0888-8809/01/$03.00/0
*Printed in U.S.A.*

Molecular Endocrinology 15(8):1381–1395
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# Multiple Promoters Exist in the Human GR Gene, One of Which Is Activated by Glucocorticoids

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**A new human GR gene sequence (hGR 1Ap/e), which is distinct from the previously identified human GR promoter and coding sequences, has been isolated and characterized. The hGR 1Ap/e sequence is approximately 31 kbp upstream of the human GR coding sequence. This sequence (2,056 bp) contains a novel promoter (the hGR 1A promoter; 1,075 bp) and untranslated exon sequence (hGR exon 1A sequence; 981 bp). Alternative splicing produces three different hGR 1A-containing transcripts, 1A1, 1A2, and 1A3. GR transcripts containing exon 1A1, 1A2, 1B, and 1C are expressed at various levels in many cancer cell lines, while the exon 1A3-containing GR transcript is expressed most abundantly in blood cell cancer cell lines. Glucocorticoid hormone treatment causes an up-regulation of exon 1A3-containing GR transcripts in CEM-C7 T-lymphoblast cells and a down-regulation of exon 1A3-containing transcripts in IM-9 B-lymphoma cells. Deoxyribonuclease I footprinting using CEM-C7 cell nuclear extract reveals four footprints in the promoter region and two intraexonic footprints. Much of the basal promoter-activating function is found in the +41/+269 sequence, which contains two deoxyribonuclease I footprints (FP5 and FP6). When this sequence is cloned into the pXP-1 luciferase reporter gene, hormone treatment causes a significant increase in luciferase activity in Jurkat T cells that are cotransfected with a GR expression vector. FP5 is an interferon regulatory factor-binding element, and it contributes significantly to basal transcription rate, but it is not activated by steroid. FP6 resembles a glucocorticoid response element and can bind GR$\beta$. This novel hGR 1Ap/e sequence may have future applications for the diagnosis, prognosis, and treatment of T-cell leukemia and lymphoma. (*Molecular Endocrinology* 15: 1381–1395, 2001)**

THE EFFECTS OF glucocorticoids (GCs) are mediated by the binding and activation of their intracellular receptor, the GR, which is a member of the steroid receptor superfamily that contains ligand-activated nuclear transcription factors. A cell's ability to respond to hormone treatment is determined by the number of functional GR molecules within the cell (1), and the steroid hormone itself can alter receptor protein levels. In most cells tested to date, GR is down-regulated in response to hormone treatment and the cells remain viable (reviewed in Ref. 2). In sharp contrast, in immature T cells, immature thymocytes, and T lymphoblasts, human (h)GR mRNA and protein are up-regulated by hormone treatment, which is followed by apoptosis or programmed cell death. The CEM-C7 cell line [derived from a T-cell acute lymphoblastic leukemia (ALL) pediatric patient] is a well established *in vitro* model system for studying GC-mediated apoptosis in T cells (3, 4). In the GC-sensitive 6TG1.1 cell line, derived from CEM-C7 cells, the observed up-regulation of the GR mRNA is a tissue-specific, primary transcriptional response (5). GC-treated CEM-C7 cells arrest in the $G_1$ phase of the cell cycle (6), and they then undergo apoptosis. A certain threshold level of intracellular GR is needed for GC-mediated cell death (7), suggesting that GC-mediated up-regulation of the GR is required for hormone-induced apoptosis. The molecular mechanisms that result in the cell type-specific autoregulation of GR mRNA and protein levels (upward or downward) are not yet completely clear.

To date, only one promoter that controls the expression of the hGR gene has been described (8). In mouse, three separate 5′-untranslated exon 1 regions were identified and designated exons 1A, 1B, and 1C (9). The mouse exon 1A-containing transcripts are expressed selectively in T cells. Therefore, to gain a better understanding of how the hGR gene is expressed and regulated in hormone treated T cells, we sought to identify hGR transcripts that may be expressed in a cell type-specific manner.

The current study shows that human GR mRNA transcripts can contain at least five different 5′-untranslated exon 1 sequences. Exons 1B and 1C share a high degree of homology with the corresponding mouse exon 1B and 1C sequences. A novel exon 1A region contains three separate alternative splice sites that give rise to three GR mRNA transcripts containing exons 1A1, 1A2, or 1A3. Thus, at least five hGR transcripts are expressed from three separate promoters. The exon 1A3-containing transcript is expressed at higher levels in cancer cell lines of hematopoietic

Abbreviations: ALL, acute lymphoblastic leukemia; BAC, bacterial artificial chromosome; CMV, cytomegalovirus; Dex, dexamethasone; GC, glucocorticoid; GRE, glucocorticoid response element; IFN, interferon; IRF-1, IFN-regulatory factor-1; IRF-E, IFN-regulatory factor element; RACE, 5′-rapid amplification of cDNA ends.

origin, and it is up-regulated by hormone in CEM-C7 cells and down-regulated by hormone in IM-9 (B lymphoma) cells. A −964 to +269 hGR 1A promoter/exon sequence confers GC-dependent up-regulation of a reporter gene in T cells. Thus, the hGR 1A promoter and exon region appear to contain sequences necessary for the cell type-specific up-regulation of GR in T cells. An interferon (IFN)-regulatory factor element (IRF-E) is important for the basal expression of the exon 1A promoter in Jurkat T cells.

GCs are routinely used in the treatment of childhood leukemia and lymphoma (reviewed in Ref. 10), and the lymphocytolytic effects of the hormone correlate with initial GR concentration (11–14). Because GC-mediated up-regulation of the GR is needed for apoptosis (7), the expression and up-regulation of the exon 1A3-containing hGR transcript may be useful in the future for the diagnosis, prognosis, and treatment of T-cell ALL.

## RESULTS

### Multiple 5′-Untranslated Exons Exist for the Human GR mRNA

To identify alternatively spliced hGR transcripts, 5′-rapid amplification of cDNA ends (RACE) was performed. A MOLT-4 human T-cell leukemia cDNA library (CLONTECH Laboratories, Inc., Palo Alto, CA) was screened using a 5′-adapter primer (AP-1; CLONTECH Laboratories, Inc.) and a 3′ gene-specific primer within exon 2 of the human GR gene. A 300- to 400-bp smear was observed on a 1.2% agarose gel (data not shown). The resulting PCR products were gel purified and subcloned into the pCR2.1 vector, and 17 clones were sequenced. Five clones, designated as exon 1C, are identical to the previously characterized untranslated human GR exon 1 (Fig. 1) (8). Eleven clones corresponded to a new hGR exon 1 region and were designated exon 1B. Exon 1B was present at position −1,111 to −1,035 bp in the published hGR promoter region. Heterogeneity at the 5′-end of the exon 1B region suggests that the exon 1B transcript may have multiple transcription start sites (data not shown). One hGR clone (54 bp in length) that was sequenced was novel, and it was designated exon 1A. In an alternative approach, a human genomic bacterial artificial chromosome (BAC) library was screened using PCR primers to the −2,101 to −1,783 bp hGR 1B promoter region. Southern blot analysis identified two BAC clones that contained the 54-bp exon 1A region (data not shown). Further sequence information for the exon 1A region was obtained by direct sequencing of the BAC clones. Additionally, the genomic organization of the hGR gene was determined using pulsed field gel electrophoresis (data not shown). The human exon 1A sequence is located approximately 25 kbp upstream of the hGR exon 1B region (Fig. 1). Exons 1A, 1B, and 1C are all spliced to the same splice acceptor site on exon 2 reported previously (15). There is an in-frame stop codon located three codons upstream of the initiator methionine ATG codon in exon 2, indicating that none of the 5′-exon 1 sequences will be translated into amino acids in the hGR protein.

### Three Untranslated Exons Derive from Alternative Splicing in the New hGR Sequence

Currently, 2,056 bp of new sequence, which contains the original 3′ 54 bp of exon 1A, have been studied (Fig. 2). This sequence is designated the hGR 1A promoter and exon (hGR 1Ap/e) sequence. Various 5′-PCR primers were designed to determine the approximate transcription start site for the exon 1A transcript. PCR primers to the +56 bp and +126 bp regions resulted in products of expected size while the primers at positions −272 and −72 bp gave no product (data not shown). This mapped the transcription start site approximately 1 kbp upstream of the exon 1A splice site. Primer extension using a primer to the +84/+103 bp site in the exon 1A region showed multiple transcription start sites in this region (data not shown) and revealed that the strongest transcription start site (indicated +1; Fig. 2) was located 981 bp upstream of

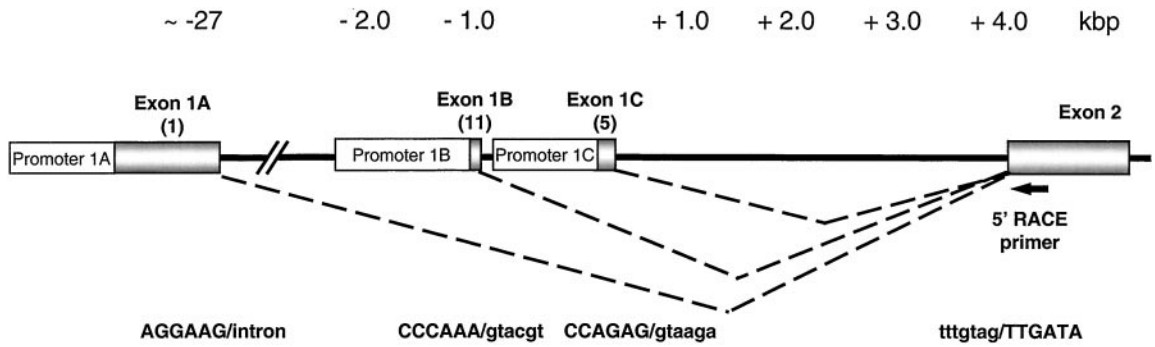


**Fig. 1.** Diagram of the hGR Gene Structure and Organization

Untranslated exons 1A, 1B, or 1C are spliced to the same splice acceptor site in exon 2. Promoters 1B and 1C are GC rich and are present in previously published sequences. The hGR 1A promoter and exon are novel, and they are located approximately 27 kbp upstream of the start site for transcription for exon 1C. The numbering system is based upon that found in Ref. 8.


