Identification, tissue expression, and glucocorticoid responsiveness of alternative first exons of the human glucocorticoid receptor

Elisabeth Presul¹, Stefan Schmidt^{1,2}, Reinhard Kofler^{1,2} and Arno Helmberg¹

¹Biocenter, Division of Molecular Pathophysiology, Innsbruck Medical University, Fritz Pregl Str 3, A 6020 Innsbruck, Austria

²Tyrolean Cancer Research Institute, Innrain 66, 6020 Innsbruck, Austria

(Requests for offprints should be addressed to A Helmberg; Email: arno.helmberg@i-med.ac.at)

Abstract

Transcripts for the human glucocorticoid receptor (NR3C1) are known to contain alternative first exons 1A1, 1A2, and 1A3 from the distal promoter or 1D, 1E, 1B, 1F, 1C, or 1H from the proximal promoter. Here, we report two additional alternative first exons identified by Rapid amplification of cDNA ends (RACE)-PCR. The first, exon 1I, starts approximately 700 bp downstream of the splice donor site of the longest form of exon 1A, 1A3, considerably extending the known distal promoter region with a region containing conserved transcription factor-binding sites as well as a potential glucocorticoid response element (GRE) that differs from the consensus GRE in only two positions. The second, exon 1J, is part of the proximal promoter region and resides between exons 1D and 1E. Since this has been determined by quantitative real-time reverse transcriptase (RT)-PCR, exon 1I is used foremost in cells of the T-lymphocyte lineage. In the T-ALL cell line CEM-C7H2, which is sensitive to glucocorticoid-induced apoptosis, transcripts containing alternative first exons from the distal as well as the proximal promoter regions were markedly autoinduced by glucocorticoid treatment, with more pronounced relative induction in the distal promoter. Neither transcript was autoinduced in the related, resistant cell lines CEM-C1, and CEM-C7R5. In contrast, the glucocorticoid-sensitive PreB697 cell line strongly autoinduced transcripts from the proximal promoter, but not transcripts from the distal promoter, to relevant levels. Therefore, the autoinductive feedback loop implicated in glucocorticoid-induced apoptosis cannot universally rely on the distal promoter of the glucocorticoid receptor.

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Introduction

Glucocorticoids (GC) induce highly diverse responses in different tissues such as brain, immune system, and metabolic organs (Sapolsky *et al.* 2000, Schimmer & Parker 2006) to cope with stress events. This tissue diversity may partially be due to tissue-specific expression from multiple promoters, resulting in multiple alternative untranslated first exons, of the glucocorticoid receptor (GR). The *GR* gene (NR3C1, OMIM+138040) contains a presently undetermined number of untranslated first exons that are spliced to a common exon 2 (Strahle *et al.* 1992, Chen *et al.* 1999, McCormick *et al.* 2000, Breslin *et al.* 2001, Zhang *et al.* 2004, Turner & Muller 2005). The resulting differences in 5' untranslated regions may add a second level of regulation due to differing posttranscriptional treatment of the resulting mRNAs, potentially influencing mRNA processing, stability, export, and translation (Ayoubi & Van De Ven 1996, Zhang *et al.* 2004). The alternative first exons identified thus far are located in two promoter regions, one proximal, spanning about 5 kb upstream of the translation start site, and the

second distal, located more than 30 kb further upstream. For the human *GR* gene, nine untranslated exons have been published thus far (Breslin *et al.* 2001, Turner & Muller 2005), 12 for the rat *GR* (McCormick *et al.* 2000, Zhang *et al.* 2004), and five for the mouse (Strahle *et al.* 1992, Chen *et al.* 1999). The splice donor sites terminating these first exons are highly conserved between man, mouse, and rat. Pronounced tissue-specific expression has been observed for both promoter regions. At the proximal promoter, rat exon 1₇ was expressed at high levels in hippocampus (McCormick *et al.* 2000). Expression of certain distally located exons, termed 1A3 in man (Breslin *et al.* 2001), 1A in mouse (Strahle *et al.* 1992), and 1₁ in rat (McCormick *et al.* 2000), is limited to thymocytes, hematopoietic cell lines, and some regions of the brain.

