Articles

Human Glucocorticoid Receptor α Transcript Splice Variants with Exon 2 Deletions: Evidence for Tissue- and Cell Type-Specific Functions†

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ABSTRACT: Alternative splicing of exon 9 in human glucocorticoid receptor (hGR) transcripts yields two native hGR transcripts and proteins, hGRα and hGR*β*. We have now identified four novel hGRα transcripts that have various deletions of exon 2 sequences. Among these hGRα splice variants, three of them, 1A1/E2dist hGRα, 1A2/E2prox hGRα, and 1A3/E3 hGRα, arise from the hGR 1A promoter, while 1B/E3 hGRα comes from the hGR 1B promoter. When fused to Flag and enhanced green fluorescent protein (EGFP) tags at the carboxy terminus, all transcript variants can be correctly translated in vitro and in vivo. The Flag-tagged hGRα protein variants can functionally bind to a glucocorticoid response element (GRE) and can mediate hormonal stimulation of a pGRE-luciferase reporter gene. Compared to the "classical", native hGRα, these four variants exhibit a cell type-specific activation of a reporter gene, and this is influenced by the hGRα 3′ untranslated region in the hGR transcript. When equal amounts of the cDNAs for these GRα variant proteins are transfected into cells, they can exhibit lower or higher transcriptional activation compared to the classical GR. Furthermore, the EGFP-tagged proteins are nuclear localized, even in the absence of hormone. Using quantitative reverse transcription PCR, we found that these transcripts exist at a low level in CEM-C7 cells and IM-9 cells, although the concentrations of the 1A3/E3 hGRα and 1B/E3 hGRα transcripts are higher than for hGR*β* transcripts, while 1A1/E2dist hGRα and 1A2/E2prox hGRα transcript levels are comparable to the 1A1 hGRα and 1A2 hGRα (without the exon 2 deletions) transcript levels, respectively. Because these novel hGR, N-terminal deleted, protein variants have altered biological activity, their expression could potentially affect the hormone sensitivity or resistance in leukemia and be useful in diagnosing hormone-sensitive or -resistant disease.

Glucocorticoids (GCs) play important roles in regulating many biological processes including hematopoiesis, immune system responses, and metabolism. Importantly, GCs are routine chemotherapeutic agents used for various hematologic malignancies that include multiple myeloma, non-Hodgkin's lymphoma, acute lymphoblastic leukemia (ALL), and chronic lymphoblastic leukemia, because of their cytolytic actions in lymphoid and leukemic cells, especially in childhood acute lymphoblastic leukemias (*1*−*3*). In these cases, glucocorticoid hormones induce the apoptosis and lysis of lymphoblast cells, the mechanisms of which are largely unknown (*4*).

Apoptosis of lymphoid malignancies induced by GCs is mediated by the glucocorticoid receptor (GR) protein (*5*), a ligand-induced transcription factor of the nuclear receptor family of transcription factors (*6*). After binding the hormone, the GR dimerizes, translocates to the nucleus, and functions as a transcription factor, e.g., by binding to glucocorticoid response element (GRE) sequences. The GR protein contains three domains: (1) an N-terminal domain, which is coded by exon 2 and contains an activation function, AF-1, segment involved in protein−protein interaction with various cofactors; (2) a DNA-binding domain (DBD), which is coded by exons 3 and 4 and is necessary for DNA binding and homodimerization; and (3) a C-terminal ligand-binding domain (LBD), which specifically binds glucocorticoid hormones (*6*) and contains a second activation function, AF-2. Two native human GR proteins have been described (hGRα, 777 amino acids, and hGR*β*, 746 amino acids), and their molecular functions have been intensively investigated in vitro and in vivo (*6*−*8*). hGRα is the active form that can functionally bind hormone and DNA, while hGR*β* cannot bind hormone, due to a defective LBD. Because hGR*β* has an intact DBD identical to hGRα, its potential dominant inhibitory effects on hGRα were suggested via competitive binding to the GRE (*8*). Some studies have identified other hGR variants deriving from mutated and deleted forms of hGR transcripts (*9*−*15*). These variants have only been

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observed in cultured cancer cell lines or cancer cells obtained from patients, raising the possibility that these transcripts occur predominantly in cancer cells. However, it is not yet completely clear if these GR variants may also be important in normal cells or in other diseased states and if they modulate the activity of the wild-type GRα protein in vivo.

hGR transcripts are coded by nine exons in the hGR gene (*16*). Exon 1 codes for a 5′ untranslated region, and the actual sequence is determined by alternative promoter usage (hGR promoter 1A, 1B, or 1C) and/or alternative splice donor sites for exon 1A (*17*, *18*). Exons 2−9 code for the hGR protein with an ATG translation start site located in exon 2 and two different exon 9 forms for the generation of either GRα- or GR*β*-encoding transcripts. At least five exon 1-containing mRNA transcripts encode the hGRα protein (1A1, 1A2, 1A3, 1B, and 1C) (*17*, *18*). Thus, including alternative splicing of exons 8 and 9α or 9*β*, there are at least 10 potential mature mRNAs for the hGR. hGR transcripts have very long 3′ untranslated regions, which are important in the posttranscriptional regulation of hGR gene expression (*19*). Although a recent paper shows that splicing factor SRp30c is required for the alternative splicing in exons 8−9 to form hGR*β* (*20*), the mechanisms involved in splicing of the hGR transcripts are largely unknown.

Here we describe four novel alternatively spliced hGRα transcripts, 1A1/E2dist hGRα, 1A2/E2prox hGRα, 1A3/E3 hGRα, and 1B/E3 hGRα, in T-cell ALL (CEM-C7) and pre-B cell leukemia (IM-9) cell lines. One of these transcripts (1A2/E2prox hGRα) is also expressed in normal human cerebellum. All of these transcripts have the potential to produce GR-related proteins different from the "classical" GRα protein. The 1A3/E3 hGRα and 1B/E3 hGRα transcripts code for an N-terminal truncated GR protein that lacks all of the exon 2-encoded sequence, including activation function 1 (AF-1), while the 1A1/E2dist hGRα and 1A2/E2prox hGRα transcripts code for GRα proteins with partial truncation of the exon 2-encoded N-terminus plus the addition of additional novel amino acid sequences encoded by exon 1A. Transient transfection experiments in various cell types (Jurkat, COS-1, and E8.2) suggested possible cell type-specific functions for the hGRα proteins coded by these alternatively spliced mRNA transcripts, as well as a different cellular localization from that of the classical hGRα in the absence of hormone. Quantitative reverse transcription PCR analysis revealed low copy numbers of these transcripts in CEM-C7 and IM-9 cell lines, although one of the forms was more abundant than hGR*β* transcripts. Further, 1A3/E3 hGRα and 1B/E3 hGRα transcript levels were upregulated by steroid in hormone-sensitive CEM-C7 cells. These observations suggest that these newly identified hGRα transcript variants may have physiologically relevant roles that may be manifested in a cell type-specific manner. Whether the variant protein products modulate the responsiveness of normal cells, or of hematological malignancies, to corticosteroids remains to be determined.

MATERIALS AND METHODS

Cell Cultures. Human CEM-C7, T-cell acute lymphoblastic leukemia cells (from Dr. E. Brad Thompson, University of Texas Medical Branch, Galveston, TX) were grown in RPMI 1640 with 10% dialyzed fetal bovine serum (D-FBS; Life Technologies, Gaithersburg, MD). Human Jurkat, T-cell acute lymphoblastic leukemia cells (ATCC, Manassas, MD) were maintained in RPMI 1640 supplemented with 10% fetal bovine serum (FBS; Life Technologies). COS-1 cells were cultured in DMEM (low glucose) containing 10% FBS. Mouse fibroblast E8.2 cells were grown in DMEM (high glucose) with 10% bovine calf serum (BCS; Life Technologies). All of the cells were grown at 37 °C in a humidified incubator under 5% CO_2.

RNA Purification and Reverse Transcription Polymerase Chain Reaction (RT-PCR). Total cellular RNA was isolated from cultured cells using Tri-Reagent following the manufacturer's protocol (Molecular Research Center, Inc., Cincinnati, OH). One microgram of total RNA was reverse-transcribed using the Advantage RT for PCR kit (Clontech, Inc., Palo Alto, CA) and an oligo(dT) (20) primer. Resultant cDNAs were amplified using the Advantage cDNA PCR kit according to the manufacturer's instructions. The PCR reaction products were resolved on 1.2% agarose gels or 5% PAGE. The primer sets hGR 1Ap/e, +126 bp (described in ref *17*)/exon 9α specific reverse primer (5′-GCCAAGTCT-TGGCCCTCTAT-3) and hGR 1Ap/e, +126 bp/hGR DBD Rev (5′-GAATCTAGATCAGAGCACACCAGGCAG-3′) were used to amplify exon 1A-containing transcripts. For exon 1B-containing transcripts, the primer set 1BCAPXhoI (5′-TGCTCTCGAGCTTCTCTCCCAGTGCGAGAGCGCG-3′) and DBD Rev was used for PCR, and the primer set hGR1CCAPXhoI (5′-TGGCTCGAGGGAGACTTTCTT-AAATAGGGGC-3′) and DBD Rev was used to amplify hGR 1C transcripts.