**Fig. 2.** Sequence of hGR 1Ap/e. 2056 Nucleotides of the Coding Strand

The putative CAP site (*bold, underlined*) is labeled +1. There are 981 nucleotides of exon 1A and 1,075 nucleotides of 1A promoter sequence depicted. FP1-FP6 (*boxes*) refer to putative transcription factor binding sites as determined by DNase I footprinting. The *bold, vertical arrows* and the *bolded letters* indicate the three alternative exon 1A splice donor sites, SD1 (exon 1A1; 212 nucleotides), SD2 (exon 1A2; 308 nucleotides), and SD3 (exon 1A3; 981 nucleotides). *Dashed arrows* lie below sequences that were used for PCR and for primer extension analysis.

the exon 1A splice donor site. Other primer extension stops were located at −10 bp, +29 bp, and +40 bp. Surprisingly, RT-PCR primers to the +56 and +126 bp regions resulted in the amplification of three products, one of the expected size and two that were smaller (Fig. 3A). To determine whether these were specific or nonspecific PCR products, they were subcloned and sequenced. Two additional splice sites were present in

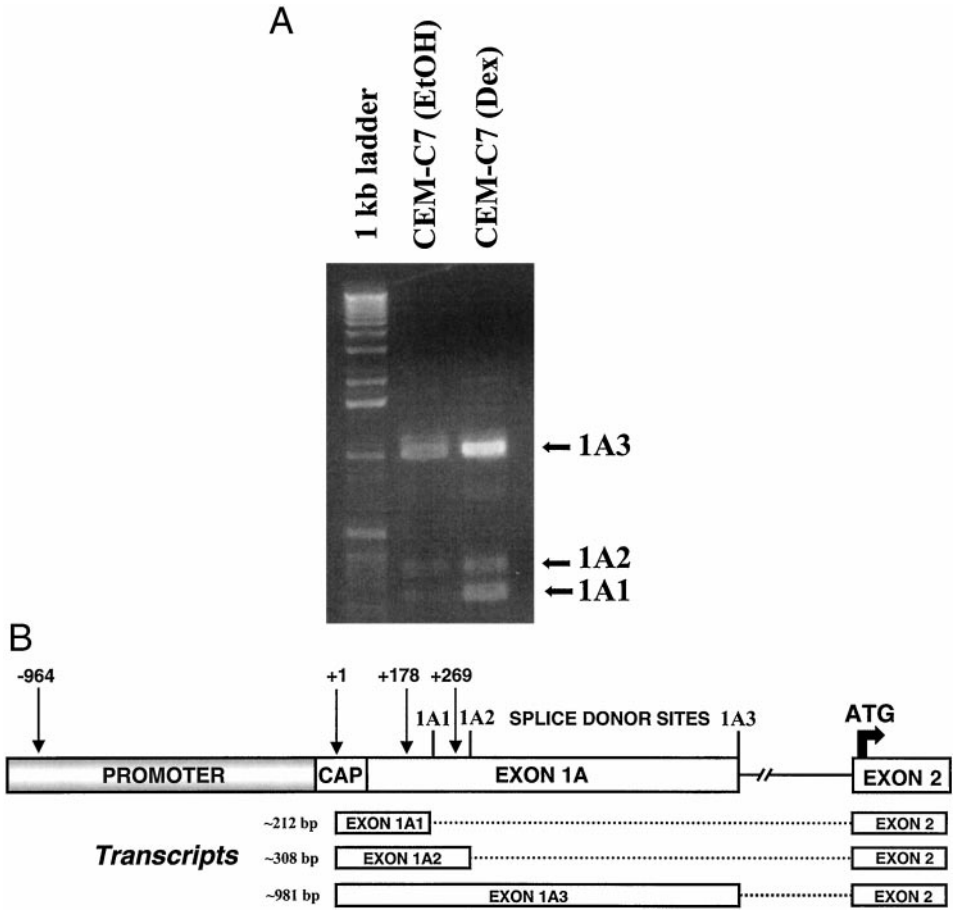


**Fig. 3.** Alternative Splicing of Exon 1A Occurs to Give Rise to Three Different 5′-Untranslated Regions

A, RT-PCR amplification was performed on RNA extracted from CEM-C7 T-lymphoblast cells as described in *Materials and Methods* and *Results*. Three distinct products were visualized upon agarose gel electrophoresis. B, Diagram of alternative splicing of the exon 1A-containing transcript. Transcription is presumably controlled by the same hGR 1A promoter using the same initiation site for transcription. Three alternative splice donor sites are used as indicated. All three untranslated exon 1A sequences are spliced to the same splice acceptor site in exon 2, as determined by direct sequencing. The initiator methionine ATG codon is found in exon 2, and an in-frame stop codon occurs three codons upstream of the ATG. The *numbers above the top bar* refer to positions that were used for analysis of hGR promoter activity.

the exon 1A region resulting in the formation of three distinct hGR transcripts: exon 1A1, spliced at position +212; exon 1A2, spliced at position +308; and, exon 1A3 (originally identified in the 5′-RACE experiment), spliced at position +981 (Fig. 3B). RT-PCR using an exon 1A3-specific upstream primer and downstream primers for each of the separate exons 2–9 of the hGR coding sequence indicated that all nine coding sequences are present in transcripts containing the untranslated exon 1A3 (data not shown).

### Cancer Cells from the Hematopoietic Lineage Express hGR 1A3 Transcripts at Higher Levels Than Other Cancer Cell Lines

To determine the significance of these multiple hGR transcripts, RT-PCR was performed with exon 1A1, 1A2, 1A3, 1B, and 1C specific primer sets to determine the expression pattern for the various hGR transcripts in a variety of cancer cell lines originating from various tissues. Nonquantitative RT-PCR indicated that the 1A3 containing hGR transcript was expressed at high levels in cell lines from the hematopoietic lineage (data not shown). To allow an estimation of the relative levels of each transcript in the various cell lines, Quantum*RNA* 18S internal control primers (Ambion, Inc., Austin, TX) were used. In a pilot experiment, exon 1B and 1C transcripts were detected in all cell lines tested (Fig. 4). Exon 1A1 and 1A2-containing transcripts were low or not detected in IM-9 (B-cell lymphoma), HL-60 (promyelocytic leukemia), 786–0 (renal carcinoma cells), WI-38 (normal human diploid fibroblasts), and human adult brain tissue (Fig. 4). They were present in varying levels in the other cell lines tested (Jurkat T-cell ALL; HeLa $S_3$ cervical carcinoma; HepG2 hepatocarcinoma; MCF-C7 breast cancer; SJSA osteosarcoma;