Activated GR influences the expression of its own gene. Negative autoregulation of *GR* expression has been observed in most tissues and cell lines (Kalinyak *et al.* 1987, Dong *et al.* 1988, Burnstein *et al.* 1991). Conversely, a few cell types, including thymocytes, some acute lymphoblastic leukaemia (ALL) cell lines, and primary ALL cells from patients, respond to GC with

positive *GR* autoregulation (Eisen *et al.* 1988, Gomi *et al.* 1990, Obexer *et al.* 2001, Tonko *et al.* 2001, Yoshida *et al.* 2002, Schmidt *et al.* 2006), although no classical GC response element (GRE) has been identified in the known *GR* promoters. Interestingly, these cells are sensitive to GC-induced apoptosis. Concordantly, GC-resistant subclones of cell lines with this phenotype lost their ability to autoinduce *GR* after GC treatment (Kofler *et al.* 2003, Riml *et al.* 2004, Schmidt *et al.* 2006), and sensitivity for GC-induced apoptosis in GC-resistant Jurkat, and CCRF-CEM-sublines could be restored by *GR* overexpression (Helmberg *et al.* 1995, Geley *et al.* 1996, Schmidt *et al.* 2006). Because of these cell-specific apoptosis-inducing properties, GC are routinely used to treat lymphoblastic malignancies. Recent studies in cell lines suggest that distinct exons 1 may contribute more to GC sensitivity than others. It was shown that a high ratio of exon 1A3 to proximal located exons 1 increased the amount of GR-B protein, translated from the second start codon (Pedersen *et al.* 2004), which is more active in transactivation than the GR-A isoform (Yudt & Cidlowski 2001). In GC-sensitive CEM-C7 cells, basal exon 1A3 levels were high and more strongly induced by GC than proximal first exons (Breslin *et al.* 2001, Pedersen & Vedeckis 2003), further suggesting that promoter usage might account for sensitivity to GC-induced apoptosis. In contrast, a recent study of promoter usage in primary childhood ALL cells found no relation to GC sensitivity/resistance (Tissing *et al.* 2006).

In this study, we (i) searched for the presence of additional exons 1 in the human *GR* gene, (ii) determined the expression levels of known *GR* exons 1 in different human tissues, and (iii) addressed the question of whether usage of the distal *GR* promoters is a requirement for GC-induced cell death.

Materials and methods

Cells

The GC-sensitive CCRF-CEM-C7H2 cell line (Strasser-Wozak *et al.* 1995) and the two GC-resistant cell lines, CEM-C1 (Norman & Thompson 1977) and CEM-C7R5 (Hala *et al.* 1996), have been described previously. GC-sensitive PreB697 c-ALL cells (Findley *et al.* 1982), recently renamed 'EU-3' by H W Findley (personal communication), were obtained from DSMZ (Braunschweig, Germany). The two non-hematopoietic adherent cell lines ONK2-H2B-GFP, an U2OS human osteosarcoma derivative (Geley *et al.* 2001) and HeLa Ohio human cervix carcinoma cells (ECACC 84121901), were provided by Dr Stephan Geley (Innsbruck Medical University, Austria). All cell lines were tested for, and found to be free of, mycoplasma

infection and their authenticity was verified by DNA fingerprinting, as detailed previously (Parson *et al.* 2005). Suspension cells were maintained in RPMI 1640 medium (Cambrex Bio Science, Verviers, Belgium), and adherent cells in DMEM (Cambrex Bio Science). Media were supplemented with 10% FCS, 2 mM L-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin (all supplements from Invitrogen) at 37 °C and 5% CO₂. Human thymic tissue, obtained from thymi removed during pediatric cardiac surgery, was kindly provided by Dr Jan Wieggers (Innsbruck Medical University, Austria). Thymocytes were dissociated by injection of supplemented RPMI 1640 medium into the thymus tissue, which was then pressed through a sieve into culture medium. The thymocytes were maintained at a density of 3–7 × 10⁶ cells/ml.

Apoptosis determination

Apoptosis was determined by FACS analysis of propidium-iodide (PI)-treated permeabilized cells (Nicoletti *et al.* 1991). Briefly, 2–5 × 10⁵ cells were pelleted, resuspended in 700 µl Triton X-100/PI-staining solution (50 µg/ml propidium-iodide, 0.1% sodium citrate, 0.1% Triton X-100), maintained for a minimum of 6 h at 4 °C protected from light and analyzed with a FACScan cytometer (Becton Dickinson Biosciences, San Jose, CA, USA) using the CellQuest Pro software (Becton Dickinson Biosciences).

RNA

Total RNA from human tissues was obtained from Clontech or prepared from human cell lines and thymus tissue using TriReagent (MRC, Cincinnati, OH, USA), according to the manufacturer's protocol. The pellet obtained from ~2 × 10⁶ to ~2 × 10⁷ cells was resuspended in 1 ml TriReagent. For the thymus, 90 mg tissue was homogenized in a glass Teflon potter with 1 ml TriReagent. After mixing with 200 µl chloroform, RNA was precipitated from the aqueous supernatant by isopropanol.

