Steroid-Responsive Sequences in the Human Glucocorticoid Receptor Gene 1A Promoter

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At least three promoters (1A, 1B, and 1C) control the expression of mRNA transcripts for the human glucocorticoid receptor (hGR) protein. An hGR 1A promoter/exon sequence (−218/+269) contains at least 12 deoxyribonuclease (DNase) I footprints that contain bound protein. Whereas four of these footprints (FP6, FP7, FP8, and FP11) contain bound hGR in protein-DNA complexes that are formed, only two (FP7 and FP11) appear to be important for the up-regulation of hGR 1A promoter/exon activity in T-lymphoblasts. Furthermore, the activity of these DNA elements depends upon the promoter context, leading to a redundant and complex regulation of expression of the hGR 1A promoter/exon. FP7 appears to be required for hormonal responsiveness in the absence of upstream sequences (+41/+191), whereas the hormonal re-

sponsiveness of FP11 requires a functional, adjacent FP12 DNA sequence. FP12 contains overlapping binding sites for the protooncogene transcription factors c-Myb and c-Ets. It seems likely that binding of either c-Myb or c-Ets to FP12 is necessary for the direct or indirect binding of the hGR to FP11 (a nonconsensus glucocorticoid response element), and the resultant steroid-responsiveness of the hGR 1A promoter/exon sequence. We propose that the identity of the accessory transcription factor bound to FP12 (c-Myb or c-Ets) may determine the nature of regulation (positive or negative) of hGR gene expression by hormone, and that this may be important for hormone-induced apoptosis in T cell acute lymphoblastic leukemia. (*Molecular Endocrinology* 18: 912–924, 2004)

GLUCOCORTICOID STEROID HORMONES play important roles in metabolism and in the immune system (reviewed in Refs. 1 and 2). They affect various biological processes and can inhibit cell proliferation, promote cell differentiation, induce G1 arrest, and trigger programmed cell death of certain types of lymphoid cells including pre-B lymphoma cells, peripheral T lymphocytes, immature thymocytes, and some leukemia cell lines (3–7). Several synthetic glucocorticoid steroids have been widely used for treatment of asthma, inflammation, and as therapeutic agents to treat certain types of leukemia and lymphoma (8–10).

Glucocorticoid ligands bind to the glucocorticoid receptor (GR) and modulate the receptor to regulate transcription of target genes that have a glucocorticoid response element (GRE) (11, 12). The GR belongs to the steroid receptor superfamily that includes the mineralocorticoid, progesterone, androgen, and estrogen receptors and others. These transcription factors all have a nonconserved N-terminal domain, a conserved DNA binding domain that binds to the hormone response elements (HREs), and a C-terminal ligand

binding domain that mediates steroid activation of receptors (13). Hormone-binding causes receptor binding to the HRE, recruitment of various coactivator proteins, changes in chromatin structure, and, finally, altered rates of gene transcription (reviewed in Refs. 14 and 15).

Natural and more potent, synthetic glucocorticoids are used as agents in cancer chemotherapy regimens for lymphomas and leukemias (3, 10, 16). In immature thymocytes and certain leukemia and lymphoma cells, the steroid causes an initial G1 arrest followed by programmed cell death, or apoptosis (4, 6, 17). However, the precise molecular mechanism by which the steroid signal triggers apoptosis is unknown. It is well established that glucocorticoids cause a down-regulation in GR mRNA and protein levels in most cell types (18, 19). Immature thymocytes and T-lymphoblasts are the exceptions, as an auto-up-regulation in GR levels occurs upon hormone treatment (20). Most importantly, auto-induction of GR expression appears to be critical for apoptosis in at least one sensitive T cell acute lymphoblastic leukemia (ALL) cell line (21). Therefore, elucidating the mechanisms that promote the auto-induction of human GR (hGR) expression will help in revealing their roles in triggering hormone-induced apoptosis.