Expression Vectors and Constructs. pCMVhGR was kindly provided by Dr. John A. Cidlowski, NIEHS, Research Triangle Park, NC. pEGFP-N1 was from Clontech (Palo Alto, CA) and pcDNA3.1+ was from Invitrogen (Carlsbad, CA). A synthetic 1× Flag coding sequence (5′-GATCCA-GATTACAAGGACGACGATGACAAGTGAGC-3′) and its complimentary oligo (5′-GGCCGCTCACTTGTCATCGT-CGTCCTTGTAATCTG-3′) were inserted at the *Bam*HI/*Not*I site of pcDNA3.1+ to generate the pcDNA3.1-Flag vector construct. A primer set for 1A-containing transcripts, 5′-GCCGGATCCAGGTTATGTAAGGGTTTGCTTTCACC-3′ (forward) and 5′-CTTGTGAGACTCCTGTAGTG-3′ (reverse, hGR cDNA 1660 AS), was used to generate the PCR amplicons 1A3/E2, 1A1/E2dist, 1A2/E2prox, and 1A3/E3. These amplicons were cloned into pCRII, digested with *Bam*HI and *Cla*I, and ligated into *Bgl*II/*Cla*I-digested pCMV hGRα to generate pCMV1A1/E2dist hGRα, pCMV1A2/E2prox hGRα, pCMV1A3/E3 hGRα, and pCMV1A3/E2 hGRα.

To generate the enhanced green fluorescent protein (EGFP) fusion expression construct p1A1/E2dist hGRα−EGFP, the 1A1/E2dist hGRα full-length cDNA sequence was amplified [forward primer, 1ACAP *Xho*I 5′-TTGTCTCGAGAGGT-TATGTAAGGGTTTGCTTTCA-3′ (*Xho*I), and reverse primer, hGR full-length rev 5′-AGGCGG***ATC***CTTTTGAT-GAAACAGAAGTTTTTTG-3′ (*Bam*HI)] with the introduction of a mutation of an in-frame cDNA stop codon (TGA to GAT; underlined, bold, and italic in the primer sequence). This was digested by *Xho*I and *Bam*HI and inserted between the *Xho*I/*Bam*HI site of pEGFP-N1. Other EGFP fusion expression constructs, p1A2/E2prox hGRα−EGFP, p1A3/E3 hGRα−EGFP, p1B/E3 hGRα−EGFP, p1C/E2hGRα−

EGFP(WT), and p1A3/E2 hGRα−EGFP(WT), were constructed by replacing the *Xho*I/*Cla*I region of p1A1/E2dist hGRα−EGFP with *Xho*I/*Cla*I-digested PCR products amplified using primer sets 1ACAPXhol/hGR cDNA1660 AS (for the 1A2/E2prox, 1A3/E3, and 1A3/E2 constructs), 1BCAPXhoI/hGR cDNA1660 AS (for the 1B/E3 construct), and hGR1CCAPXhoI/hGR cDNA1660 AS [for the 1C/E2 (WT) construct], respectively. The cloned cDNAs were also transferred from pEGFP fusion constructs (*Nhe*I/*Bam*HI fragments) into pcDNA3.1-Flag (*Nhe*I/*Bam*HI) to yield C-terminal Flag-tagged fusion protein products. All constructs were sequenced to ensure the correct insertion.

Transient Transfection and Luciferase Reporter Gene Analysis. Cells were transfected using Superfect (Qiagen, Valencia, CA). A GR transactivatable luciferase reporter expression construct, pGRE (Clontech), was cotransfected with the alternatively spliced hGR cDNA variant expression constructs and/or pCMVhGRα. A β-galactosidase expression construct (pCMV-β-galactosidase) was included for transfection efficiency normalization. Ethanol or dexamethasone (1 μM) was added 24 h after transfection, and cells were collected for analysis after another 24 h of incubation. Cell lysates were assayed for luciferase and β-galactosidase activity on an Ascent luminometer (Lab System, Franklin, MA).

Western Blot Analysis. For protein analysis, cells were collected and lysed with 1× Laemmli SDS loading buffer with a protease inhibitor cocktail (catalog no. P 8340; Sigma, St. Louis, MO). Proteins were resolved on SDS−PAGE (10%) or Ready-gel Tris-HCl gels (4%−20%; Bio-Rad, Hercules, CA) and transferred to Immobilon-nitrocellulose (NC) membranes (Millipore, Bedford, MA). The membranes were blocked and blotted using a standard ECL western blotting method (Amersham, Piscataway, NJ). Rabbit polyclonal anti-hGR antibodies were obtained from Santa Cruz Biotechnologies (Santa Cruz, CA), anti-Flag M2 mouse monoclonal antibodies were from Sigma, and rabbit polyclonal anti-Flag antibodies were purchased from Affinity BioReagents (Golden, CO).

In Vitro Translation and Gel Shift Assays. Using the TNT T7 transcription−translation kit (Promega, Madison, WI), hGRα variants that were inserted in the pcDNA3.1-Flag vector were translated according to the manufacturer's instructions for western blot and gel shift banding assays. For gel shift analysis, a ^{32}P end-labeled, consensus GRE (glucocorticoid response element: 5′-AGAGGATCTGTACAGGATGTTCTAGAT-3′) was used, and anti-Flag antibodies (Affinity BioReagents) were used for the supershift assays as described previously (*17*). Complexes were resolved on 4% PAGE, and the gels were dried for autoradiography.

EGFP Fusion Protein Expression and Detection. Mouse E8.2 cells were transiently transfected with EGFP fusion hGR expression constructs using the Superfect transfection reagent (Qiagen). After 24 h of incubation, cells were observed using a fluorescent microscope at an emission light wavelength of 488 nm for green fluorescence.

Quantification of Transcripts. Total RNA was isolated from cells by guanidinium thiocyanate−phenol−bromochloropropane extraction using TriReagent according to the guidelines of the manufacturer. Quantification of GR transcripts in samples of total RNA was done by real-time quantitative RT-PCR (QRT-PCR) with Taqman probes (Applied Biosystems, Foster City, CA) containing 6-carboxyfluorescein at the 5′ end and the quencher 6-carboxy-*N*,*N*,*N*′,*N*′-tetramethylrhodamine at the 3′ end, as described previously (*21*). The sequences of the primers and Taqman probes are listed in Supporting Information (Table S1). RNA standards were generated as follows. DNA fragments encompassing the QRT-PCR amplicons were generated by PCR using primers listed in Table S1. DNA fragments were cloned into the pCRII-TOPO vector using a TOPO TA cloning kit from Invitrogen (Carlsbad, CA). RNA was generated by in vitro transcription of *Hin*dIII- or *Bam*HI-digested plasmid DNA using the MEGAscript T7 kit from Ambion Inc. (Austin, TX), followed by gel purification as previously described (*21*). RNA standards were quantified by fluorometry using the RiboGreen RNA quantitation reagent and kit from Molecular Probes (Eugene, OR) as previously described (*21*). For the quantification of GR transcripts, the samples were diluted to ca. 40 ng of total RNA/μL, and RNA standards were diluted in 40 ng of yeast RNA/μL. GR transcript concentrations were normalized relative to total RNA. Total RNA was determined by measuring 18S rRNA by real-time QRT-PCR relative to known quantities of Taqman control total human RNA purchased from Applied Biosystems. Pre-developed Taqman assay reagents (PDAR) from Applied Biosystems for measuring 18S rRNA were used.

Table 1: Concentration of GR Transcripts in Lymphoblast Cells[a]

	transcript concentration (fmol/g of RNA)			
splice	CEM-C7/ EtOH	CEM-C7/ DEX	IM-9/ EtOH	IM-9/ DEX
exon 1A1/E2dist	<0.25	<0.25	<0.25	<0.25
exon 1A2/E2prox	<2.5	<2.5	<2.5	<2.5
exon 1A3/E3	0.59	4.6	<0.25	<0.25
exon 1B/E3	25	67	7.3	7.3
exon 8/exon 9α	4.6×10^3	2.0×10^4	6.6×10^3	3.2×10^3

[a] Transcript concentrations were determined in CEM-C7 and IM-9 cells treated for 24 h with 1 μM DEX or the ethanol vehicle. The values are the averages of four samples.