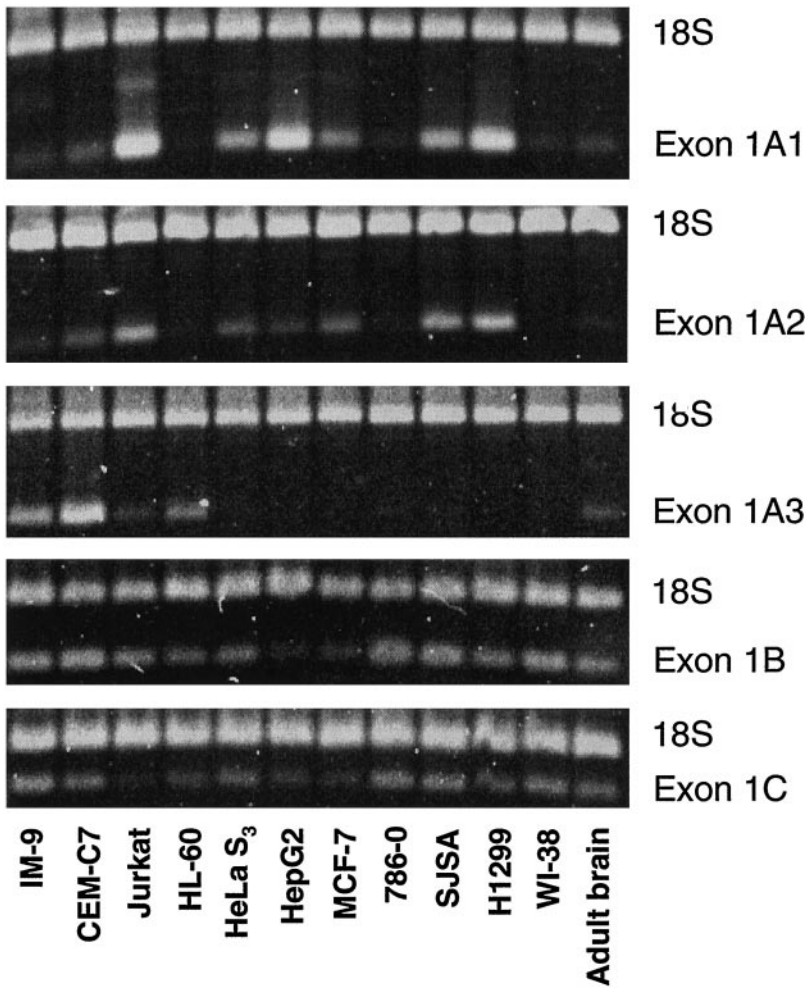


**Fig. 4.** Cell-Type Selective Expression of the hGR Transcripts

Quantitative RT-PCR was performed using primers specific for the hGR transcripts containing exon 1A1, 1A2, 1A3, 1B, or 1C, as described in *Materials and Methods* and *Results*. Human cell lines tested were: IM-9 (B-cell lymphoma); CEM-C7 (T-cell acute lymphoblastic leukemia, ALL); Jurkat (T-cell ALL); HL-60 (acute myeloid leukemia, AML-M2); HeLa $S_3$ (cervical carcinoma); HepG2 (hepatocarcinoma); MCF-7 (breast carcinoma); 786–0 (kidney carcinoma); SJSA (osteosarcoma); H1299 (lung carcinoma); and WI-38 (normal human diploid fibroblast). Adult brain RNA was also analyzed. 18S rRNA was used as an internal control.

H1299 lung cancer). Most notably, the expression of exon 1A3 transcripts was highest in cells of hematopoietic origin (Fig. 4), *i.e*. in CEM-C7, IM-9, HL-60, and (to a much lesser extent) Jurkat cells (Fig. 4). Jurkat cells are known to lack functional GR. Whether or not this is partly due to low GR protein levels resulting from low levels of expression of various GR transcripts (including 1A3) requires further study. Expression of the 1A3-containing transcripts was also detected in human adult brain tissue.

Quantitative RT-PCR was performed repeatedly on five different cell lines, three of the hematopoietic lineage (IM-9, CEM-C7, Jurkat) and two that were nonhematopoietic (HeLa, WI-38). 1A1, 1A2, 1B, and 1C transcripts were seen at various levels in these cell lines, while the hematopoietic cells showed a higher level of exon 1A3-containing transcripts than nonhematopoietic cells (Fig. 5).

### Glucocorticoid Hormone Regulates Exon 1A-Containing hGR Transcripts

Using an hGR probe to coding sequences, it was shown that the hGR mRNA is up-regulated in CEM-C7 cells and down-regulated in IM-9 cells (5). Thus, these two cell lines were chosen for the study of hormonal regulation of the various hGR transcripts.

To quantify the individual hGR transcripts, the Quantum*RNA* 18S RT-PCR system was used as described above. Exon 1A3-containing transcripts were up-regulated 2.5-fold ($P < 0.05$) in 1 $\mu$M dexamethasone (Dex)-treated CEM-C7 cells and down-regulated 69% ($P < 0.05$) by hormone-treatment in IM-9 cells (Fig. 6). Exon 1B-containing transcripts were slightly up-regulated 1.4-fold ($P < 0.05$) in CEM-C7 cells and were unchanged in IM-9 cells (Fig. 6). The expression level of the exon 1C-containing transcript did not

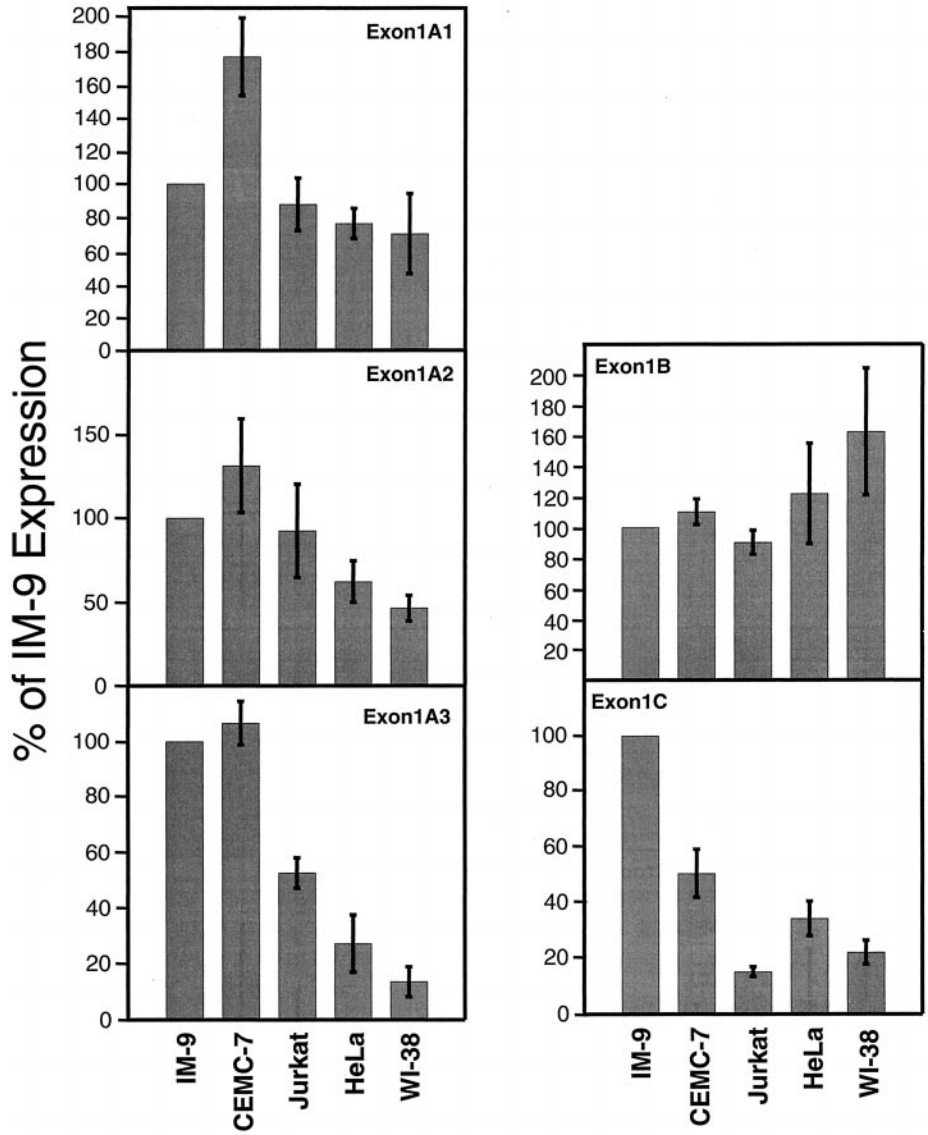


**Fig. 5.** Quantitation of the Expression of hGR 1A1, 1A2, 1A3, 1B, and 1C Transcripts in Various Cancer Cell Lines

Quantitative RT-PCR was performed on four different samples of the five cell lines listed. Values obtained upon densitometry for these transcripts were normalized to the respective 18S rRNA value in the same sample. For each of the four experiments, the individual measurement of a transcript in a cell line (*e.g*. 1A3/18S, CEM-C7 cell) was divided by the IM-9 value for the same transcript (1A3/18S, IM-9 cells). These ratios were multiplied by 100 to give the percent value, with IM-9 cells equal to 100%. The four individual percent values for each transcript in each cell line were then averaged to obtain the mean, and the SEM was calculated.

change in hormone-treated CEM-C7 or IM-9 cells (Fig. 6). Exon 1A1 and 1A2-containing transcripts were up-regulated approximately 2.1-fold and 3-fold, respectively, by hormone in CEM-C7 cells (Fig. 3A and data not shown). The basal expression levels of exon 1A1- and 1A2-containing transcripts in IM-9 cells were too low (Fig. 4) to accurately determine whether they are also down-regulated by hormone treatment. From these studies, it appears that the exon 1A-containing transcripts are the major hormone-responsive (both up-regulated and down-regulated) hGR mRNA species.