At least three promoters control hGR gene expression, 1A, 1B, and 1C (22). Promoters 1B and 1C seem to be housekeeping gene promoters because they are GC rich and expressed in most if not all cells (22). Initial

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studies suggested that the hGR 1A promoter is active mainly in hematopoietic cells (22). Although various transcription factors (Sp1, AP-2, YY1) bind to and presumably influence the expression of the GR gene in the 1B and 1C promoters, no GRE and other GRE-like regulatory element were found in these promoters. Furthermore, luciferase reporter genes linked to promoters 1B plus 1C did not respond to hormone stimulation when transiently transfected into T cells (our unpublished observations). On the contrary, the 1A promoter (which is located about 25 kb upstream of the 1B promoter) is significantly stimulated by the synthetic glucocorticoid, dexamethasone (DEX), in CEM-C7 T-ALL cells and when it is incorporated into a luciferase reporter construct and transiently transfected into Jurkat T-ALL cells (22). Elucidation of the molecular mechanism for steroid activation of the hGR 1A promoter has been challenging. Six DNase I footprints were found in the hormone-responsive region of 1A promoter/exon sequence, 3 of which were identified as binding sites for nuclear factor-κB (p65), interferon regulatory factor (IRF) 1/2, and GR. We suggested that the GR binding element (FP6) in the human 1A promoter/exon was the potential target site of hormonal responsiveness (22). Here we show that other DNA elements existing downstream of FP6 mediate
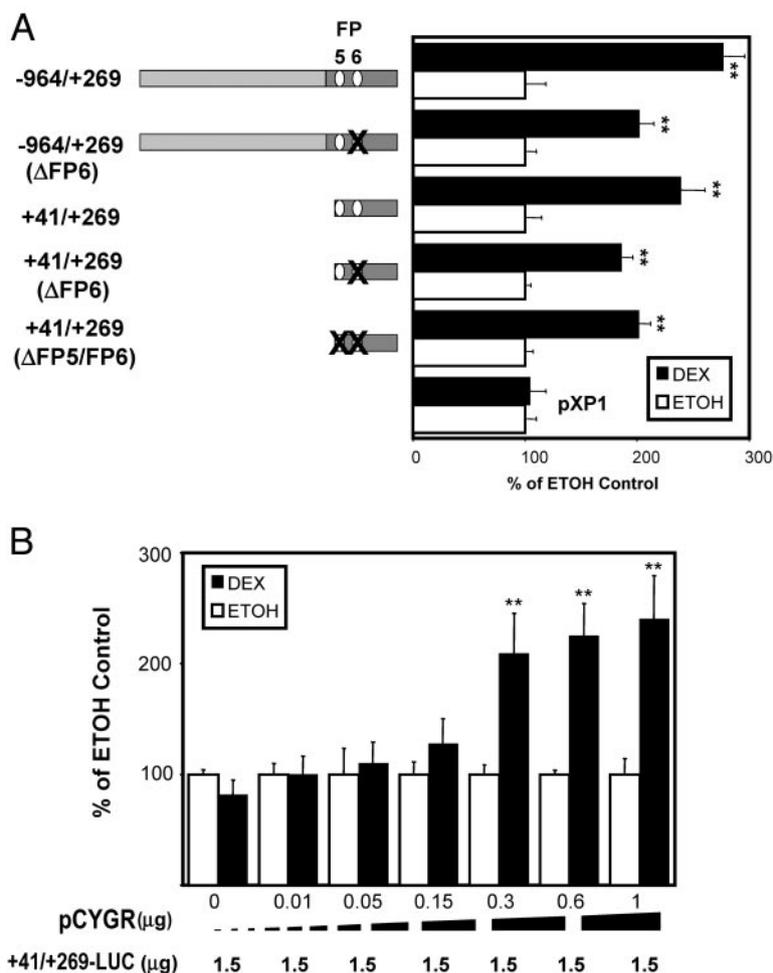

Fig. 1. Hormone Responsiveness of the hGR 1A Promoter/Exon

hGR 1A promoter-luciferase reporter constructs were cotransfected with a CMV-β-galactosidase expression construct (for normalizing transfection efficiency). For each set, the normalized, DEX-treated sample luciferase activities are expressed as a percentage of the mean value of ethanol-treated samples. A, Hormone-induced promoter activity in deletions of FP5 and/or FP6. *In vitro* deletions were performed in either the −964/+269 or +41/+269 hGR 1A promoter/exon linked to the luciferase reporter gene. DEX (1 μM) treatment was for 24 h. The pCYGR human GRα expression vector (1 μg), was included in each transfection reaction. **, $P < 0.01$ for the reporter activity in DEX-treated samples *vs.* ethanol controls. B, The hormone responsiveness of the hGR 1A promoter/exon requires the expression of functional hGRα protein in Jurkat cells. Increasing amounts of pCYGR were cotransfected with the hGR 1A +41/+269 promoter/exon reporter construct. The same amount of total transfected DNA was maintained in each experiment using an empty vector. **, $P < 0.01$ for an increase in activity for the hormone-treated sample *vs.* the ethanol-treated control value. Results of three or four separate experiments were used for calculating the means and SEM in all experiments.