RESULTS

Identification of New, Alternatively Spliced hGR Transcripts. RT-PCR amplification of cDNA from DEX-treated, T-cell ALL CEM-C7 cells using hGR 1A promoter-specific primer/hGR E3 AS primer sets revealed several PCR amplicons smaller than the expected 1A/E3 transcripts (Figure 1, arrows b, c, and d). DNA sequencing showed that these smaller amplicons were identical to the normal hGR transcript cDNA sequence except that the exon 2 coding sequence was partially (Figure 2A, 1A1/E2dist, 1A2/E2prox) or completely (Figure 2A, 1A3/E3) deleted. Subsequently, we identified a 1B/E3 variant in CEM-C7 cell samples, in which exon 1B is directly spliced to exon 3 (Figure 1, arrow e, 1B/E3; see Figure 2A). Using the hGRα-specific exon 9 primer, hGRα AS, we also amplified the full-length, specific hGRα variants corresponding to the shorter amplicons b, c, d, and e shown above (Figure 1), and these were confirmed by sequencing. Some of these variants also exist in IM-9 B-cells (Figure 1, panel B, band labeled b, 1A1/E2dist). This observation suggested that, besides native, classical hGRα transcripts that have been described before, there are also other types of hGRα transcript variants. As these variant

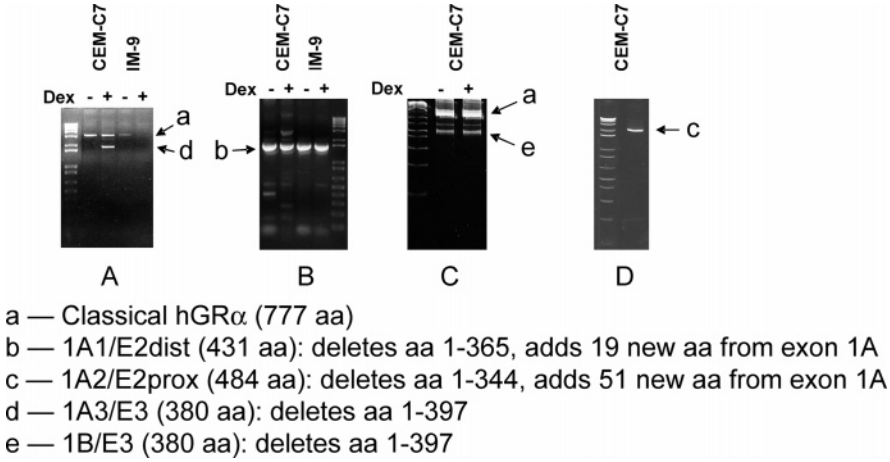

FIGURE 1: RT-PCR amplification of novel, alternatively spliced hGR mRNA transcripts in leukemia cell lines. EtOH- and DEX-treated CEM-C7 and/or IM-9 cell cDNA samples (as indicated above the gels) were amplified using (A) an exon 1A3-specific hGR 1A544 sense upstream primer and an hGR exon 9α downstream primer, (B) an upstream 1A FP6 sense primer and an hGR exon 9α downstream primer, (C) an exon 1B CAP upstream primer and an hGR E3 antisense downstream primer, and (D) an exon 1A2/E2prox-specific upstream primer and an hGR E9α antisense downstream primer. The lower case letters in each panel refer to the alternatively spliced transcripts that are defined below the gels.

transcripts would encode proteins without the AF-1 function (Figure 2A), they might modulate the glucocorticoid responsiveness of the cell. Therefore, cDNAs for these newly identified hGRα transcript variants were cloned and investigated to determine if they could produce protein products and if these novel proteins had biological activity and specific functions.

The Variant hGR Transcripts Use Functional Alternative Translation Start Codons Located in Exon 1A or Exon 3. Because the reported protein translation start codon exists in exon 2 of classical hGR transcripts, the deletion of exon 2 sequences could have caused the defective initiation of translation or the termination of hGRα protein translation by frame-shift mutation. However, computer analysis of the full-length sequence of these alternatively spliced, partial exon 2 deletion variants (1A2/E2prox and 1A1/E2dist) revealed a unique upstream (exon 1A +154) initiator ATG codon in exon 1A (Figure 2). Thus, this alternative splicing could give rise to an N-terminal partially deleted hGRα protein with an additional 19 (1A1/E2dist) or 51 (1A2/E2prox) amino acids coded for by exon 1A and added in-frame to the remainder of hGRα (Figure 2B). The predicted molecular masses of the GRα proteins derived from the 1A1/E2dist and 1A2/E2prox transcripts are approximately 47 and 53 kDa, respectively. For the 1A3/E3 and 1B/E3 transcripts, an ATG is located immediately downstream of the exon 1/exon3 splice, and it could potentially serve as a translation start site to give a completely exon 2-deleted, N-terminal truncated, hGRα protein with a molecular mass of about 42 kDa (Figure 2B).

To determine whether these transcripts can be functionally recognized by the cellular translational machinery and be translated into variant hGR proteins, we fused a Flag tag or EGFP tag to the C-terminus of the predicted truncated protein, using fusion protein expression constructs driven by the CMV promoter (for eukaryotic cells) or the T7 promoter (for bacterial expression and in vitro translation). The CMV-driven constructs were transfected into the mouse E8.2 cell line that does not express any GR mRNA or protein (*22*). Western blots using the EGFP-tagged hGRα protein variants and an EGFP sequence-specific antibody (Figure 3A) demonstrated that the predicted alternative initiator ATGs could be used and that these exon 2-deleted hGR transcripts were correctly translated into the predicted C-terminal EGFP-tagged protein. Using an in vitro rabbit reticulocyte lysate transcription/translation system and T7 promoter-driven constructs, these hGRα variant proteins were also successfully translated into Flag-tagged proteins (Figure 3B). These observations suggest that alternative usage of the two predicted new translation ATG start codons in these transcripts is possible and that the 1A1/E2dist, 1A2/E2prox, 1A3/E3, and 1B/E3 hGRα transcripts that we identified can be translated in vitro and in vivo into hGRα protein variants that diverge at the N-terminus from the classical hGRα protein.

The hGRα Protein Variants Can Functionally Bind to a Consensus GRE. We tested the DNA-binding activity of in vitro translated, Flag-tagged, 1A3/E2 GRα and 1C/E2 hGRα (both classical hGRα; control), 1A3/E3 hGRα, 1A2/E2prox hGRα, 1A1/E2dist hGRα, and 1B/E3 hGRα proteins. The gel shift analysis clearly showed the binding of these Flag-tagged hGRα protein variants to a consensus GRE in vitro (Figure 4, S), and the anti-Flag antibody can supershift the protein−GRE complexes into slower migrating complexes (Figure 4, SS). Shifted bands were also seen in the control samples (no plasmid, pcDNA3.1-Flag), and this most likely represents endogenous rabbit GR protein in the rabbit reticulocyte lysate used for the coupled transcription−translation reaction. To support this interpretation, the anti-Flag antibody could not supershift this band (Figure 4), as the endogenous rabbit GR is not Flag-tagged. Thus, like the classical hGRα protein, these hGRα variants are able to functionally bind to a glucocorticoid response element (GRE) that mediates the response of promoters to hormonal regulation. This was not surprising, because in all of these variants the DNA-binding domain, the dimerization domain, and the C-terminal ligand-binding domain are intact, and these are critical for the protein−DNA interaction at the GRE sequence (*23*, *24*).

Transcriptional Activity of hGR Protein Variants on a GRE-Containing Promoter. We next determined if the hGRα variant proteins have biological activity. A luci-