### The hGR 1Ap/e Sequence Has Promoter Activity

hGR 1Ap/e fragments were cloned in front of the luciferase reporter gene to determine whether any sequences confer basal promoter activity and respond to hormone treatment. Because CEM-C7 cells show very low transient transfection efficiencies, Jurkat cells were chosen. However, Jurkat cells lack functional hGR protein; therefore, an hGR cDNA expression plasmid was cotransfected along with the various hGR 1Ap/e-luciferase constructs. The −964/+269 bp hGR 1Ap/e fragment was able to drive the expression of the

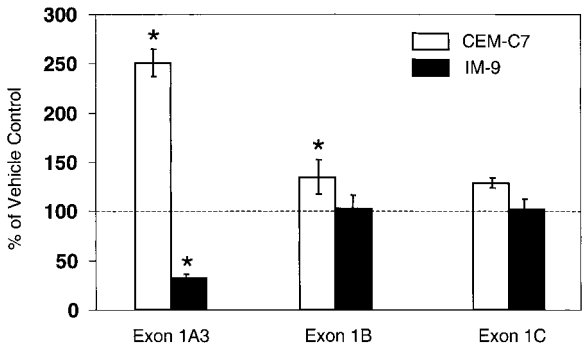


**Fig. 6.** Identification of hGR Transcripts That Are Hormone-Regulated

CEM-C7 cells and IM-9 lymphoblasts were treated for 24 h with ethanol vehicle alone or with 1 $\mu$M Dex. Total RNA was extracted and was analyzed using quantitative RT-PCR. The signal, as determined by densitometric scanning, for the hormone-treated sample was then compared with that of the ethanol vehicle control and expressed as a percentage of the control value. The *dashed line* represents the level of each transcript in the non-hormone-treated sample in each cell line, and it was set at 100% to allow comparison of the effects of hormone treatment. The data are from three separate experiments and represent the mean ± the SEM. *, $P < 0.05$ for the hormone-treated sample *vs.* the respective ethanol control value.

luciferase gene, while, as expected for an authentic promoter, luciferase activity was undetectable when the promoter region was cloned in the opposite orientation, +269/−964 bp, with respect to the luciferase gene (Fig. 7A). Hormone treatment of the −964/+269 bp hGR 1Ap/e-luciferase construct resulted in an approximately 2-fold increase in the measured luciferase activity (Fig. 7B) in CEM-C7 cells. This induction was dependent on the cotransfection of the hGR expression plasmid (data not shown). This stimulatory response is specific for T cells, as no increase in luciferase activity was observed when IM-9 cells were transfected with the −964/+269 reporter construct and then treated with Dex (Fig. 7B), even though IM-9 cells have sufficient endogenous GR to down-regulate endogenous GR 1A3 transcripts (Fig. 6). Further deletions to yield the −301/+269 bp and +41/+269 bp constructs showed no change in overall basal activity in CEM-C7 cells, and they were still hormone-responsive (Fig. 7, A and B). A deletion yielding the −619/+178 bp construct only retained 35% of the full-length basal promoter activity and no longer was induced by hormone treatment. A smaller deletion construct (+179/+269) had a very low level of basal activity (~15% of full-length activity; Fig. 7A) but still was induced by hormone (data not shown). Thus, the region between +41/+178 bp is critical for basal activity of hGR 1Ap/e, and the region between +179/+269 contains the hormone-responsive *cis*-acting elements.

To assay for enhancer activity, the +41/+269 and +179/+269 regions were cloned in both orientations into the pTAL plasmid, which contains a heterologous, TATA-box containing promoter. The +41/+269 element increased promoter activity in the 3′ to 5′ direction but not in the normal orientation (Fig. 8). The +179/+269 fragment demonstrated enhancer activity and increased transcription in both orientations. Unfortunately, Dex treatment of the pTAL plasmid backbone alone caused an inhibition of luciferase expression, so that the hormone responsiveness of these two hGR 1A elements could not be accurately tested using this heterologous promoter.

### At Least Six DNase I Footprints Occur in the hGR 1Ap/e Sequence, One of Which is an IRF-E

Four DNase I footprints (FP1–FP4) were observed upstream of position +1 (Fig. 2; data not shown). Because the deletion experiments indicated that the +41/+269 bp exon 1A fragment was important for both basal and hormone-induced gene expression, nuclear extracts from ethanol (control) or Dex-treated CEM-C7 cells were used to footprint this region. Both the coding and noncoding strands were analyzed. The protected patterns were identical using either control or Dex-treated extracts. Two separate protected sites or footprints were consistently observed in the +41/+269 bp exon 1A region, FP5 at position +102 to +125 bp, and FP6, which was quite extensive (Fig. 9A).

Computer analysis (16) of the FP5 region revealed a high degree of homology to the consensus IRF-E. To identify the proteins that specifically interacted with FP5, EMSAs were performed using ethanol (control) or Dex-treated CEM-C7 nuclear extracts with oligonucleotides from FP5 (+100 to +130 bp) and a consensus IRF-E. Similar results were observed with the control and hormone-treated nuclear extracts. EMSAs using the consensus IRF-E oligonucleotide yielded a single major protein-DNA complex (Fig. 9B). At least four distinct complexes, the major band of which comigrated to the same location as the consensus IRF-E complex, were observed with the FP5 oligonucleotide (Fig. 9, B and C). Addition of either an IFN-regulatory factor-1 (IRF-1) antibody or an IRF-2 antibody to the binding reaction resulted in a supershift complex using both the consensus IRF-E and FP5 oligonucleotides and CEM C7 cell nuclear extract (Fig. 9, B and C). Competition with unlabeled FP5 or the consensus IRF-E gave similar competition patterns, and a decrease in binding was seen with as little as a 50-fold excess of unlabeled oligonucleotide (Fig. 9, B and C). When the IRF-E was deleted from the competing FP5 oligonucleotide, it no longer was an effective competitor (data not shown). Therefore, FP5 in hGR exon 1A has all of the characteristics of an authentic IRF-E.

### The IRF-E Contributes to Basal Promoter Activity but Not to Hormone Responsiveness

*In vitro* site-directed deletion was performed to determine the overall contribution of the IRF-E (FP5) to basal or hormone-induced activity. Deletion of FP5