the auto-induction process in this promoter. We mapped the functional glucocorticoid response elements (units) in the 1A promoter, mapped the DNA sites at which the GR proteins bind, and identified other transcription factors that may contribute to the hormonal response by binding to adjacent elements. This leads to a hypothesis that protein-protein interaction between the GR and a member of the c-Myb and/or c-Ets family of transcription factors controls hGR 1A promoter activation. These results may help in understanding the mechanism whereby T cell leukemias are either sensitive or resistant to hormone therapy and may lead to new therapeutic approaches for the treatment of leukemia patients.

RESULTS

The hGR 1A Promoter/Exon Is Activated by Hormone in T-Lymphoblasts

Previous studies showed that the hGR 1A promoter is induced by DEX treatment in the human T cell ALL cell line, CEM-C7 (22). An intraexonic sequence, +41/+269 showed promoter activity and mediated hormonal stimulation in the Jurkat ALL cell line when inserted in a promoterless reporter plasmid pXP1 (Fig. 1A), whereas the further upstream sequence (−964/+41) did not respond to hormone (22). Thus, the DEX response of the +41/+269 fragment was further investigated to locate the sequence(s) required for the hormone response. Two DNase I footprints were previously described (22). FP5, which is an interferon regulatory factor-element (IRF-E) (23), has no effect on the DEX response. Although FP6 binds hGR proteins *in vitro* (22), deletion of FP6 from the 1A +41/+269 sequence did not abolish the hormone responsiveness (Fig. 1A). The same result was observed when FP6 was deleted from the 1A −964/+269 fragment (Fig. 1A). These observations suggested that one or more elements exists downstream of FP6 in the +41/+269 sequence and mediate(s) the DEX response in the 1A

promoter, and that FP6, at least alone, is not the sole functional GRE that mediates the hormonal response of the hGR 1A promoter in T cells.

The hormonal auto-induction of 1A promoter activity in T cells is clearly GR dependent. In Jurkat cells, which do not have functional endogenous GR protein, the +41/+269 reporter construct showed no response to DEX treatment without cotransfection with a GR cDNA expression plasmid, pCYGR (Fig. 1B), whereas the hormonal response showed a proportional dose dependence on the amount of pCYGR that was cotransfected (Fig. 1B).

There Are Six Additional DNase I Footprints in the hGR 1A +187/+269 Sequence

Using DEX (1 μM)-treated CEM-C7 nuclear extracts, DNase I footprinting was done to identify possible protein binding elements that mediate hormone stimulation in the +187/+269 region. This DNA fragment is highly protected by protein complexes because six DNase I footprints (FP7–FP12) were identified in this region (Fig. 2). Comparing these footprint sequences with known consensus transcriptional factor binding DNA core sequences, no conserved positive GREs were found. However, FP8 shares a homologous core sequence to a negative GRE in the POMC gene (24) and a consensus core sequence for the Nur77 and COUP TF proteins (25). FP12 contained overlapping conserved binding sequences for c-Myb and c-Ets (reviewed in Ref. 26).