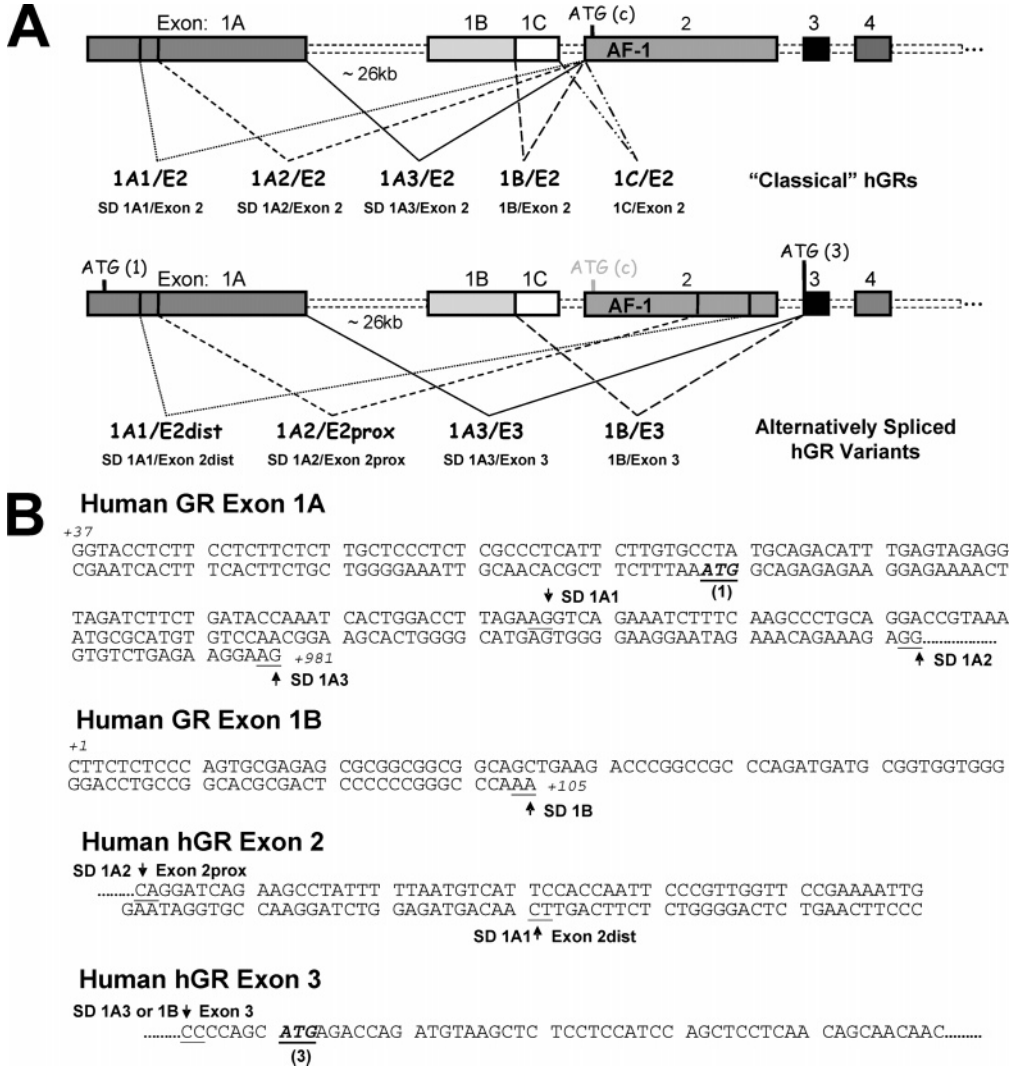

FIGURE 2: Alternative splicing of hGR mRNA transcripts. (A) Alternative splicing of hGR gene transcripts to generate hGR transcripts. The top panel shows the splicing of different, untranslated exon 1 sequences to a common splice acceptor site at the beginning of exon 2. Because there is an in-frame stop codon upstream of the ATG initiator methionine in exon 2, none of the exon 1 sequences contribute peptide sequence to the classical GR. The lower panel shows alternatively spliced transcripts described here. Because the in-frame stop codon is deleted in the splicing out of most of exon 2 for the 1A1/E2dist and 1A2/E2prox transcripts, the GR proteins derived from these two transcripts have novel, additional amino acids derived from the translation of exon 1A in the amino terminus of the GR variants. (B) Nucleotide sequences of exon 1A and 1B spliced to exon 2 and exon 3 to generate 1A1/E2dist, 1A2/E2prox, 1A3/E3, and 1B/E3 hGR splicing variants. Abbreviations: AF-1, activation function 1 of the glucocorticoid receptor; ATG (c), the initiator methionine used to code for the classical, wild-type GR; ATG (1), cryptic initiator methionine coded by exon 1A; ATG (3), cryptic initiator methionine coded by exon 3.

ferase reporter expression construct, pGRE, in which the luciferase gene is driven by a GRE and a TATA-box-like promoter (GRE-TAL), was used in transient cotransfection experiments with hGRα protein expression constructs to determine the transactivation and/or transrepression activities of the hGRα variant proteins compared to the classical hGRα. Three cell lines were used, Jurkat (a T-cell ALL line), COS-1 (African green monkey kidney cells), and E8.2 (mouse fibroblasts).

Initially, Flag-tagged hGRα variant expression plasmids were used. Equivalent amounts of cDNA were transfected for all constructs. As expected, the classical hGRα protein significantly mediated the hormonal responsiveness. In all three cell lines, no DEX response was obtained without transfection of hGRα expression constructs (Figure 5A, compare Vector with 1C/E2 hGRα-Flag samples). In Jurkat and COS-1 cells (which contain endogenous nonfunctional GR protein), transfection with 1A1/E2dist hGRα and 1B/E3 hGRα cDNAs resulted in activity similar to that of classical hGRα in transactivating the GRE-TAL promoter, while less potent activation, compared to classical 1C/E2 hGRα transfection, was obtained with the 1A2/E2prox hGRα and 1A3/E3 hGRα cDNAs (Figure 5A). However, in E8.2 cells (which lack any detectable GR protein), transfections with the cDNAs of all of the hGR protein variants caused less transactivation than with the classical 1C/E2 hGRα. Of these, 1A1/E2dist hGRα displayed the lowest activity (Figure 5A). These observations suggest that, in the absence of any endogenous GRα protein (functional or nonfunctional), the hGR protein variants with exon 2 deletions are able to mediate a specific hormonal response via a GRE but that they may be less active than the classical hGRα protein. Because of their lower transactivation properties, proteins coded by these transcripts could potentially function as partial competitive inhibitors in cells containing endogenous native hGRα. In addition, these functional variant GRα proteins

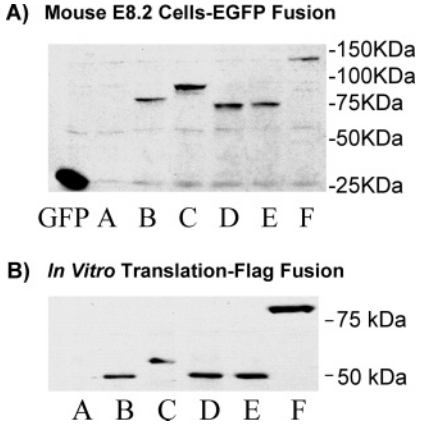

FIGURE 3: Translation of EGFP-tagged and Flag-tagged hGR alternatively spliced variants. (A) Mouse E8.2 cells were transfected with cDNA expression plasmids for the EGFP-tagged classical hGRα protein (lane F) and the variant proteins. As a control, cells were also transfected with the GFP-containing vector not containing any fused hGR sequences (GFP). After the cells were extracted, western blotting was performed using an anti-EGFP-specific antibody. (B) Flag-tagged classical hGRα and hGRα variant cDNAs were in vitro transcribed and translated using a TNT T7 transcription/translation kit from Promega. In all of these cases, protein concentrations from cell lysates or translation mixtures were measured using a Bio-Rad Dc protein assay kit, and 40 μg of total protein per sample was subjected to SDS−PAGE. After electrophoresis, the proteins were transferred to nitrocellulose membranes and blotted with the appropriate antibody. Lanes contained samples using the following constructs: (A) vector alone; (B) 1A1/E2dist hGRα; (C) 1A2/E2prox hGRα; (D) 1A3/E3 hGRα; (E) 1B/E3 hGRα; (F) 1C/E2 classical GRα.

may complement nonfunctional GRα mutants, and this could give rise to the cell type-specific activity seen in the Jurkat and COS-1 cells. Finally, these GRα variants can repress basal GRE-containing promoter activity in the absence of ligand, as most dramatically seen for 1A1/E2dist hGRα in Jurkat and COS-1 cells (Figure 5A).

While these Flag-tagged hGRα protein variants showed less or, at most, the same activity as classical hGRα, other experiments using the pCMVhGRα vector (containing the normal GR mRNA 3′ untranslated region) showed that transfection with the 1A1/E2dist hGRα (pCMV1A1/E2dist hGRα) and 1A3/E3 hGRα (pCMV 1A3/E3 hGRα) causes a more potent transactivation (assayed with the GRE-TAL promoter) compared to classical hGRα (pCMV hGRα) (Figure 5B). In Jurkat cells, 1A1/E2dist hGRα appears to be about three times more potent in stimulating the GRE-TAL promoter upon hormone treatment than classical hGRα (1C/E2 hGRα, Figure 5B). 1A2/E2prox hGRα and 1A3/E3 hGRα also significantly activate the GRE-TAL promoter upon DEX treatment (Figure 5B), although the degree of activation was comparable to classical 1C hGRα. No DEX response (repression or activation) was observed on the GRE-TAL promoter with the pCMV 5 empty vector (Figure 5B), as no functional GR protein is expressed in Jurkat cells. Similar to results for the Flag-tagged hGRα splice variant proteins, lower basal reporter gene activities (no added DEX) were also observed for Jurkat cells transfected with pCMV1A1/E2dist hGRα and pCMV1A2/E2prox hGRα expression constructs. The increased transactivation obtained with the 1A1/E2dist hGRα cDNA is surprising, because activation function 1 (AF-1), which has been reported to mediate most of the transactivation of the GR (*25*, *26*), is absent in 1A1/E2dist hGRα.