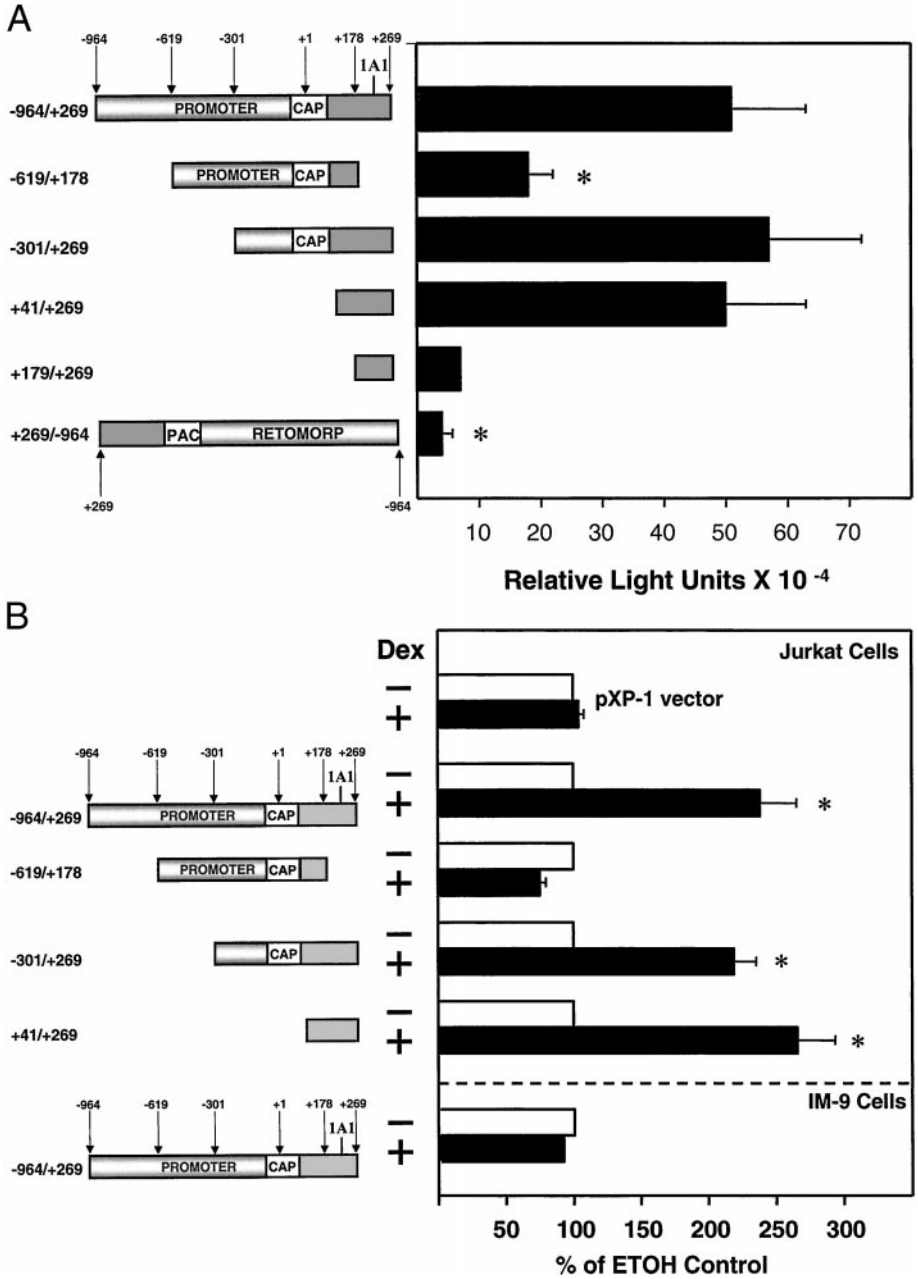


**Fig. 7.** Deletion Analysis of the Basal and Hormone-Induced Activity of the hGR 1A Promoter

Deletions were performed on the hGR promoter region linked to a luciferase reporter gene in the pXP-1 expression vector. The various constructs were cotransfected into Jurkat cells with a $\beta$-galactosidase construct driven by a constitutive promoter [cytomegalovirus (CMV)] to normalize for transfection efficiency. A, Basal promoter activity. The relative light units are the normalized value for the basal hGR 1A promoter constructs. Shown are the means and SEMs from the results of four separate experiments, except for the +179/+269 construct, which is the average of two experiments. *, $P < 0.05$ for the deletion constructs *vs.* the −964/+269 full length promoter value. B, Hormone-induced promoter activity. The experiments were performed as described in panel A, except that a constitutive GR expression vector was also cotransfected to reconstitute functional GR activity in the Jurkat cells. The hormone-treated pXP-1 vector control, normalized luciferase activity, was divided by the value for the ethanol-treated vector alone control and the value set at 100%. The other normalized values for the hGR 1A promoter constructs are then expressed as a percentage of the pXP-1 value. Shown are the means and SEMs of the means from four separate experiments. *, $P < 0.05$ for an increase in activity for the hormone-treated sample *vs.* the respective ethanol control value. IM-9 cells were treated in the same manner, except the constitutive hGR expression plasmid was not cotransfected because these cells contain functional, endogenous GR protein.

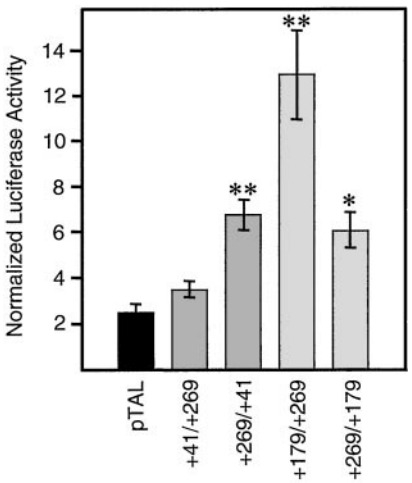


**Fig. 8.** Assay for Enhancer Activity of the hGR 1A Sequence

Two hGR 1A sequences were cloned into the heterologous expression plasmid pTAL in the normal 5′- to 3′-orientation (+41/+269; +179/+269) or in the reversed orientation (+269/+41; +269/+179). These were transfected into Jurkat cells and luciferase activity was assayed as described in the legend to Fig. 7 and in *Materials and Methods*. *, $P < 0.05$; **, $P < 0.01$, compared with the pTAL plasmid alone.

(TTCACTTCT) resulted in an approximately 55% decrease in basal activity of the full-length promoter (Fig. 10A). However, the FP5 deletion promoter construct was still responsive to hormone (Fig. 10B).

### FP6 Resembles a Glucocorticoid Response Element (GRE)

EMSAs were performed using the FP6 oligonucleotide. Numerous protein-DNA complexes were observed (Fig. 11), consistent with the extensive DNase I footprint that was seen (Fig. 9A). Competition assays using 50- or 1,000-fold molar excess of an unlabeled consensus GRE effectively competed away these complexes, suggesting that FP 6 bound the GR. The cognate FP6 oligonucleotide effectively competed the binding (data not shown), while the deletion of the sequences AGAAAA and TCTTCT from FP6 diminished its ability to compete out some of the bands, even at a 1,000-fold molar excess (Fig. 11). No supershift occurred using a GR$\alpha$ antibody, while a supershifted complex was obtained using a GR$\beta$ antibody. The ability of an anti-GR$\beta$ antibody to supershift one of these bands indicates that FP6 is a putative GRE. There are two reasons why the anti-GR$\alpha$ antibody did not cause a supershift. First, this antibody does not cause a good supershift even when a consensus GRE is used, indicating that this antibody does not function well in this assay. This is the most likely explanation. Second, it is possible that GR$\beta$ binds more avidly to the noncanonical FP6 GRE than does GR$\alpha$. We are not aware of any report of a GR$\beta$-specific GRE.

## DISCUSSION

In most cell types tested to date, GR mRNA and protein are down-regulated by GCs. Conversely, treatment of immature T lymphocytes and thymocytes and T lymphoblasts with hormone results in an up-regulation of GR mRNA and protein, and these cells undergo programmed cell death or apoptosis. GR up-regulation in T lymphoblasts is essential for hormone-mediated apoptosis (7). Therefore, we examined the regulation of the hGR gene in T cells to determine the molecular mechanisms involved in the cell type-specific expression and up-regulation of GR in these cells.

At least three separate promoters were identified for the mouse GR gene (9). All three mouse GR transcripts encoded an identical protein product and only differed in their 5′-exon 1 untranslated regions, designated exons 1A, 1B, and 1C. More recent studies indicate that there are at least five mouse GR untranslated exon 1 sequences arising from at least four separate promoters (17, 18), and the rat GR gene codes for at least 11 alternative first exons arising from multiple promoters (19). Thus, multiple promoters and untranslated first exons for the GR gene appear to be common.

The hGR gene contains at least three promoters whose utilization gives rise to at least five separate transcripts containing different 5′-untranslated first exons. The human exon 1A region utilizes three separate splice donor sites (1A1, 1A2, and 1A3) located within approximately 1 kbp of sequence. 1B- and 1C-containing transcripts were detected in all cell types tested, consistent with the fact that the GR is ubiquitously expressed. This also agrees with the fact that the proximal GR promoter/exon region (1B and 1C) is very GC rich, contains a CpG island characteristic of housekeeping genes, lacks TATA boxes and CAAT boxes, and has numerous Sp1-binding sites (20). There are 3 YY1 sites, four Sp1 sites, and one unidentified footprint upstream of the exon 1B transcription start site, and the hGR promoter 1B sequence can drive transcription in the absence of the promoter 1C sequence (Ref. 21 and Nunez, B. S., and W. V. Vedeckis, in preparation). Thus, there appear to be at least two constitutive hGR promoters that occur in a region proximal to the hGR-coding exons. In contrast, exon 1A3-containing transcripts were expressed at highest levels in cancer cells of hematopoietic origin. Exon 1A1 and 1A2 transcripts have a broader expression pattern, even though they presumably share a common promoter with exon 1A3, suggesting that tissue-specific alternative splicing and not promoter usage may result in the relative abundance of the hGR exon 1A3 transcript in blood cells. Further analysis of the tissue-specific splicing of these various transcripts is needed to resolve these questions.

The exon 1A1-, 1A2-, and 1A3 (but not exon 1B or 1C)-containing transcripts were up-regulated by hor-