Rapid amplification of cDNA ends (RACE) PCR

All primers are listed in Table 1. One microgram of CCRF-CEM-C7H2 total RNA or poly A⁺ human placenta control RNA, 1 µl of 10 µM linker oligonucleotide (SMART III; Clontech), 0.5 µl random hexamer primers (500 ng/µl), and nuclease-free water to 5 µl were incubated for 2 min at 72 °C and put on ice for 2 min. After addition of 2 µl 5 × first-strand buffer, 1 µl 20 mM dithiothreitol (DTT), 1 µl dNTP-Mix (each 10 mM), and 1 µl PowerScript reverse transcriptase (Clontech), the solution was incubated for 1 h at 42 °C.

Table 1 Primer sequences

	Primer	Sequence	localization	Paired primer(s)/probe
Application	SMART III	5'-AAGCAGTGGTATCAACGCAGAGTGGCCATTATGGCCGGG-3'	—	
	5'-PCR primer	5'-AAGCAGTGGTATCAACGCAGAGT-3'	—	
	hGR 2R511	5'-AGGAAAGGCTGATTTTGGCCCTGCTGT-3'	Exon 2	hGR 2R511, hGR 2RBA
Hybridization	hGR 2RBA	5'-CAGGAGTTAATGATTCTTTGGAGTCC-3'	Exon 2	5'-PCR primer
	HP1A1	5'-AGATCTTCTGATACCAAATCACTGGA-3'	Exon 1A1	5'-PCR primer
	HP1A2	5'-ATGAGTGGGGAAGGATAGAAACA-3'	Exon 1A2	
	HP1I	5'-AGGAAGCTGCCAGAACCATCTAC-3'	Exon 1I	
	HP1D	5'-CTTAACGCCGCCCCAGA-3'	Exon 1F	
	HP1J	5'-TGGACGCGGGCCCT-3'	Exon 1J	
Hybridization and qPCR ¹	hGR 2RBA	5'-CAGGAGTTAATGATTCTTTGGAGTCC-3'	Exon 2	hGR 1BF/hGR FAM 1B2
	hGR 2R	5'-TTCTTTGGAGTCCATCAGTGAATATC-3'	Exon 2	hGR 1CF/hGR FAM 1C2
	hGR 1CF	5'-GGGAAGTGCAGGACGGTG-3'	Exon 1C	hGR 2R/hGR FAM 1C2
	hGR 1BF	5'-TGCCGACGCGCA-3'	Exon 1B	hGR 2RBA/hGR FAM 1B2
	hGR 1A3F	5'-GCCTGGCTCCTTCTCTCAA-3'	Exon 1A3	1A3Rq/1A3Sq
	11Fq	5'-AAGGAAGGAGAGCAGTCAGGAA-3'	Exon 1I	1A3Rq/1A3Sq
qPCR ¹	1DFq	5'-AAATCATAATCTGTGCCGCACA-3'	Exon 1D	1A3Rq/1A3Sq
	1JFq	5'-AACTTGGACGCGGGCC-3'	Exon 1J	1A3Rq/1A3Sq
	1FFq2	5'-CTGGAGAAACTCGGTGGCC-3'	Exon 1F	1A3Rq/1A3Sq
	1A3Rq	5'-CATCACATCTCCCTCTCCTG-3'	Exon 2	hGR 1A3F, 1FFq2, 11Fq, 1JFq, 1DFq/1A3Sq
	hGR FAM 1C2	5' FAM-CGAGCGGCTCCTCTGCCAGAGT-TAMRA 3'	Exon 1C/2	hGR 1CF, hGR 2R
	hGR FAM 1B2	5' FAM-CCCGGGCCCAAATGATATTCAGT-TAMRA 3'	Exon 1B/2	hGR 1BF, hGR 2RBA
	1A3Sq	5' FAM-AGAAGAAAACCCAGCAGTGTGCTTGC-TAMRA 3'	Exon 2	hGR 1A3F, 1FFq2, 11Fq, 1JFq, 1DFq, 1A3Rq
	hGR1275F	5'-GAACCTTCCCTGGTCAACAGTT-3'	Exon 2	hGR1358R/hGR FAM
	hGR1358R	5'-GAGCTGGATGGAGGAGAGCTT-3'	Exon 3	hGR1275F/hGR FAM
	hGR FAM	5' FAM-TGGCTATTCAAGCCCCAGCATGAGA-TAMRA 3'	Exon 2/3	hGR 1275F, hGR1358R
	18S fwd	5'-CCATTGGAACGTCTGCCCTAT-3'	—	18S rev/18S probe
	18S rev	5'-TCACCGTGGTCACCATG-3'	—	18S fwd/18S probe
	18S probe	5' FAM-ACTTTTCATGGTAGTCGCCGTGCCT-Dabcyl 3'	—	18S fwd, 18S rev

qPCR¹: quantitative real-time reverse transcriptase (RT)-PCR.