As in Jurkat cells, transfection with the 1A1/E2dist hGRα cDNA caused higher transactivation in COS-1 cells and E8.2 cells, although it was even more potent (~6-fold) than classical 1C hGRα (Figure 5B) in COS-1 cells and less potent (~2-fold) in E8.2 cells. In addition, while 1A3/E3

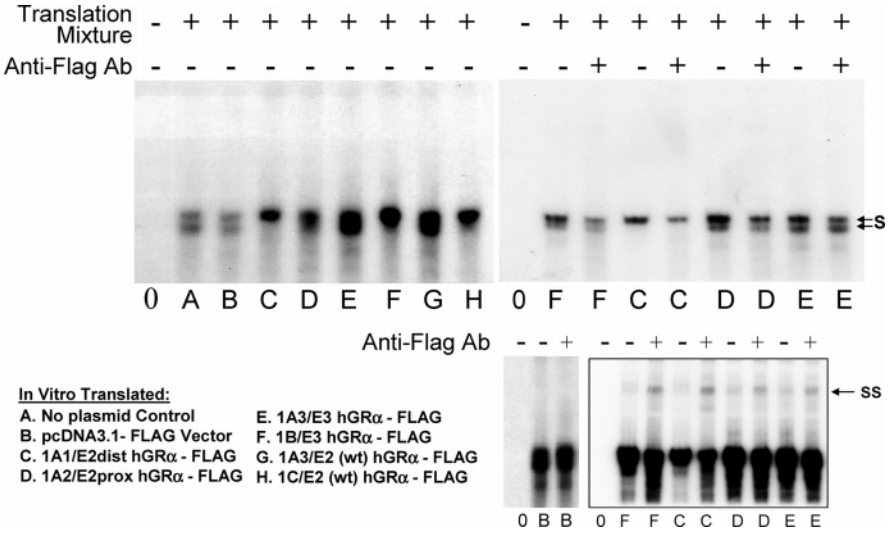

FIGURE 4: DNA-binding activity of Flag-tagged hGRα variants as analyzed by EMSA. For the electrophoretic mobility shift assays (EMSAs), 2 μL of in vitro translated protein mixture was added to the DNA-binding mix, as described in Materials and Methods. A consensus GRE, end-labeled with ^{32}P using T4 polynucleotide kinase, was added to the binding mix at 50 fmol per reaction. Incubations were done at room temperature for 30 min before the samples were loaded on prechilled 4% PAGE gels, followed by electrophoresis at 4 °C. For supershift analyses, 2 μg of anti-Flag antibody was added to each DNA-binding reaction mix containing the in vitro translated protein, and this was incubated in room temperature for 45 min prior to addition of the ^{32}P-labeled GRE oligo. The various samples used are indicated in the figure. Abbreviations: S, ^{32}P-labeled GRE−protein complexes; SS, supershifted bands obtained after addition of an anti-Flag tag antibody. The smaller panel (below, right) is a darker exposure of the upper right-hand panel to more clearly show the supershifted bands. The small panel (bottom, left) is a very overexposed control gel in which the pcDNA3.1-Flag vector (no fused hGRα sequences) was added to the transcription−translation reaction. The shifted DNA bands are likely due to endogenous rabbit GR present in the rabbit reticulocyte lysate used for the reaction. As this endogenous GR is not Flag-tagged, the addition of an anti-Flag antibody caused no supershift in the band.

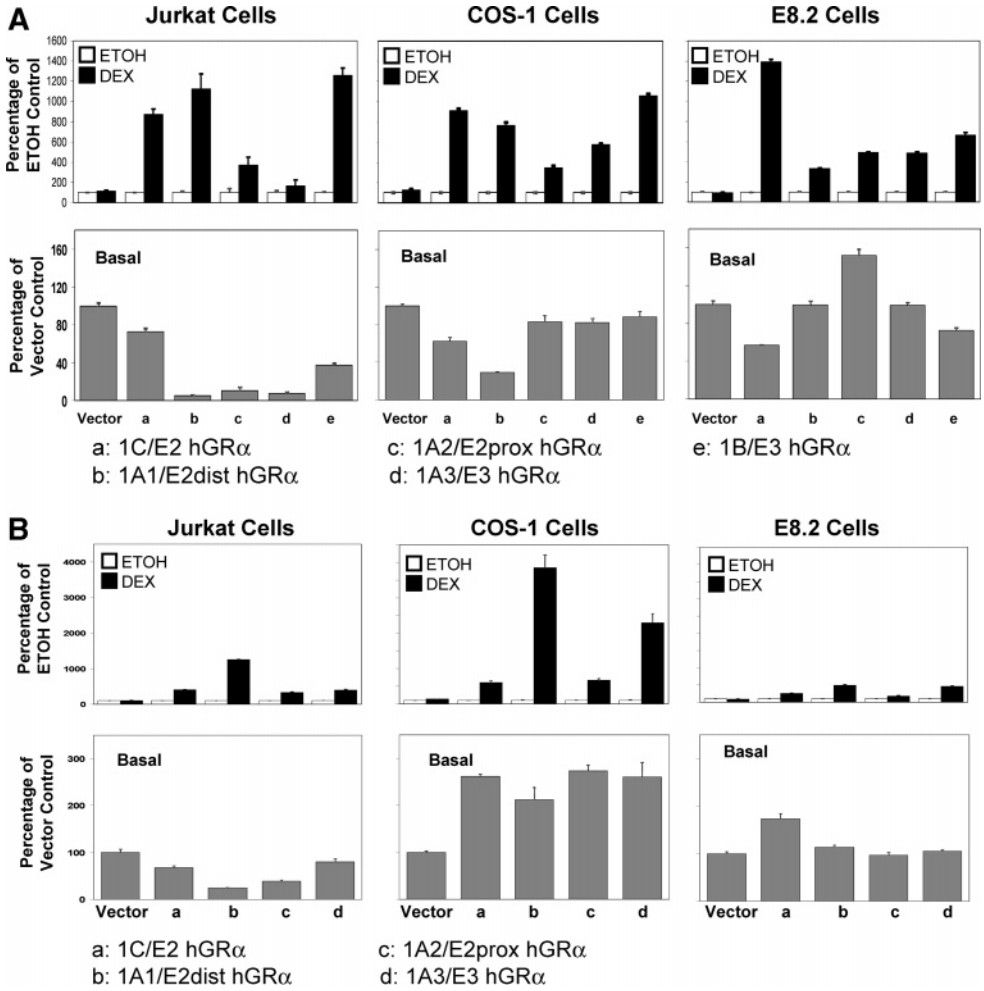

FIGURE 5: Responsiveness of a GRE-containing luciferase reporter gene to variant hGRα proteins. (A) 1.5 μg of the pGRE luciferase reporter gene was cotransfected along with vector alone (pcDNA3.1-Flag) or with expression vectors containing cDNAs for the various alternatively spliced hGRα protein variants containing the Flag tag at the carboxy teminus. Three cell lines were used: Jurkat T-lymphoblasts, COS-1 African green monkey kidney cells, and mouse E8.2 fibroblasts. Experimental details are found in Materials and Methods. (B) 1.5 μg of the pGRE reporter gene was transiently transfected to cells along with 1.5 μg of pCMV empty vector or pCMV constructs containing cDNAs that code for the various alternatively spliced hGRα protein variants. 1 μg of the pCMV-β-gal construct was included in all the transfection reactions to normalize the transfection efficiency. The luciferase activity of each transfection was measured with a luminometer and normalized by its own β-gal activity. Each transfection was performed three or four separate times. The values are the means, and the error bars are ± SEM. In the DEX treatment experiments, each normalized luciferase activity value from the EtOH-treated sample was set at 100%, and the DEX-treated sample value is shown as the percentage of the respective EtOH- (vehicle-) treated samples. To plot basal promoter activity, the values from the pCMV 5, empty vector-transfected, control samples were set to 100%, and the values for the hGR variant-transfected cells are given as the percentage of the respective empty vector control value.

hGRα behaved like the classical hGRα protein in transactivating the GRE-TAL reporter gene in Jurkat cells, transfection with its cDNA demonstrated more activity than the classical receptor cDNA in both COS-1 and E8.2 cells (Figure 5B). These results imply potential cell type-specific actions of 1A3/E3 hGRα. 1A2/E2prox hGRα had transactivating properties similar to classical hGRα in all three cell lines. The effects of the hGRα variant proteins on basal promoter activity of GRE-TAL (no hormone added) differed greatly among the cell types, being mostly inhibitory in Jurkat cells, mostly stimulatory in COS-1 cells, and (with the exception of the classical GRα protein) without effect in E8.2 cells.

Taken together, these studies show functional cell type-specific differences in the transcriptional activity of these hGRα variants on a GRE-containing promoter. The reason for the differences in activity between constructs using the pcDNA3.1-Flag (Figure 5A) and pCMVhGRα (Figure 5B) backbones is unclear. One possibility is that the pCMVhGRα backbone constructs contain the full-length, native hGR 3′ untranslated region (UTR) (*8*), and these transcripts more closely resemble natural hGRα transcripts than the pcDNA3.1-Flag construct, which contains the SV40 3′ UTR.