Multiple DEX-Responsive Regions Exist in the hGR 1A Promoter/Exon Sequence

To identify the glucocorticoid-responsive sequences in the hGR 1A promoter/exon, we constructed a series of 5′ and 3′ deletions of the +41/+269 luciferase reporter plasmid. Both 5′ and 3′ regions of the +41/+269 fragment contribute to basal promoter activity (Fig. 3A). 5′ Deletions of all of FP5 and one half of FP6 (+179/+269) or of both sequences (+191/+269) reduced basal activity, whereas the further deletion of
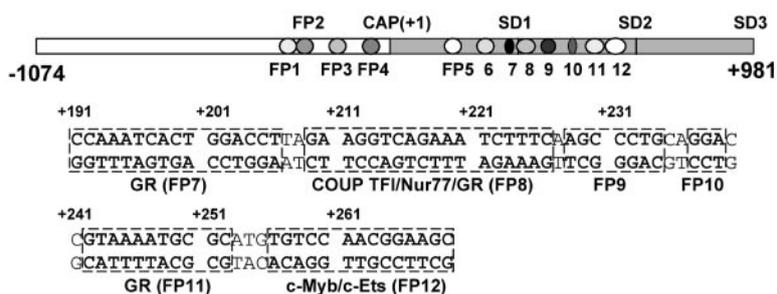
Fig. 2. hGR 1A Promoter/Exon Structure
   Shown is the arrangement of identified DNase I footprints in the human GR 1A promoter/exon. Additional protein binding footprints (FP7–FP12) downstream of FP1–6 that were described previously (22) are noted by the *boxed* regions of the sequence shown. SD1, SD2, and SD3 refer to the splice donor sites used to generate hGR transcripts containing exon 1A1, 1A2, and 1A3, respectively (22). CAP (+1) is the most upstream transcription initiation site.
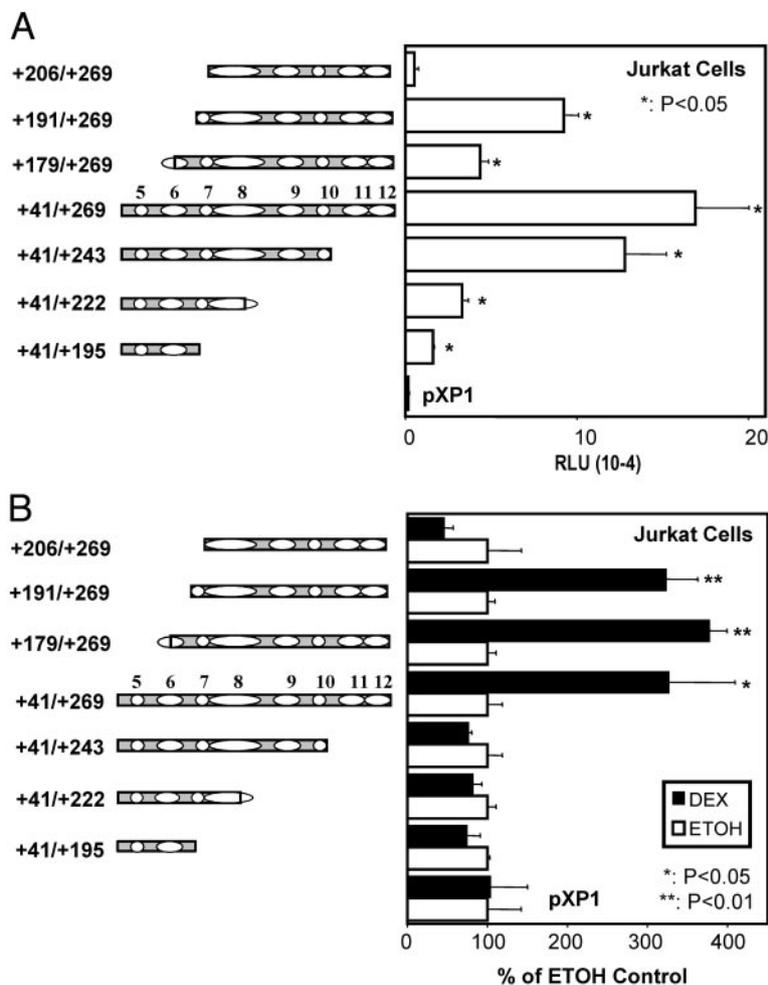
Fig. 3. Basal Promoter Activity and DEX Responsiveness of 5′ and 3′ End Deletions of the hGR 1A Promoter/Exon in Jurkat Cells

5′ or 3′ End sequentially deleted hGR 1A +41/+269 promoter/exon sequences were inserted into the pXP1 luciferase reporter gene, and the resultant 1A promoter/exon reporter constructs were transfected into Jurkat cells to assess basal promoter activity and hormone responsiveness. All transfections included 1.0 μg of the pCYGR plasmid. A, Basal promoter activities of various 5′ and 3′ deletions of the hGR 1A +41/+269 promoter/exon sequence. *, $P < 0.05$ for promoter activity that is significantly higher compared with the pXP1 vector control. B, DEX-induced activity in the hGR 1A promoter/exon 5′ and 3′ end-deletion reporter constructs. Values are expressed as the percentage of the DEX-treated compared with the respective ethyl alcohol (ETOH) controls. **, $P < 0.01$ and *, $P < 0.05$ for the DEX-induced values compared with the vehicle controls.