First-strand cDNA corresponding to 40 ng total RNA or 20 ng control Poly A⁺ RNA (Clontech), was PCR amplified with 200 µM of each dNTP, 200 nM gene-specific reverse primer (hGR 2R511), 200 nM 5' PCR primer (Clontech), 2.5 or 3.5 mM MgCl₂, 5 µl 10× Core Buffer (Brilliant Blue Core Reagent Kit; Stratagene Europe, Amsterdam, The Netherlands), and 2.5 U Sure Start Taq Polymerase (Stratagene Europe) at the following cycling parameters: 10 min at 95 °C, 30 cycles of 15 s at 95 °C, 30 s at 61 or 65 °C, 1 min at 72 °C, and 1 cycle of 10 min at 72 °C. PCRs were run on a Bio-Rad iCycler or an MWG Hybaid Thermocycler and the products analyzed on 2% agarose gels. Reactions containing 2.5 and 3.5 mM MgCl₂ amplified with an annealing temperature of 61 and 65 °C showed a clear band of about 500 bp that was gel purified with QIAquick gel extraction kit (Qiagen) and reamplified with a nested gene-specific PCR primer (hGR 2RBA) and the 5' PCR primer according to the above PCR amplification protocol using 2.5 mM MgCl₂ and 60 °C annealing temperature. The resulting ~250 bp PCR product was cloned with the pGEM-T vector system (Promega). The experiment was repeated with CCRF-CEM-C7H2 cells treated for 8 h with 10⁻⁷ M dexamethasone and some technical modifications. For PCR amplification, the above protocol with 3.5 mM MgCl₂ and an annealing temperature of 68 °C was applied.

PCR products were cloned without further purification into pGEM-T vector. To eliminate clones with known hGR exons 1, replica copies of the resulting colonies were differentially hybridized with ³²P-labeled hGR exon 2 and hGR exons 1 oligodeoxynucleotides (ODNs). Clones corresponding to potentially novel exons 1 were grown, and the plasmids were purified with the Wizard Plus Minipreps DNA purification system (Promega) and sequenced (MWG, Ebersberg, Germany).

Quantitative real-time RT-PCR

First-strand cDNA was synthesized from 500 ng total RNA in 20 µl using random hexamer primers (Promega) and the Superscript II Reverse Transcriptase RNase H⁻ Kit (Invitrogen) according to the manufacturer's protocol.

PCR amplification was performed using the TaqMan assay (Applied Biosystems, Foster City, CA, USA). C_T values of the target gene were normalized to the corresponding 18S RNA C_T values. Mean normalized expression (MNE) was calculated by the method described by Muller *et al.* (2002), which is a modified comparative C_T-method (User Bulletin #2; Applied Biosystems) that accounts for amplification efficiencies.

To quantify hGR exons 1, the amplicon was positioned at the exon 1/exon 2 boundary, and for total GR at the exon 2/exon 3 boundary. Primers and TaqMan probes were designed with the Primer Express Software (Applied Biosystems). The TaqMan probes contained 6-carboxy-fluorescein (FAM) at the 5' end and 6-carboxy-*N,N,N',N'*-tetramethyl-rhodamine (TAMRA) at the 3' end; only the 18S RNA probe contained the quencher 4-(4'-dimethylaminophenylazo) benzoic acid (DABCYL) at the 3' end. Primers and probes were obtained from MWG (Ebersberg, Germany) or Microsynth (Balgach, Switzerland), and the sequences are listed in Table 1. The probes for measuring total hGR, exons 1C, and 1B straddled the exon/exon boundary, and all remaining hGR exons were analyzed using a probe in exon 2. First-strand cDNA was PCR amplified using the Brilliant Blue Core Reagent Kit (Stratagene Europe). Each 25 µl amplification reaction contained 2.5 µl cDNA (corresponding to approximately 20 ng RNA), 700 nM forward primer, 700 nM reverse primer, 250 nM TaqMan probe (100 nM TaqMan probe for 18S amplification), 2.5 µl 10× Core buffer, 2.5 mM MgCl₂, 0.2 mM of each dNTP, 0.6 nM reference dye and 1.25 U Sure Start Taq. Reactions were carried out in triplicate in 96-well plates with the ABI Prism 7700 Sequence Detection system (Applied Biosystems) in a two-step amplification protocol: 10 min at 95 °C, 30 cycles of 15 s at 95 °C, and 1 min at 60 °C. Data were analyzed with the Sequence Detection Software (Applied Biosystems).