Cellular Localization of hGRα Protein Variants. Because of the unusual activities of some of the hGRα variant proteins, we wished to determine their subcellular localization both in the presence and in the absence of hormone. Thus, we used hGR fusion proteins containing EGFP at the C-terminus of the hGR proteins. Initially, we transfected p1C/E2 hGRα−EGFP, which expresses the classical hGRα−EGFP fusion protein, into HeLa and COS-1 cells and treated these with ethanol vehicle control or 1 μM DEX. As expected, HeLa and COS-1 cells transiently transfected with classical hGRα−EGFP exhibited fluorescence throughout the cell in the absence of DEX (Figure 6A). However, DEX treatment caused an accumulation of hGRα−EGFP in

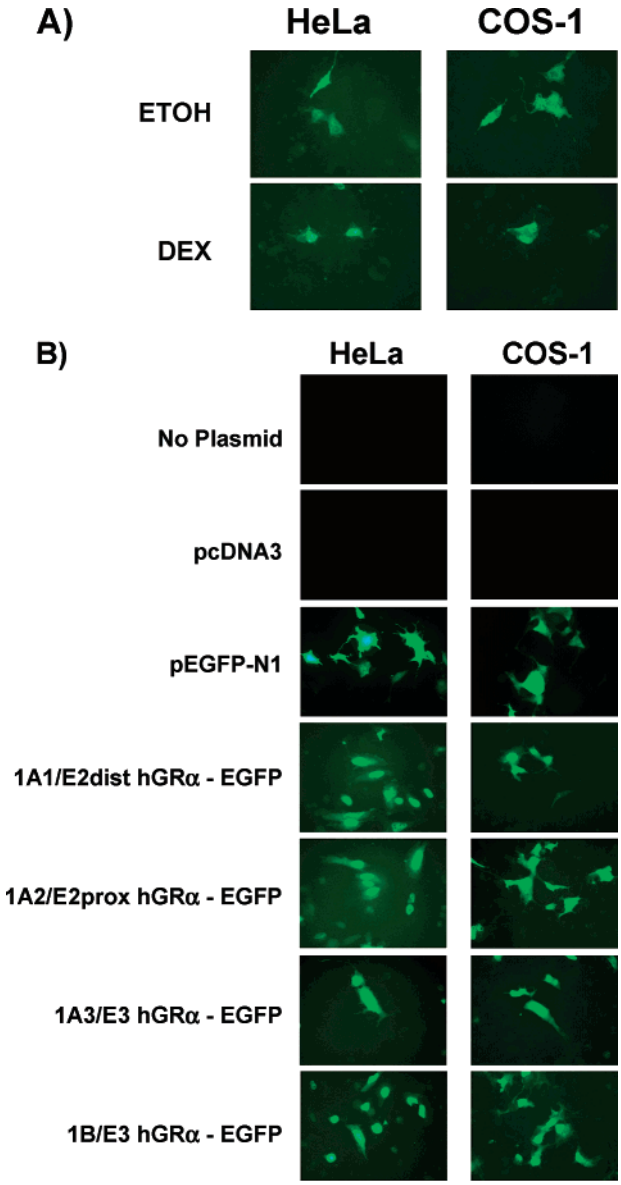

FIGURE 6: Cellular localization of hGRα protein variants. HeLa cells and COS cells were transfected by EGFP-tagged hGRα variant expression constructs as described in Materials and Methods. The cells were observed via fluorescence microscopy 24 h after transfection. Panel A: a plasmid containing EGFP-tagged, wild-type, classical hGRα, p1C/E2 hGRα−EGFP, was transfected into either HeLa cells or COS-1 cells. The cells were treated with either ethanol vehicle (EtOH) or 1 μM DEX. Panel B: HeLa or COS-1 cells were untransfected (No Plasmid) or were transfected with an empty vector (pcDNA3), a plasmid containing EGFP alone (pEGFP-N1), or plasmids containing cDNAs coding for the various alternatively spliced hGRα protein variants that were tagged at the carboxy terminus with EGFP. No hormone was added to these cultures.

the nucleus along with a decrease of cytoplasmic fluorescence. Therefore, the fusion of EGFP at the C-terminus of hGRα does not interfere with its cellular location or with its hormone-induced nuclear translocation.

Using C-terminal EGFP fusion expression constructs, the cellular localization of the hGRα variant proteins was then determined. In striking contrast to the classical hGRα protein, all of the N-terminal, exon 2-deleted, hGRα−EGFP fusion proteins were nuclearly localized in both HeLa cells and COS-1 cells even in the absence of hormone (Figure 6B). Because the EGFP tag does not itself affect the hGRα cellular location (Figure 6A), the absence of the N-terminal segment that is coded for exon 2 of the hGR gene results in the constitutional nuclear location of these hGRα variant proteins.

Cellular Levels of Alternatively Spliced hGR Variants in CEM-C7 Cells and IM-9 Cells. The concentrations of the alternatively spliced variants and of the GR transcript containing the splice site between exon 8 and exon 9α (hGRα-encoding transcripts) were determined in CEM-C7 cells and IM-9 cells treated for 24 h with either 1 μM DEX or ethanol vehicle (Table 1). In CEM-C7 cells, the concentrations of the 1A1/E2dist and the 1A2/E2prox transcripts were too low to be accurately determined by the QRT-PCR assays (less than 0.25 fmol/g of RNA and 2.5 fmol/g of RNA, respectively), compared to 4.6×10^3 fmol/g of RNA for total hGRα transcripts in the absence of DEX. In CEM-C7 cells, 1B/E3 is the most abundant exon 2-deleted transcript, being present at 25 fmol/g of RNA in the absence of DEX (Table 1). In IM-9 cells, except for the 1B/E3 transcript (7.3 fmol/g of RNA), the levels of alternatively spliced hGRα transcripts were too low to be accurately measured, while total hGRα transcripts were present at a concentration of 6.6×10^3 fmol/g of RNA in the absence of DEX. Thus, these alternatively spliced transcripts each constitute less than 1% of the transcripts with the exon 8− exon 9α splice site. Similarly to total hGRα-encoding transcripts, the 1B/E3 and 1A3/E3 transcripts were significantly upregulated by DEX in CEM-C7 cells. However, while the hGRα-encoding transcripts were significantly downregulated by DEX in IM-9 cells, the 1B/E3 transcript failed to show simultaneous downregulation.

Finally, we wished to determine if any of these alternatively spliced GRα transcripts were present in any normal human tissues. In a preliminary experiment, the specific expression of the 1A2/E2prox transcript was detected in human cerebellum (data not shown), but not in eight other tissues, suggesting a possible specific, physiologically relevant role for this transcript in cerebellum. However, the levels of this transcript in this tissue were still low, being, at most, about 0.1% of the total GRα transcripts.

DISCUSSION

The cellular hGR protein level is directly related to the responsiveness of cells to glucocorticoids (*27*). Furthermore, the cellular hGRα level is critical in determining the sensitivity of leukemia and multiple myeloma patients during hormone treatment or combination chemotherapy (*28*−*30*). Patients that exhibit hormone resistance may have not only abnormally low intracellular levels but also defective, mutated GR proteins (*29*, *31*).

A naturally occurring splice variant, GR*β*, has been extensively studied. While hGRα mediates steroid signaling and the normal cellular response, studies on the role of hGR*β* as a possible dominant negative inhibitor in this process have led to inconclusive or contradictory results in vitro and in vivo (*8*, *14*, *32*−*34*). No other forms of functional hGR have been reported in normal tissues and cells. However, several hGR forms with alternative splicing have been found in cancer cells, and these could contribute to the development of hormone resistance in certain types of hematologic malignancies. For example, human GR P, which is coded

by exon 2 through a part of intron 7, is a C-terminal truncated protein found in tumor cells (*11*). It has been recently reported that GR P is found in most hematological malignancies and, given its transcriptional regulatory abilities, it may be important in determining hormonal sensitivity in multiple myeloma patients (*35*). An hGRγ splice variant is present in a corticotroph adenoma (*12*). This variant has an amino acid (arginine) coded by an intron inserted in the DNA-binding domain, and it can comprise up to 8% of the total hGRα amount in cells. The alternatively spliced GR variants that we have discovered in CEM-C7 human T-cell ALL cells are potential candidates for novel hGR isoforms that could alter the cellular responsiveness to steroid therapy.

The GR protein contains three distinct functional domains (*6*). The amino-terminal domain contains the AF-1, or tau1, domain that is reported to be responsible for the majority of the transactivation properties of the GR (*6*, *24*−*26*, *36*, *37*). This region, which is deleted in the splicing variants that we have described here, acts as an interaction platform for a number of proteins that affect gene transcription (*38*, *39*). However, the N-terminal deleted variant GRα proteins were capable of significant steroid-dependent transactivation of a GRE-containing reporter gene, suggesting that the AF-2 activation function contributes significantly to the hormonal response.