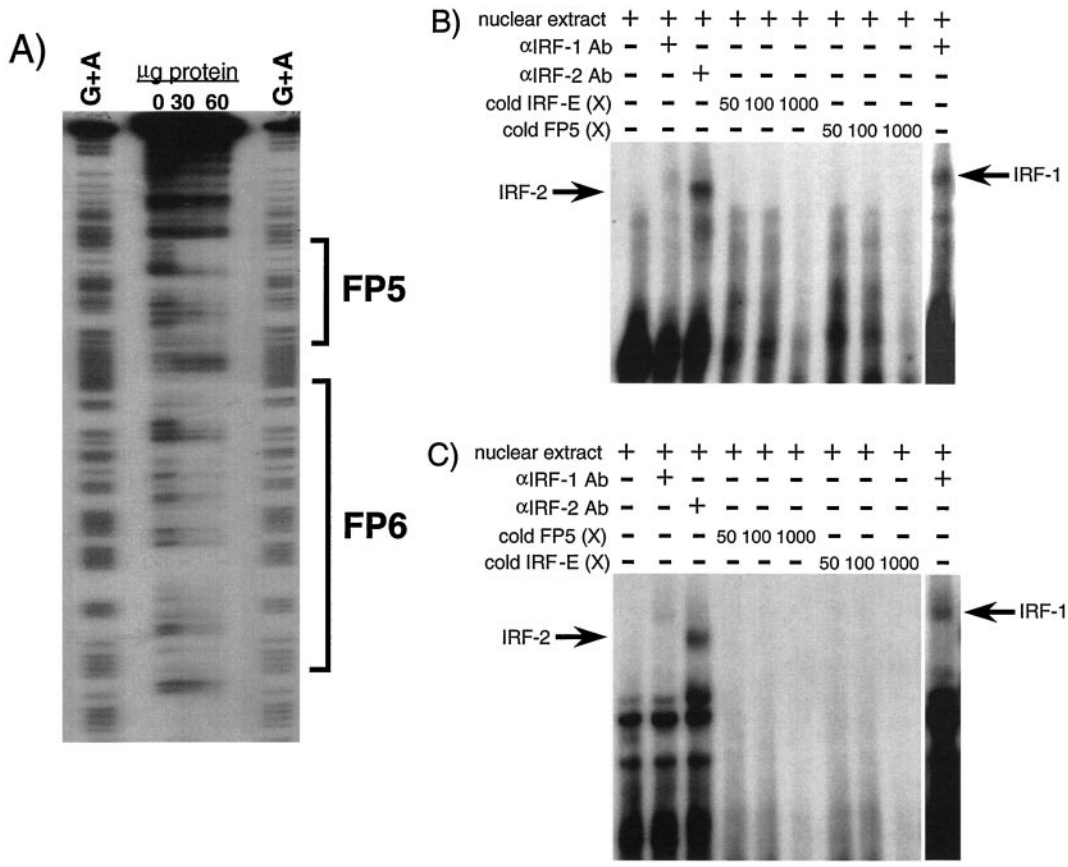


**Fig. 9.** Protein Binding Regions of the hGR Exon 1A Region

A, DNase I footprinting was performed on the +41/+269 fragment of the hGR 1Ap/e sequence in the presence of 0, 30, or 60 μg of CEM-C7 cell nuclear protein. Two footprints were revealed, FP5 (+102/+125) and FP6. B, EMSA using a consensus IRF-E. A consensus IRF-E oligonucleotide was $^{32}$P-labeled and mixed with nuclear extracts obtained from CEM-C7 cells that were treated for 24 h with 1 μM Dex. Gel shift analysis was performed on the complex formed. Antibodies to IRF-1 or IRF-2 were added as indicated. *Arrows* indicate the supershifted complexes seen with the IRF-1 and IRF-2 antibodies, respectively. Fifty-, 100-, or 1,000-fold excess of unlabeled consensus IRF-E or FP5 was added in competition experiments. The *dark band at the bottom* of the gel is the major DNA-protein complex. The *rightmost* lane is an overexposure of the second lane to more clearly show the IRF-1 supershifted complex. C, The same experiment was performed as in panel B except that a $^{32}$P-labeled FP5 oligonucleotide was used instead of the consensus IRF-E sequence.

mone in CEM-C7 cells, and exon 1A3-containing transcripts were down-regulated in IM-9 cells, while transcripts with exon 1B or 1C were not. The absence of down-regulation for the 1B- and 1C-containing transcripts was surprising, because an internal control region (coding for amino acids 550–697) is clearly involved in GC-mediated down-regulation in transfection experiments (22). One possibility is that exon 1 sequences may also contribute to hormone-mediated down-regulation. The cDNA construct used in Ref. 22 began at exon 2; *i.e.* it lacked any exon 1 sequences. Perhaps under normal *in vivo* circumstances exon 1B and exon 1C inhibit hormone-mediated GR down-regulation caused by the intragenic hGR DNA sequences, and that this inhibition is lost when an exon 2–9 cDNA construct is used. Our results suggest that the exon 1A3-containing transcript is the down-regulated species of hGR mRNA in B cells. However, when the −964/+269 hGR 1Ap/e sequence was placed into a luciferase expression vector, no hormone-mediated decrease in luciferase expression occurred in IM-9 cells. Thus, both exon 1A3 sequences and the intragenic GR cDNA control region may normally be required *in vivo* for hormone-mediated down-regulation.

The −964 to +269 bp hGR 1Ap/e fragment has basal promoter activity and is affected by hormone treatment in Jurkat T cells. Four of the six DNase I footprints identified to date are located in the promoter region (FP1–FP4), while two are intraexonic (FR5, FP6). Visual inspection reveals that FP4 contains a TATA box-like sequence at position −60, and FP2 contains a sequence resembling a CAAT box at position −195.

The intraexonic location of FP5 and FP6 was surprising. The fact that the +41/+269 fragment conferred basal activity and hormone inducibility to the pXP-1 luciferase expression vector may mean that a cryptic promoter in the vector can be activated by this

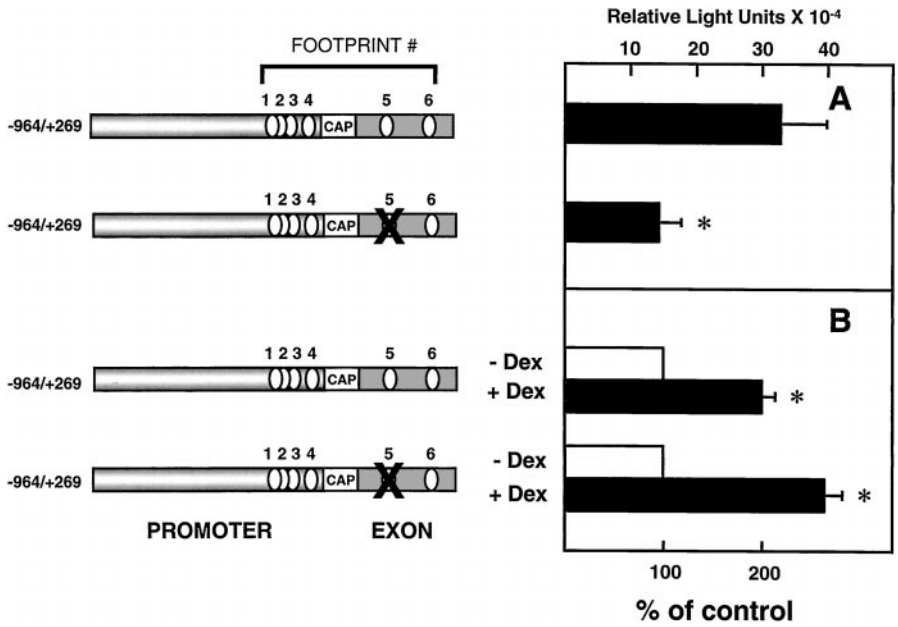


**Fig. 10.** Deletion Mutagenesis of FP5

*In vitro* mutagenesis was performed on the −964/+269 hGR 1Ap/e sequence cloned into the pXP-1 luciferase reporter gene to delete the core sequence (5′-TTCACTTCA-3′) of the IRF-E in FP5. The undeleted and deleted hGR 1Ap/e constructs were then cotransfected with a constitutive hGR cDNA expression vector and a β-galactosidase normalization vector into Jurkat cells as described in the legend to Fig. 8. Shown are the mean and the SEM for three separate experiments. A, Effect of FP5 deletion on basal promoter activity. *, $P < 0.05$ for a decrease in activity for the deletion construct *vs.* the −964/+269 full length promoter value. B, Effect of FP5 deletion on hormone-induced hGR 1Ap/e promoter activity. The *open bar* is the value obtained in the absence of hormone, and it is set at 100%, even though the absolute activity of the FP5-deleted construct was considerably lower (see panel A) than for the undeleted construct. Shown are the mean and the SEM for three separate experiments. *, $P < 0.05$ for an increase in activity for the hormone-treated sample *vs.* the respective ethanol control value.

intraexonic piece of DNA. The +179/+269 sequence demonstrates enhancer activity. Internal control regions for eukaryotic genes have recently become better characterized. For example, a downstream promoter element, or DPE, has been identified in many TATA-less promoters (23), although this sequence does not occur in hGR 1Ap/e. Also, ER factor-1 regulates ER transcription by binding to an intraexonic element in the ER gene that codes for an untranslated exon 1 (24). The FP5 and FP6 intraexonic sequences in hGR 1Ap/e may be additional examples of this gene-regulatory mechanism.

FP5 is an IRF-1 binding site that can bind IRF-1 and IRF-2, both of which are transcription factors that were identified based on their ability to regulate the expression of the IFN-β gene in response to IFNs (25, 26). IRF-1 and IRF-2 bind the same DNA site (26–28); IRF-1 is an activator of type 1 IFNs and IFN-inducible genes, whereas IRF-2 represses the effect of IRF-1 (29, 30). However, IRF-2, also known as histone nuclear factor M, does play a positive role in the cell cycle regulation of the human histone $H_4$ gene *FO108* (31), and IRF-2 directs muscle cell-specific expression of vascular adhesion molecule-1 in the absence of cytokine stimulation (32). The roles of IRF-1 and IRF-2 in regulating *in vivo* expression of exon 1A-containing transcripts of the hGR are under current investigation.