FP7 (+206/+269) completed abolished basal activity. 3′ Deletion of FP 11 and FP12 (+41/+243) caused a modest and not significant decrease in basal promoter activity, whereas further deletion of FP9 and FP10 without (+41/+222) or with (+41/+195) deletion of a portion of FP8 caused a substantial decrease in basal activity. Thus, it appeared that, minimally, FP7, FP9, and/or FP10, and perhaps FP8 contributes to basal activity for the 1A sequence.

5′ Deletions of all of FP5 and one half of FP6 (+179/+269) or of both sequences (+191/+269) had no effect on the hormonal response (Fig. 3B), whereas the further deletion of FP7 (+206/+269) completely abrogated steroid responsiveness. However, basal promoter activity is also lost when FP7 is deleted, and this could be of primary importance. The 3′ deletion of FP 11 and FP12 (+41/+243) totally abolished DEX induc-

tion of luciferase gene expression and further 3′ deletions were similarly unresponsive to hormone.

These results indicated a considerable complexity in the elements that are involved in both basal and hormone-induced promoter activity. Because there may be interactions between a number of these elements and because terminal deletions remove multiple elements, *in vitro* site-directed mutagenesis of individual elements was performed.

FP11 and FP12 Largely Mediate the DEX Response of the hGR 1A Promoter/Exon Sequence

FP7, FP8, FP11, and FP12 were deleted from two different parental constructs, +41/+269, which had maximal basal and hormone-induced promoter activ-

ity, and +191/+269, which had substantially lower basal activity, but was still hormone inducible. In contrast to the 5′ deletion of FP7 that included FP5 and FP6, the internal deletion of FP7 in the +41/+269 construct had little effect on basal promoter activity (Fig. 4A). Internal deletion of FP8 resulted in a loss of about 40% of basal activity (Fig. 4A), whereas the internal deletion of either FP11 or FP12 resulted in even greater decreases in basal promoter activity. Internal deletions of these footprints in the +191/+269 reporter gene gave somewhat different results (Fig. 4B). As was found for the +41/+269 construct, deletion of FP11 or FP12 caused substantial decreases in basal activity. However, the deletion of FP8 in the +191/+269 reporter did not lower basal expression as had occurred in the longer reporter construct. Even more dramatically, the deletion of FP7 caused a large decrease in basal activity in the +191/+269 construct, whereas it only caused a modest decrease in the +41/+269 construct. One possible explanation is that FP5, FP6, and FP7 each can contribute to basal promoter function, and that FP7 deletion only has a dramatic effect when it occurs in a promoter that also lacks FP5 and FP6 (the +191/+269 construct). It should be noted that the basal activity of the parental

+191/+269 reporter is only about 25% of that seen for the parental +41/+269 construct (compare the x-axes in Fig. 4, A and B, *left panels*).