The GR variants containing the natural hGR 3′ UTR displayed particularly interesting properties (Figure 5B). Transfection with the 1A1/E2dist hGRα caused transactivation of the reporter gene to a higher level than the classical hGRα in all cell lines tested. However, the magnitude of this increased response, compared to the classical hGR varied widely in the Jurkat (~3-fold), COS-1 (~6-fold), and E8.2 (~2-fold) cell lines. Jurkat cells have two mutant GR proteins expressed at relatively normal levels, and COS-1 cells have low, but detectable levels of simian GR, while E8.2 cells are almost completely devoid of any GR protein (*22*, *40*). This appears to be comparable to results obtained for the S49 mouse lymphoma nt^i GR mutant, which is similar to the complete exon 2-deleted (1A3/E3, 1B/E3) mutants that we have found here. The nt^i mutant is less transcriptionally active in CV-1 cells that have low endogenous GR (*41*), while it has activity higher than the wild-type receptor when it is expressed in S49 cells that contain relatively high levels (30% immunologically cross-reactive receptor) of nonfunctional (<2% of hormone-binding activity) GR (*42*). The interpretation of these results was that the AF-1 negative/hormone-binding positive nt^i mutant was complemented with endogenous AF-1 positive/hormone-binding negative endogenous GR in these cells by the formation of heterodimers between these mutant GR proteins. A similar situation may exist here. Thus, heterodimers between 1A1/E2dist variant proteins and endogenous GR in Jurkat and COS-1 cells may result in a greater transactivation than classical GRα homodimers. Because E8.2 cells essentially lack any endogenous GR protein, the intrinsic activity of the various, individual, alternatively spliced, GRα variant proteins is probably that seen in the E8.2 cell line. Conversely, 1A2/E2prox hGRα is indistinguishable from the classical hGRα in all cell types (Figure 5B), while 1A3/E3 hGRα has activity equivalent to classical hGRα (in Jurkat cells) or an apparent higher activity in COS-1 and E8.2 cells. These observations suggest that these hGRα variants coded by the alternatively spliced hGRα mRNA retained full hGRα function and yet can be modulated in specific cells in a way different from classical hGRα, perhaps by other transcription cofactors present in these cells. Currently, it is not known what roles, if any, are played by the unique N-terminal peptides coded for by hGR 1A exon sequences in 1A1/E2dist hGRα (19 amino acids) and 1A2/E2prox hGRα (51 amino acids).

Besides their intrinsic receptor activity, and their ability to interact with endogenous GR protein present in the cells, other reasons for the altered activity of the hGRα variants are possible. For example, the different 3′ UTRs present in the Flag-tagged construct (SV40 3′ UTR, Figure 5A) and the nontagged GR proteins (hGR 3′ UTR, Figure 5B) could affect the stabilities or translational efficiencies of the various transcripts, resulting in nonequivalent expression levels of the hGR protein variants. For example, the hGR 3′ UTR appears to be important in controlling the half-life of hGR transcripts (*19*). This could be the reason that pCMVhGRα-based hGRα variant expression constructs behaved differently from the pcDNA3.1-Flag-based constructs in mediating the transactivation of GRE-containing promoter.

Perhaps most dramatically, the hGRα variants lacking AF-1 were located in the nucleus without bound ligand. This constitutive nuclear location may be the reason for the surprising transrepression (most evident for Flag-tagged GR in Jurkat cells) or transactivation (most clearly seen for Flag-tagged GR in E8.2 cells) observed for basal promoter activity in the absence of hormone. Previous studies have shown that overexpression of mouse GR protein in CHO cells causes a hormone-independent nuclear localization of receptor (*43*, *44*). We do not believe that this phenomenon occurs here, as cells transfected with the native, classical hGR expression construct displayed normal cytoplasmic localization without DEX but nuclear localization when DEX was added. The cause for the nuclear localization of the variant GRα proteins is not yet clear. It does not appear to be a function of the unique exon 1A-derived polypeptide sequences at the amino termini of the 1A1/E2dist or 1A2/E2prox variants, as the 1A3/E3 hGRα and 1B/E3 hGRα proteins also were nuclearly localized in the absence of hormone. One possible explanation is that the GR N-terminal acidic region reduces nonspecific DNA binding and that this reduces nonspecific nuclear accumulation (*24*). Furthermore, the transcriptional effect of these GRα variant proteins could be promoter-specific, as was found for tau1-deleted mutants of the GRα protein (*36*). The effect of these variant GRα proteins on the expression of endogenous hormonally regulated genes remains to be determined.

The alternatively spliced GRα variants are present at low concentrations in CEM-C7 and IM-9 cells, relative to the total concentration of GRα transcripts. Thus, the potential physiological or pathological roles are not yet clear. Also, the relationship between the transcript and protein levels for the GRα variants is not known. Specific antibodies to detect the endogenous GRα variant proteins are unavailable. Therefore, it is possible that the intracellular protein levels could be biologically relevant, for example, if protein stability and half-lives were greater for the variant GR proteins than for the classical GRα protein. Because the alternatively spliced transcripts were detected in a cancer cell line, it is not yet known if these variant GRα proteins are present in normal tissues to a significant extent. However, preliminary

studies have shown that normal human cerebellum does specifically contain the 1A2/E2prox transcript. It is possible that this GRα variant plays a specific, physiologically relevant role in this tissue. Finally, additional studies are needed to assess the presence and cellular levels of these variant GRα transcripts and proteins in acute lymphoblastic leukemia patients. Because of the novel and atypical biological activity of the GRα variant proteins on regulating transcription of a GRE-containing reporter gene, it is possible that they play an important role in the hormonal responsiveness (resistance or hypersensitivity) to hormone therapy. If they do, then the detection of these transcripts in leukemia patients may be useful diagnostically, and new treatment regimens might be developed to improve clinical treatment based upon the GRα protein variant that is present in the individual patient.

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SUPPORTING INFORMATION AVAILABLE

One table showing nucleotide sequences of primers and probes used for real-time QRT-PCR and for RNA standards. This material is available free of charge via the Internet at http://pubs.acs.org.