Sequences and software

Human *GR* (NR3C1) promoter sequences (4552 bp distal, 5163 bp proximal) were extracted from the *Homo sapiens* chromosome 5 genomic contig NT_029289, gi 37550092 (last update 29-Aug-2006). The region complement (3943329.3948491) corresponds to the proximal *hGR* promoter (translation initiation codon ATG is at position 3943340), the region complement (3975640.3980191) corresponds to the distal *hGR* promoter.

X66367 (Strahle *et al.* 1992) contains the mouse *GR* (Nr3c1) proximal promoter sequence, the distal promoter sequence (4550 bp) was obtained from *Mus musculus* chromosome 18 genomic contig NT_039674, gi 94404691 (last update 28-Apr-2006), region complement (36643735.36648284). The *GR* translation initiation codon ATG is at position 36613206 on the complementary strand of this chromosome contig.

The corresponding rat sequences are AJ271870 (McCormick *et al.* 2000) for the proximal *GR* (Nr3c1) promoter and 4665 bp distal promoter sequence, extracted from *Rattus norvegicus* chromosome 18 genomic contig NW_047512, gi 62664370 (last update 22-Jun-2006), region complement (1160301.1164965).

The position of the rat *GR* translation initiation codon ATG is 1127464 on the complementary strand of this contig.

NNSPLICE 0.9 (Reese *et al.* 1997) is available at http://www.fruitfly.org/seq_tools/splice.html MatInspector is available at <http://www.genomatix.de/>

Results

RACE-PCR on CCRF-CEM-C7H2 RNA revealed two novel hGR exons 1, termed 1I and 1J

To identify the hGR exons 1 expressed in the GC-sensitive T-ALL cell line CCRF-CEM-C7H2, RACE-PCR was performed on total RNA from untreated and GC-treated (8 h with 10⁻⁷ M dexamethasone) cells as detailed in Materials and methods. RACE products were ligated into the pGEM T vector and screened for the presence of known alternative first exons with ³²P-labeled oligonucleotide probes specific for hGR exons 2 and 1 A1, A2, A3, B, and C (Breslin *et al.* 2001). Sequencing plasmid inserts containing exon 2, but none of the tested first exons, revealed two novel first exons, termed 1I and 1J, in addition to the recently published exon 1F (Turner & Muller 2005) and some scrambled sequences. Spatial relations between the novel and previously described exons 1 are shown in Fig. 1. Exon 1I maps to the distal promoter region downstream of exons 1A3, 1J to the proximal promoter region. As detailed in Table 2, exons 1A3 and 1C were predominant among the RACE-PCR products, followed in frequency by exons 1B, 1I, 1F, and 1J.

Basal expression levels of alternative first exons in tissues and cell lines: distal exon 1I is expressed predominantly in the T-cell lineage

To determine the tissue-dependent usage of the newly identified versus previously known exons, we performed quantitative real-time RT-PCR on total *GR* (exon 2/exon 3 boundary), all alternative first exons detected by our RACE-PCR and exon 1D. We assayed total RNA from a panel of tissues and a number of GC-sensitive and -resistant cell lines. Mean normalized expression levels (MNE) are shown in Fig. 2.

The newly identified proximal exon, 1J, was expressed at significantly higher levels in cultured cells than in tissues. Marked expression of exon 1D was found in testis. Compared with the other first exons, upstream exon 1I showed an expression pattern similar to that of exon 1A3. Among the tissues assayed, the highest levels were reached in the thymus. Comparing different CCRF CEM sublines, basal expression levels of the distally located exons 1I and 1A3 showed some correlation with GC sensitivity. While expression levels of proximally

Figure 1 Mapping of newly identified exons 1I and 1J to the *hGR*. Published exons 1C (Zong *et al.* 1990), 1A1, 1A2, 1A3, 1B (Breslin *et al.* 2001), 1D, 1E, 1F, and 1H (Turner & Muller 2005) are indicated. Filled arrows mark exons 1 that could be detected by 5' RACE in total RNA of CCRF-CEM-C7H2 cells. The sequence of the RACE product corresponding to exon 1J is (–3993) ACCGGGAACCTTGGACGCGGGCCCTGGCCGGGGTGAAGAA-GAG (–3951), the respective sequence of 1I is shown in Fig. 4.

