the GR gene, but also the different start sites and exons 1 resulting in differences in secondary structure of the pre-mRNAs might alter the elongation speed at which mRNAs are formed. This could also influence splicing regulation. These 5' differences are restricted to the non-coding region and do not influence the open reading frame, which starts in exon 2. If alternative promoter usage and splice site selection for GR expression are linked through either transcription factor recruitment or elongation speed by means of such a coupling remains to be proven. Any supporting evidence should first rule out the possibility that alternative splice variants are the consequence of important changes in pre-mRNA secondary structure resulting from the different exon 1 sequences.

Nonetheless, our data demonstrate that GR- α expression is related to the use of 1C promoter and that expression of GR-P is related to the use of 1B promoter. The regulation of GR splice variants through the multiple promoters and the cell-type-specific expression of transcription factors is another way in which cells and tissues can be sensitized to the actions of GCs and the functions of the different splice variants.

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