Computer analysis suggested that FP6 contains an apparent half-GRE, and this sequence can bind GRβ. However, studies to determine regions of the hGR 1Ap/e sequence that confer hormone responsiveness have not yet yielded clear-cut results. For example, deletion of 6 or 12 nucleotides of the apparent GRE in FP6 did not completely abolish Dex responsiveness (our unpublished observations). There is a broad region of DNase I protection in the FP6 footprint, so other transcription factors may also bind this sequence. Computer analysis of the +41/+269 region revealed that there are four additional potential half-GRE binding sites, but these have not yet been characterized. Because of the complexity of the +41/+269 sequence, a detailed analysis of the regions of the exon 1A sequence that contribute to the hormonal activation of GR 1A promoter and GR up-regulation is required and ongoing. Finally, FP3 resembles a nuclear factor-κB binding site, and preliminary studies show that the nuclear factor-κB can bind to this sequence (Geng, C.-D., and W. V. Vedeckis, unpublished observations).

During the course of these studies, two reports appeared for the mouse GR gene that are similar to ours for the hGR gene (17, 18). These studies detected mouse GR transcripts 1A and 1B, plus two new transcripts, 1D and 1E. Thus, there are four mouse GR

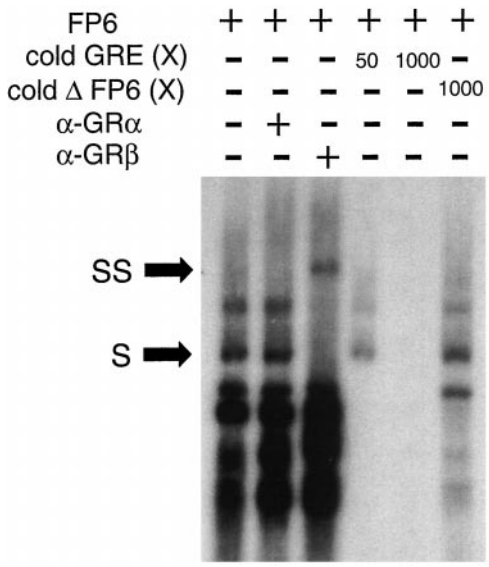


**Fig. 11.** EMSA of FP6

Radiolabeled FP6 was incubated with nuclear extract of CEM-C7 cells that had been treated with 1 $\mu$M Dex for 24 h. A complex EMSA is obtained. Addition of a GR$\alpha$ antibody did not cause a clear supershift, while addition of a GR$\beta$ antibody caused a distinct supershift (SS) with a concomitant loss of a lower band (S). A 50-fold or 1,000-fold excess of unlabeled consensus GRE was added as indicated. A 50- or 1,000-fold excess of unlabeled FP6 completely eliminated the shifted bands (data not shown), while an FP6 oligonucleotide from which the putative GRE was deleted did not compete as effectively for all of the bands.

transcripts, 1B, 1C, 1D, and 1E, that originate in the proximal promoter, while the exon 1A transcript originates far upstream, as originally shown by Strähle *et al.* (9). The 1A transcript was expressed at high levels in mouse S49 lymphoma cells that were enriched in the membrane-associated GR believed to be involved in hormone-mediated apoptosis (17). Computer comparison of the hGR 1Ap/e sequence with the mouse 1A sequence indicated 60.6% sequence identity in the promoter region and 61.6% identity for exon 1A (data not shown). The 100 bp between +104 and +203 in hGR 1Ap/e [which contains the IRF-E (FP5) and FP6] is 73.5% identical to the corresponding region in the mouse 1A sequence (data not shown). Positions +111 to +122 in the minus strand of FP5 of the hGR 1Ap/e sequence contain a perfect direct repeat, AAGTGA, of an IRF-E sequence (26, 27), and, except for a transition in one nucleotide, this is completely conserved in the mouse 1A sequence. Although Chen *et al.* (17, 18) did not study the hormonal regulation of any of the transcripts in their studies, it is likely that the mouse 1A transcript will also be selectively up-regulated by glucocorticoids in mouse T-cell leukemia and lymphoma as well.

Autoinduction of GR levels is essential for apoptosis in human T lymphoblasts (7), and this effect appears to be mediated specifically via the exon 1A hGR transcript. Thus, the present findings may have clinical relevance for T-cell ALL patients. RT-PCR of the exon 1A3-containing GR transcripts may help in diagnosing elevated blast levels in peripheral blood leukocytes at initial presentation and early relapse. *Ex vivo* challenge of peripheral blood leukocytes with Dex may be useful for prognosis, *e.g.* if T-cell ALL blasts show up-regulation of exon 1A3-containing transcripts, this would indicate a hormonally responsive phenotype. Determining transcriptional activators of the hGR 1Ap/e sequence may elucidate signal transduction pathways leading to new therapeutic approaches for up-regulating GR levels by treatment with biological response modifiers (such as IFNs, cytokines, lymphokines, etc.) that could lead to increased steroid sensitivity. Further detailed studies are required to analyze the possible clinical applications of the hGR 1Ap/e sequence.

## MATERIALS AND METHODS

### Cell Culture

Human CEM-C7 ALL cells (a kind gift from Dr. E. Brad Thompson, University of Texas Medical Branch, Galveston, TX) were grown in RPMI 1640 (Life Technologies, Inc., Gaithersburg, MD) supplemented with 10% dialyzed, heat-inactivated FBS (Life Technologies, Inc.). Human Jurkat ALL cells (ATCC, Manassas, VA) were grown in RPMI 1640 (Life Technologies, Inc.) supplemented with 10% FBS (Life Technologies, Inc.). Cells were treated with either 1 $\mu$M Dex (Sigma, St. Louis, MO) or ethanol (0.01% vehicle alone).

### 5′-RACE

A MOLT-4 (human T-cell ALL) Marathon ready double-strand cDNA library (CLONTECH Laboratories, Inc.) was used in the 5′-RACE reaction. 5′-RACE was performed using a 5′-AP-1 primer (CLONTECH Laboratories, Inc.) (5′-CCATCCTAATACGACTCACTATAGGGC-3′) and two separate 3′-primers to exon 2 of the human GR gene, exon 2 primer no. 1 (5′-GGGTTTTATAGAAGTCCATCACATCTCC-3′), and exon 2 primer no. 2 (5′-CGACAGCCAGTGAGGGTGAAGACG-3′). All custom-made oligonucleotide primers were obtained from Sigma Genosys (The Woodlands, TX). A control 5′-RACE reaction was performed with the 5′-AP-1 primer and a 3′-glyceraldehyde-3-phosphate dehydrogenase primer (5′-GACCACAGTCCATGACATCACT-3′). The RACE reaction contained 0.5 ng MOLT-4 cDNA library, 10 $\mu$M each of the 5′- and 3′-primers, 0.2 mM deoxynucleoside triphosphates (CLONTECH Laboratories, Inc.), 1X Advantage *Taq* PCR buffer (CLONTECH Laboratories, Inc.), and 1X Advantage *Taq* DNA polymerase (CLONTECH Laboratories, Inc.) in a 50-$\mu$l reaction. The PCR reaction was performed in a 9600 Thermocycler (Perkin-Elmer Corp., Norwalk, CT) with an initial denaturation of 94 C for 1 min, five cycles of 94 C for 10 sec, 72 C for 3 min; five cycles of 94 C for 10 sec, 70 C for 3 min, and 25 cycles of 94 C for 10 sec, 68 C for 3 min. The PCR products were analyzed on a 1.2% agarose/1× Tris-borate-EDTA gel containing 0.1 $\mu$g/ml ethidium bromide. The band of interest was excised from the gel and purified using the GENECLEAN III kit (BIO 101, Vista, CA). The gel-purified DNA was ligated into the pCR2.1 TA cloning vector (Invitrogen, Carlsbad, CA). Clones were sequenced using the 3′-exon 2 human GR gene primers (nos. 1 and 2) using a Thermosequenase Kit (Amersham Pharmacia Biotech, Arlington Heights, IL).