located exons in resistant CEM-C1 and CEM-C7R5 cells were 28–70% of those in sensitive CEM-C7H2 cells, the corresponding values were 6–35% for the two distally located first exons. Specifically, the expression levels of exon 1I in CEM-C7H2 were about sevenfold higher than in CEM-C1 cells, and 1A3 levels differed by a factor of about 3 between the two cell lines. There was also a difference in 1I levels between the two GC-resistant cell lines, CEM-C1, which expresses functional GR at a low level, and CEM-C7R5, which is deficient in ligand binding (Fig. 2). Interestingly, very low basal expression levels of exons 1I and 1A3 were found in GC-sensitive PreB697 cells.

Glucocorticoid regulation of alternative first exons

As detailed in the introduction, there is growing evidence for correlation of GC sensitivity and GR autoupregulation after GC treatment. To further address this issue, we analyzed the CCRF-CEM T-ALL and the PreB697 B-ALL model for changes in expression of total GR and the seven exons 1A3, 1B,

1C, 1D, 1F, 1I, and 1J after treatment with 10^{-7} M dexamethasone using quantitative real-time RT-PCR with 18S RNA as reference. Total GR and all proximal alternative first exons were markedly induced by GC in the two sensitive cell lines, CEM-C7H2 and PreB697, but not at all or only slightly in GC-resistant CEM-C1 and CEM-C7R5 cells (Fig. 3). In contrast, we observed a marked difference in GC regulation at the distal promoters between the two sensitive cell lines. Distally located exons 1I and 1A3 were strongly induced in CEM-C7H2 cells, but negligibly in PreB697 cells (Fig. 3).

In silico splice site prediction in human, mouse, and rat GR promoter regions

Some of the previously known human exons have exact correlations in mouse and rat. To look for potential additional human splice sites and to determine whether exons 1I and 1J might also be used in mouse and rat, we analyzed the proximal and the distal GR promoter regions of man, mouse, and rat for potential splice sites

Table 2 Summary of the two RACE experiments

	Clones with hGR exon 1	1C ^{a,b}	1B ^{c,d}	1A3 ^d	1I	1F ^b	1J
CEM-C7H2 untreated							
Clones	67 (of 130)	13	5	41	4	3	1
In %	100	19.4	7.5	61.2	6	4.5	1.5
CEM-C7H2 8 h dex							
Clones	15 (of 446)	6	3	5	1	–	–
In %	100	40	20	33.3	6.7		
Exon length							
Longest RACE product		73 nt	79 nt	168 nt	111 nt	71 nt	43 nt
Published		120 nt ^a 306 nt ^b	117 nt ^c	981 nt ^d		62 nt ^b	

RACE products from untreated CEM-C7H2 cells were purified from the gel and reamplified with nested primers prior to cloning in pGEM-T vector. RACE products from GC-treated cells (8 h with 10^{-7} M dexamethasone, dex) were cloned without further purification or amplification.

^aZong *et al.* 1990.
^bTurner & Muller (2005).
^cNunez & Vedeckis (2002).
^dBreslin *et al.* (2001).

Figure 2 Mean normalized expression (MNE) values of total hGR and seven transcripts containing alternative hGR exons. Normalization refers to 18S RNA in all human cell lines and tissues, calculated with consideration of amplification efficiencies according to Muller *et al.* (2002). Expression levels as a percentage of CEM-C7H2 MNE are indicated at the bottom of the bars.

Figure 3 Glucocorticoid responsiveness of individual alternative first exons. Basal (filled bars) and induced (open bars) mean normalized expression (MNE) levels of total hGR and transcripts containing the indicated alternative first exons were determined by quantitative real-time RT-PCR in GC-sensitive CEM-C7H2 and PreB697, and GC-resistant CEM-C1 and CEM-C7R5 cells. Cells were induced with 10^{-7} M dexamethasone (Dex) for the indicated durations; all expression data were normalized to 18S RNA.

using NNSPLICE 0.9 (Reese *et al.* 1997) and aligned them using the Align X software of the Vector NTI suite (Invitrogen). Aligned splice sites predicted by the algorithm are summarized in Table 3. Purely sequence-based predictions for the human promoter included scores of 0.94, 0.96, and 1.0 for known exons 1A3, 1B, and 1C respectively, which were present with high frequency in our RACE clones. For the newly identified exons described here, the actual splice sites were predicted with a score of 0.77 for 1J and a perfect score of 1.0 for 1I. The splice site of the recently reported exon 1F (Turner & Muller 2005), which we also found in our RACE, was predicted with a score of 0.49. Another splice site, predicted with a score of 0.94, corresponds to that of the recently reported exon 1D (Turner & Muller 2005). A total of six additional splice sites (1P1–1P6, Table 3), five in the distal and one in the proximal promoter, were predicted in the human

sequence, and remain to be experimentally confirmed. Out of these sites, 1P3 in the distal and 1P6 in the proximal promoter had promising correlates in both mouse and rat.