REFERENCES

1. Galili, U. (1983) Glucocorticoid induced cytolysis of human normal and malignant lymphocytes, *J. Steroid Biochem. 19*, 483−490.
2. Nanni, P., Nicoletti, G., Prodi, G., Galli, M. C., De Giovanni, C., Grilli, S., Lollini, P. L., Gobbi, M., Cavo, M., and Tura, S. (1982) Glucocorticoid receptor and in vitro sensitivity to steroid hormones in human lymphoproliferative diseases and myeloid leukemia, *Cancer 49*, 623−632.
3. Distelhorst, C. W. (1988) Glucocorticosteroids induce DNA fragmentation in human lymphoid leukemia cells, *Blood 72*, 1305−1309.
4. Greenstein, S., Ghias, K., Krett, N. L., and Rosen, S. T. (2002) Mechanisms of glucocorticoid-mediated apoptosis in hematological malignancies, *Clin. Cancer Res. 8*, 1681−1694.
5. Nazareth, L. V., Johnson, B. H., Chen, H., and Thompson, E. B. (1996) Transfected glucocorticoid receptor and certain GR fragments evoke cell death in malignant lymphoid, not myeloid cell lines, *Leukemia 10*, 1789−1795.
6. Weinberger, C., Hollenberg, S. M., Rosenfeld, M. G., and Evans, R. M. (1985) Domain structure of human glucocorticoid receptor and its relationship to the v-erb-A oncogene product, *Nature 318*, 670−672.
7. Wright, A. P., Zilliacus, J., McEwan, I. J., Dahlman-Wright, K., Almlof, T., Carlstedt-Duke, J., and Gustafsson, J. A. (1993) Structure and function of the glucocorticoid receptor, *J. Steroid Biochem. Mol. Biol. 47*, 11−19.
8. Oakley, R. H., Sar, M., and Cidlowski, J. A. (1996) The human glucocorticoid receptor beta isoform. Expression, biochemical properties, and putative function, *J. Biol. Chem. 271*, 9550−9559.
9. Powers, J. H., Hillman, A. G., Tang, D. C., and Harmon, J. M. (1993) Cloning and expression of mutant glucocorticoid receptors from glucocorticoid-sensitive and -resistant human leukemic cells, *Cancer Res. 53*, 4059−4065.
10. Moalli, P. A., Pillay, S., Krett, N. L., and Rosen, S. T. (1993) Alternatively spliced glucocorticoid receptor messenger RNAs in glucocorticoid-resistant human multiple myeloma cells, *Cancer Res. 53*, 3877−3879.
11. Krett, N. L., Pillay, S., Moalle, P. A., Grepp, P. R., and Rosen, S. T. (1995) A variant glucocorticoid receptor messenger RNA is expressed in multiple myeloma patients, *Cancer Res. 55*, 2727−2729.
12. Rivers, C., Levy, A., Hancock, J., Lightman, S., and Norman, M. (1999) Insertion of an amino acid in the DNA-binding domain of the glucocorticoid receptor as a result of alternative splicing, *J. Clin. Endocrinol. Metab. 84*, 4283−4286.
13. Hillman, A. G., Ramdas, J., Multanen, K., Norman, M. R., and Harmon, J. M. (2000) Glucocorticoid receptor gene mutations in leukemic cells acquired in vitro and in vivo, *Cancer Res. 60*, 2056−2062.
14. de Lange, P., Koper, J. W., Brinkmann, A. O., de Jong, F. H., and Lamberts, S. W. (1999) Natural variants of the beta isoform of the human glucocorticoid receptor do not alter sensitivity to glucocorticoids, *Mol. Cell. Endocrinol. 153*, 163−168.
15. Beger, C., Gerdes, K., Lauten, M., Tissing, W. J., Fernandez-Munoz, I., Schrappe, M., and Welte, K. (2003) Expression and structural analysis of glucocorticoid receptor isoform gamma in human leukaemia cells using an isoform-specific real-time polymerase chain reaction approach, *Br. J. Haematol. 122*, 245−252.
16. Encio, I. J., and Detera-Wadleigh, S. D. (1991) The genomic structure of the human glucocorticoid receptor, *J. Biol. Chem. 266*, 7182−7188.
17. Breslin, M. B., Geng, C.-D., and Vedeckis, W. V. (2001) Multiple promoters exist in the human GR gene, one of which is activated by glucocorticoids, *Mol. Endocrinol. 15*, 1381−1395.
18. Nunez, B. S., and Vedeckis, W. V. (2002) Characterization of promoter 1B in the human glucocorticoid receptor gene, *Mol. Cell. Endocrinol. 189*, 191−199.
19. Schaaf, M. J., and Cidlowski, J. A. (2002) AUUUA motifs in the 3′ UTR of human glucocorticoid receptor α and β mRNA destabilize mRNA and decrease receptor protein expression, *Steroids 67*, 627−636.
20. Xu, Q., Leung, D. Y. M., and Kisich, K. O. (2003) Serine-arginine-rich protein p30 directs alternative splicing of glucocorticoid receptor pre-mRNA to glucocorticoid receptor {beta} in neutrophils, *J. Biol. Chem. 278*, 27112−27118.
21. Pedersen, K. B., and Vedeckis, W. V. (2003) Quantification and glucocorticoid regulation of glucocorticoid receptor transcripts in two human leukemic cell lines, *Biochemistry 42*, 10978−10990.
22. Housley, P. R., and Forshoefel, A. M. (1989) Isolation and characterization of a mouse L cell variant deficient in glucocorticoid receptors, *Biochem. Biophys. Res. Commun. 164*, 480−487.
23. Hollenberg, S. M., Giguere, V., Segui, P., and Evans, R. M. (1987) Colocalization of DNA-binding and transcriptional activation functions in the human glucocorticoid receptor, *Cell 49*, 39−46.
24. Danielsen, M., Northrop, J. P., Jonklaas, J., and Ringold, G. M. (1987) Domains of the glucocorticoid receptor involved in specific and nonspecific deoxyribonucleic acid binding, hormone activation, and transcriptional enhancement, *Mol. Endocrinol. 11*, 816−822.
25. Hollenberg, S. M., and Evans, R. M. (1988) Multiple and cooperative trans-activation domains of the human glucocorticoid receptor, *Cell 55*, 899−906.
26. Dahlman-Wright, K., Almlof, T., McEwan, I. J., Gustafsson, J. A., and Wright, A. P. (1994) Delineation of a small region within the major transactivation domain of the human glucocorticoid receptor that mediates transactivation of gene expression, *Proc. Natl. Acad. Sci. U.S.A. 91*, 1619−1623.
27. Vanderbilt, J. N., Miesfeld, R., Maler, B. A., and Yamamoto, K. R. (1987) Intracellular receptor concentration limits glucocorticoid-dependent enhancer activity, *Mol. Endocrinol. 1*, 68−74.
28. Geley, S., Hartman, B. L., Strasser-Wozak, E. M., Kapelari, K., and Kofler, R. (1996) Resistance to glucocorticoid-induced apoptosis in human T-cell acute lymphoblastic leukemia CEM-C1 cells is due to insufficient glucocorticoid receptor expression, *Cancer Res. 56*, 5033−5038.
29. Moalli, P. A., and Rosen, S. T. (1994) Glucocorticoid receptors and resistance to glucocorticoids in hematologic malignancies, *Leuk. Lymphoma 15*, 363−374.
30. Ramdas, J., Liu, W., and Harmon, J. M. (1999) Glucocorticoid-induced cell death requires autoinduction of glucocorticoid receptor expression in human leukemic T cells, *Cancer Res. 59*, 1378−1385.
31. Sluyser, M. (1994) Hormone resistance in cancer: the role of abnormal steroid receptors, *Crit. Rev. Oncog. 5*, 539−554.

32. de Castro, M., Elliot, S., Kino, T., Bamberger, C., Karl, M., Webster, E., and Chrousos, G. P. (1996) The non-ligand binding beta-isoform of the human glucocorticoid receptor (hGR beta): tissue levels, mechanism of action, and potential physiologic role, *Mol. Med. 2*, 597−607.
33. Hecht, K., Carlstedt-Duke, J., Stierna, P., Gustafsson, J.-A., Bronnegard, M., and Wikstrom, A.-C. (1997) Evidence that the beta-isoform of the human glucocorticoid receptor does not act as a physiologically significant repressor, *J. Biol. Chem. 272*, 26659−26664.
34. Strickland, I., Kisich, K., Hauk, P. J., Vottero, A., Chrousos, G. P., Klemm, D. J., and Leung, D. Y. M. (2001) High constitutive glucocorticoid receptor {beta} in human neutrophils enables them to reduce their spontaneous rate of cell death in response to corticosteroids, *J. Exp. Med. 193*, 585−594.
35. de Lange, P., Segeren, C. M., Koper, J. W., Wiemer, E., Sonneveld, P., Brinkmann, A. O., White, A., Brogan, I. J., de Jong, F. H., and Lamberts, S. W. J. (2001) Expression in hematological malignancies of a glucocorticoid receptor splice variant that augments glucocorticoid receptor-mediated effects in transfected cells, *Cancer Res. 61*, 3937−3941.
36. Delaunay, F., Liden, J., Gustafsson, J. A., and Okret, S. (1996) Glucocorticoid receptor lacking the tau 1 transactivation domain is a gene-specific regulator of the wild-type glucocorticoid-receptor activity, *Eur. J. Biochem. 242*, 839−845.
37. Giguere, V., Hollenberg, S. M., Rosenfeld, M. G., and Evans, R. M. (1986) Functional domains of the human glucocorticoid receptor, *Cell 46*, 645−652.
38. Almlof, T., Wallberg, A. E., Gustafsson, J. A., and Wright, A. P. (1998) Role of important hydrophobic amino acids in the interaction between the glucocorticoid receptor tau 1-core activation domain and target factors, *Biochemistry 37*, 9586−9594.
39. Hittelman, A. B., Burakov, D., Iniguez-Lluhi, J. A., Freedman, L. P., and Garabedian, M. J. (1999) Differential regulation of glucocorticoid receptor transcriptional activation via AF-1-associated proteins, *EMBO J. 18*, 5380−5388.
40. Pedersen, K. B., Geng, C.-d., and Vedeckis, W. V. (2004) Three mechanisms are involved in glucocorticoid receptor auto-regulation in a human T-lymphoblast cell line, *Biochemistry 43*, 10851.
41. Dieken, E. S., Meese, E. U., and Miesfeld, R. L. (1990) nt^i glucocorticoid receptor transcripts lack sequences encoding the amino-terminal transcriptional modulatory domain, *Mol. Cell. Biol. 10*, 4574−4581.
42. Dieken, E. S., and Miesfeld, R. L. (1992) Transcriptional transactivation functions localized to the glucocorticoid receptor N terminus are necessary for steroid induction of lymphocyte apoptosis, *Mol. Cell. Biol. 12*, 589−597.
43. Sanchez, E. R., Hirst, M., Scherrer, L. C., Tang, H. Y., Welsh, M. J., Harmon, J. M., Simons, S. S. J., Ringold, G. M., and Pratt, W. B. (1990) Hormone-free mouse glucocorticoid receptors overexpressed in Chinese hamster ovary cells are localized to the nucleus and are associated with both hsp70 and hsp90, *J. Biol. Chem. 265*, 20123−20130.
44. Martins, V. R., Pratt, W. B., Terracio, L., Hirst, M. A., Ringold, G. M., and Housley, P. R. (1991) Demonstration by confocal microscopy that unliganded overexpressed glucocorticoid receptors are distributed in a nonrandom manner throughout all planes of the nucleus, *Mol. Endocrinol. 5*, 217−225.

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