### RT-PCR

Total RNA was collected from 24 h ethanol (control) or 1 $\mu$M Dex-treated cells using TriReagent (Molecular Research Center, Inc., Cincinnati, OH). For RT-PCR, 1 $\mu$g total RNA was reverse transcribed using a random hexamer primer (RT-for-PCR kit, CLONTECH Laboratories, Inc.), and the resulting cDNA was diluted to 100 $\mu$l final volume. PCRs were carried out in the linear range of amplification for each primer set, exon 1A1/exon 2 (30 cycles), exon 1A2/exon 2 (34 cycles), exon 1A3/exon 2 (29 cycles), exon 1B/exon 2 (27 cycles), and exon 1C/exon 2 (25 cycles), using the same reaction conditions described for 5′-RACE. PCR was performed in a 25 $\mu$l reaction volume with an initial denaturation step of 94 C for 2 min followed by 94 C for 30 sec, 68 C for 30 sec, and 72 C for 30 sec with the exception of the exon 1A1/exon 2 and exon 1A2/exon 2 primer sets, for which the annealing temperature was 64 C for 30 sec. Quantu*mRNA* 18S internal control primers (1:9 primer-competitor ratio) (Ambion, Inc.) were included in each reaction to normalize the data. Primer sets used for RT-PCR were: exon 1A3, 5′-GCTTCATTAAAGTGTCTGAGAAGG-3′; exon 1B, 5′-GCAACTTCTCTCCCAGTGGCG-3′; and exon 1C, 5′-CTTAAATAGGGCTCTCCCCC-3′. The 3′-primer for all of the primer sets was hGR exon 2 primer no. 2. Primers to determine the 5′-end of the exon 1A transcripts were: GR 1Ap/e, −272 bp 5′-GGTAACCAAGGCATCACACT-3′; GR 1Ap/e, −72 bp 5′-GATGACACAGACTAATAACCAATG-3′; GR 1Ap/e, +56 bp 5′-TTGCTCCCTCTCGCCCTCATTC-3′; GR 1Ap/e, +126 bp 5′-CTGGGGAAATTGCAACACGC-3′; GR 1Ap/e, +222 bp 5′-CTTTCAAGCCCTGCAGGACC-3′; GR 1Ap/e, +455 bp 5′-TCTGCCTGGGGGAATATCTGC-3′; and GR 1Ap/e, +955/+981 bp 5′-GCTTCATTAAAGTGTCTGAGAAGGAAG-3′. The downstream primer used was the hGR exon 2 primer no. 2. RT-PCR for the exon 1A splice variants, exon 1A1 and 1A2, was performed using a 5′-upstream primer in the exon 1A region common to all three splice variants and a 3′-primer that spans the specific exon 1A1 or 1A2/exon 2 splice junction. The RT-PCR primers for the exon 1A1 splice variant were 5′-primer +56 bp and downstream primer 5′-GTGAATATCAACTTCTAAGGTCCAGTG-3′. RT-PCR primers for the exon 1A2 splice variant were 5′-primer +126 bp and downstream primer 5′ GTGAATATCAACTCTTTCTGTTTC 3′.

### DNA Constructs

The −964 to +269 hGR 1A promoter/exon region was amplified using the 5′-PCR primer, 5′-CCAGCAGTTTTATAGAAGCTAACAC-3′, and the 3′-PCR primer, 5′-GCTTCCGTTGGACACATGCG-3′. The resulting PCR product was subcloned into the pCR II TA TOPO cloning vector (Invitrogen). The −964/+269 hGR 1A p/e fragment was liberated from the pCR II TOPO vector using *Eco*RI, Klenow treated, and subcloned into the 5′ to 3′ or 3′ to 5′ orientation with respect to the luciferase gene in the pGL3 Basic vector (Promega Corp.). The pGL3 Basic vector was prepared by *Xba*I digestion and Klenow treatment to create flush ends. The −964/+269 or +269/−964 hGR 1A p/e constructs were liberated from the pGL3 Basic vector by *Nhe*I followed by Klenow treatment and *Hin*dIII digestion and ligation into the *Sma*I/*Hin*dIII digested pXP-1 luciferase vector (ATCC). Deletion constructs of the pXP-1 −964/+269 hGR 1A p/e region were created by restriction endonuclease digestion at various sites. *Bgl*II digestion of the pXP-1 −964/+269 construct results in the liberation of a −964/−619 and a −618/+178 fragment. The pXP-1 +179/+269 hGR 1A p/e fragment and −618/+178 *Bgl*II inserts were gel purified and re-ligated to create the pXP-1 −618/+178 and +179/+269 constructs. The −301/+269 hGR 1A p/e construct was created by digesting the pXP-1 −964/+269 1A promoter/exon construct with *Bam*HI/*Sac*I. The resulting pXP-1 −301/+269 vector fragment was gel purified and subsequently treated with $T_4$ DNA polymerase (NEB) to create blunt ends before re-ligation. The pXP-1 plasmid containing the −964/+269 fragment was digested with *Kpn*I and the pXP-1 plasmid containing the +41/+269 1A fragment was gel purified and religated to create the pXP-1 +41/+269 construct. The −964/+41 GR 1A p/e pXP-1 construct was generated by double-digestion of the pXP-1 plasmid containing the −964/+269/fragment with *Kpn*I/*Hin*dIII. The resulting −964/+41 GR 1Ap/e pXP-1 construct was gel purified and treated with $T_4$ DNA polymerase (Life Technologies, Inc.) before subsequent re-ligation. The pTAL plasmid (CLONTECH Laboratories, Inc.) was digested with *Sma*I and phosphatase-treated. The +41/+269 and +179/+269 fragments were prepared as described above, Klenow treated, and blunt-end ligated into the *Sma*I-cleaved pTAL. Orientation was confirmed by DNA sequencing.

### Transient Transfections

Jurkat and IM-9 cells were seeded at $2 \times 10^6$ cells/ml in six-well culture dishes. Transient transfections into Jurkat cells were performed using 2.5 $\mu$g DNA (1 $\mu$g luciferase construct in either the pXP-1 or pGL3 Basic vector, 1 $\mu$g pCYGR human GR expression plasmid (a kind gift from Dr. Jawed Alam, Alton Ochsner Medical Foundation, New Orleans, LA), and 0.5 $\mu$g pCMV-$\beta$-galactosidase plasmid), 6 $\mu$l Plus reagent, and 6 $\mu$l lipofectamine reagent (Lipofectamine-Plus reagent, catalogue no. 10964–013, Life Technologies, Inc.) according to the manufacturer's instructions. Transfections into IM-9 cells were performed with 2 $\mu$g DNA (1.5 $\mu$g luciferase construct in the pGL3 Basic vector, 0.5 $\mu$g pCMV-$\beta$-galactosidase vector), 6 $\mu$l Plus reagent, and 6 $\mu$l lipofectamine reagent. Twenty-four hours post transfection the cells were treated with either 0.01% ethanol (control) or 1 $\mu$M Dex. Forty-eight hours post transfection the cells were assayed for luciferase and $\beta$-galactosidase activities in a Dynatech ML 2250 luminometer as previously described (21).

### DNase I Footprint Analysis

DNase I footprint analysis was performed as previously described (21) except that the DNase I concentration for the zero protein control was 11 ng/$\mu$l. The nuclear extracts used for footprinting were prepared as described elsewhere (33). Briefly, nuclear extracts were prepared from 1 liter CEM-C7 cell cultures treated for 24 h with 0.01% ethanol (control) or 1 $\mu$M Dex. The DNA fragment used in the footprinting reaction was the pGL3 basic vector containing the+41/+269 hGR 1Ap/e fragment. Both the coding and noncoding strands were labeled by digestion with the restriction endonuclease *Bam*HI (followed by a subsequent *Hin*dIII digestion) or *Hin*dIII (followed by a subsequent *Bam*HI digestion) and filled-in using the Klenow fragment of DNA polymerase (New England Biolabs, Inc., Beverly, MA).

### EMSAs

The nuclear extract used for EMSAs was the same as used in DNase I footprint analysis. EMSAs were performed as previously described (21). Supershift assays using either the IRF-1 antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA; catalogue no. sc 497) or IRF-2 antibody (Santa Cruz Biotechnology, Inc., Cat. No. sc 498) were performed by adding 4 $\mu$g of the antibody to the binding reaction 1 h before the addition of the labeled oligonucleotide. The reaction was then incubated at room temperature for an additional 15 min before resolving on a gel. The consensus IFR-E oligonucleotide (catalogue no. sc 2575) and consensus GRE oligonucleotide (catalogue no. sc 2545) was purchased from Santa Cruz Biotechnology, Inc. Oligonucleotides for exon 1A FP5 were 5′-GTAGAGGCGAATCACTTTCACTTCTGCTGGG-3′ and 5′-

CCCAGCAGAAGTGAAAGTGATTCGCCTCTAC-3′ and exon 1A FP6 5′-GAGAAGGAGAAAACTTAGATCTTCTGATACCAA-3′ and 5′-TTGGTATCAGAAGATCTAAGTTTTCTCCTTCTC-3′. Anti-GR antibodies used were GR$\alpha$-specific (Santa Cruz Biotechnology, Inc., catalogue no. sc-1002) and GR$\beta$-specific (Affinity BioReagents, Inc. Golden, CO; catalogue no. PA3–514).

### *In Vitro* Site-Directed Mutagenesis

Site-directed mutagenesis was performed using the MutaGene *in vitro* Mutagenesis Kit (Bio-Rad Laboratories, Inc., Hercules, CA). Twelve picomoles of the FP5 deletion oligonucleotide (5′-GCAATTTCCCCAGCAGTGATTCGCCTCTA-CTC-3′) was used in the mutagenesis reaction.

## Acknowledgments

We are thankful to Dr. Margaret DeAngelis and Dr. Mark A. Batzer (Department of Pathology, Louisiana State University Health Sciences Center, New Orleans, LA) for assistance in the BAC screening, and to Joelle Finley for maintaining the cell cultures.

Received June 5, 2000. Accepted May 30, 2001.

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This work was supported by NIH Grant DK-47211 (to W.V.V.) and a grant from the Louisiana State University School of Medicine Institutional Research Enhancement Fund.

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## Note Added in Proof

The GenBank accession number for the hGR 1Ap/e sequence is AF395116.

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