Discussion

Our search for additional promoters/first exons of the human *GR* utilized RACE-PCR to identify three exons that could be verified by intron-spanning real-time PCR. One, exon 1F, was reported by Turner & Muller (2005) during the progress of our work, and herein we report two other, previously unknown, exons: 1J in the proximal and 1I in the distal promoter region. This increases the number of known alternative first exons to 11, similar to the present number (12) in the rat *GR* gene (McCormick *et al.* 2000, Zhang *et al.* 2004),

Table 3 Score of aligned splice sites in human, mouse, and rat *GR* promoter regions

Splice donor site <i>hGR</i> gene		Position relative to <i>hGR</i> ATG	Calculated score (NNSPLICE 0.9)		
			Human	Mouse	Rat
Exon (predicted exon)					
Distal GR promoter		−36851 to −32300			
Predicted h 1P1	TAAAACGATAGT	−36010	0.58	0.32	0.10
h 1A1	ACCTTAGAAGGT	−34462	0.53	0.37	0.07
h 1A2	CAGAAAGAGGGT	−34365	0.99	No spl. site	No spl. site
h 1A3/m 1A/r 1 ₁	GAGAAGGAAGGT	−33693	0.94	1.00	1.00
Predicted h 1P2	GCTGGGTAAAGT	−33654	0.97	<0.01	0.06
Predicted h 1P3	TCACTCAGAGGT	−33614	0.67	0.76	0.81
Predicted h 1P4	TATTCAGCAAGT	−33470	0.88	0.79	<0.01
h 1I	CATGAGAGAGGT	−32880	1.00	No spl. site	0.08
Predicted h 1P5	TACAGATCAGGT	−32755	0.96	0.03	0.03
Proximal GR promoter		−5151 to +12			
h 1D	GCCGCACAAGGT	−4311	0.94	0.72	0.21
r 1 ₄		−4247	No spl. site	0.11	0.44
h 1J	GGAAGAAGAGGT	−3951	0.77	0.55	0.55
h 1E/m 1D/r 1 ₅	TCCTTCGAGTGT	−3780	<0.01	0.32	<0.01
h 1B/m 1B/r 1 ₆	CGGGCCCAAAGT	−3537	0.96	0.38	0.38
h 1F/r 1 ₇	GAGAGACCAGGT	−3147	0.49	0.15	0.15
r 1 ₈	TCACCAACAGGT	−2946	0.08	0.14	0.14
r 1 ₉	GACGGATTCTGT	−2685	<0.01	0.10	0.10
h 1C/m 1C/r 1 ₁₀	TCTGCCAGAGGT	−2372	1.00	0.97	0.97
h 1H/m 1E/r 1 ₁₁	AACTCAACAGGT	−1763	0.53	0.53	0.53
Predicted h 1P6	AAATTTGGAGGT	−575	0.93	0.96	0.96

Human (h), mouse (m), and rat (r) proximal and distal GR promoter sequences were analyzed with NNSPLICE 0.9. (Reese *et al.* 1997) and aligned using ALIGN X (Vector NTI Suite 6, Invitrogen). Aligned *hGR* splice sites that correspond to a known exon 1 of the three species or have a score >0.4 in the human gene (predicted exons) are shown.

although thus far no direct mouse or rat correlates of exons 1I or 1J have been reported.

Is there a possibility that mouse or rat use these exons? Comparing the splice sites of the two newly identified human exons with predicted splice sites in mouse and rat, the splice donor site of proximal exon 1J seemed to be conserved, with scores of 0.55 in both mouse and rat. Further arguing in favor of a functional role of exon 1J in rodents is the fact that the region between exon 1J and the upstream exon 1D is conserved, showing 68% identity between man, mice, and rat.

For distal exon 1I, the software predicted a splice site with a weak score of 0.08 in rat, but no splice site in the mouse. Alignment of the promoter/exon 1I (P/EII) region, defined as the 702 bp sequence between the exon 1A3 splice site and the start of exon 1I, in man, mouse, and rat (Fig. 4) revealed low overall homology (about 50%) with one exception. The first 250 bp of this region, ~75% conserved between man and rodents (disregarding a microsatellite in the rat), contain conserved transcription factor-binding motifs like E4BP4 (Cowell *et al.* 1992), GATA (Ko & Engel 1993), IRF3 (Lin *et al.* 2000), ISRE (Levy *et al.* 1988), OCT1 (Groenen *et al.* 1992), Pit1 (Mangalam *et al.* 1989), and COMP1 (Funk & Wright 1992). While it seems unlikely that mouse or rat uses an exact correlate of 1I, a regulatory role of the P/EII region in these

species is suggested by these conserved elements between 1A3 and 1I.

The distal promoter region has thus far been defined by the three forms of exon 1A, about 34 kb upstream of the common exon 2 (Breslin *et al.* 2001). Multiple gene regulatory elements were analyzed in terms of 12 footprints within and upstream of exon 1A3 (Geng & Vedeckis 2004). Our results indicate that the distal promoter region has to be markedly extended downstream to include alternative first exon 1I. In addition to the conserved-binding elements mentioned above, this region contains a predicted GRE with the sequence GGTAAnnnAGTTCT, which differs in only two positions from the classical consensus GRE, GGTA-CAnnnTGTTCT (Beato *et al.* 1989). If functional, this GRE may be important in conjunction with the conserved E4BP4/NF-IL3A-binding site. As shown by Medh *et al.* (2003), E4BP4, which is repressive in most cell lines (Cowell *et al.* 1992, Wallace *et al.* 1997), but is transactivating in lymphoid cell lines (Zhang *et al.* 1995, Cowell 2002), is strongly induced by GC in sensitive CEM-C7 sublines, but not in resistant CEM-C1 sublines.

In a series of studies over the last few years, Vedeckis *et al.* have developed a model to explain the auto-inductive GR feedback loop in cells prone to glucocorticoid apoptosis. The model consists of three elements.

The first element is a marked difference in glucocorticoid autoregulation of GR transcripts depending on the cellular background: transcripts containing all alternative first exons are induced by GC in CEM-C7 cells, but downregulated in IM-9 B-lymphoma cells (Breslin *et al.* 2001). This effect is most pronounced for distal exon 1A3 (Pedersen & Vedeckis 2003).

Secondly, glucocorticoid regulation of 1A3 depends on a DNA element within 1A3 that can be bound by either c-Myb or c-Ets (PU.1). This site is adjacent to a non-consensus glucocorticoid-response element. In cells where c-Myb dominates over PU.1, 1A3 is auto-induced by GC, whereas in cells expressing mainly PU.1, 1A3 is autorepressed (Geng & Vedeckis 2004, 2005).

The third element, Pedersen *et al.* (2004) hypothesizes, may be a shift to a more active GR isoform caused by an upstream open reading frame in 1A3. The hGR exon 1A3 transcript contains an upstream open reading frame (uORF) with a termination codon that overlaps the start codon of the GR-A isoform. In human estrogen receptor α , it was shown that uORFs that terminate close to the initiation codon of the main ORF have an inhibitory effect on translation (Kos *et al.* 2002). Analogously, translation of the hGR 1A3 uORF might impair translation initiation at the first hGR AUG and promote translation initiation at the second AUG, leading to a higher ratio of GR-B:GR-A isoforms. Since the GR-B isoform is transcriptionally more active (Yudt & Cidlowski 2001), transcripts originating from 1A3 could enhance the feedback loop by this mechanism.

Taken together, this model attributes the induction of apoptosis by GC mainly to the distal GR promoter, specifically to exon 1A3, although its validity in childhood ALL has recently been questioned (Tissing *et al.* 2006).

As far as CEM-C7 cells are concerned, our results are generally compatible with this model. We find strong autoinduction of 1I-containing GR transcripts mirroring 1A3-containing transcripts. In addition, a similar uORF exists in 1I-containing transcripts as in 1A3-containing transcripts. This open reading frame starts at nt 49 of exon 1I, stops at nt 45 of hGR exon 2, and codes for a peptide of 35 AA. The ORF overlaps the first hGR AUG (nt 14–16 of hGR exon 2) and terminates before the second hGR AUG, which is positioned at nt 92–94 of hGR exon 2. Therefore, point 3 of the model mentioned above might also pertain to exon 1I.

However, our results in PreB697 cells raise a note of caution. Although PreB697 cells undergo apoptosis in response to GC, autoinduction of either exon from the distal promoter does not lead to relevant transcript levels. Instead, PreB697 cells induce all proximal exons to considerable levels, suggesting that induction of distal exon(s) may be the mechanism that induces

apoptosis in T-cells, but is not a universal mechanism of inducing apoptosis by GC.

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