Similarities and differences between the longer (+41/+269) and shorter (+191/+269) reporter constructs were also seen in the hormonal responsiveness after internal deletions (Fig. 4, *right panels*). The deletion of FP8 had little effect on the hormonal response in either reporter construct. This was somewhat surprising because EMSA results indicated that this element can be bound by GRα, GRβ, and COUP TFI (data not shown). Deletion of FP11 or FP12 completely abolished the ability of the reporter gene to be stimulated by hormone in T-lymphoblasts in both reporter gene constructs, pointing to the fundamental importance of these sites in the stimulation of the 1A promoter by steroid hormone. Deletion of FP7, again, showed a dramatic difference between the longer and shorter reporter constructs. Deletion of FP7 from the +41/+269 construct had no effect; the 1A promoter was still hormone responsive. On the contrary, FP7 deletion from the +191/+269 reporter gene resulted in a total loss of hormonal sensitivity. There are two possibilities for this discrepancy. First, it is possible that this is a redundant promoter, such that elements up-
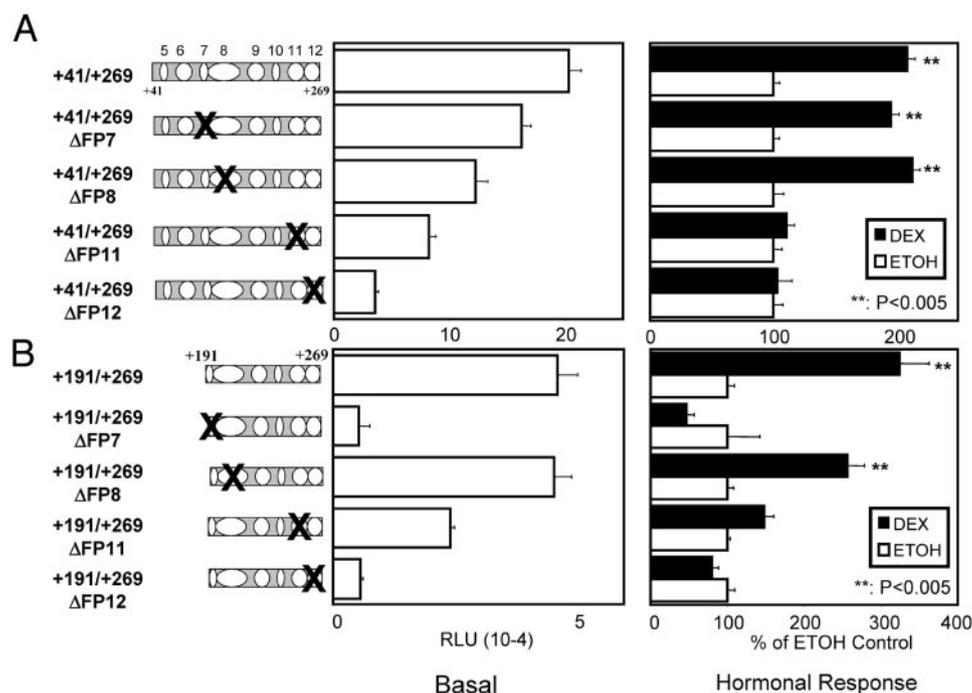

Fig. 4. Basal Promoter Activity and Hormone-Responsiveness of the hGR 1A Promoter/Exon Containing Internal Deletions

Experiments were carried out essentially as described in the legend to Fig. 3. A, Basal promoter activity and hormone-responsiveness was determined in constructs containing internal deletions of FP7, FP8, FP11, and FP12 in the hGR 1A +41/+269 promoter/exon in Jurkat cells. In the hormone-responsiveness studies, results are depicted as the percent of ethanol-treated, vehicle controls for each respective hGR 1A +41/+269 reporter construct. B, Basal promoter activity and hormone-responsiveness was determined in constructs containing internal deletions of FP7, FP8, FP11, and FP12 in the hGR 1A +191/+269 promoter/exon in Jurkat cells. These experiments were in a manner identical with those in A, except that the parental plasmid extended from +191 to +269. The construct labeled +191/+269 ΔFP7, and the data presented are the same as those for the construct labeled +206/+269 in Fig. 3B because FP7 is located at the 5′ end of the +191/+269 parental construct. **, $P < 0.005$ for the DEX-stimulated promoter activity compared with the respective ethyl alcohol (ETOH) controls.

stream of FP7 can render the gene hormone responsive in the absence of FP7, but that steroid stimulation of the promoter is absolutely dependent upon FP7 if the upstream regions are not present. This may not necessarily indicate a GRE in the upstream sequences but merely reflect the need for a minimal amount of basal promoter activity to allow a substantial hormone response. Second, the very low level of basal promoter activity in the FP7-deleted +191/+269 reporter gene might prevent detection of any stimulatory effect of hormone. This low basal activity in the absence of FP7 also makes it difficult to determine whether the apparent down-regulation by DEX observed when FP7 is deleted is real or not.

Taken together, the results in Figs. 3 and 4 suggest that all of the sequences in the hGR 1A promoter that are bound by proteins contribute in some way to basal and/or hormone-induced activity, but that individual results depend upon other sequences present in the promoter-reporter gene construct. Most notably: 1) FP11 and FP12 are important for both basal promoter activity as well as hormonal responsiveness; 2) FP8 is not involved in the DEX response and has limited effects on basal promoter activity; 3) FP7 is not absolutely required for the DEX response of the 1A promoter, provided that upstream sequences (FP5 and FP6) are present, but it is necessary if these sequences are absent. Alternatively, FP11/12 may be the important hormone-responsive sequences, but the contribution of FP5, FP6, and/or FP7 to the basal promoter activity may be required for FP11/12 to manifest hormone responsiveness.

Hormone Treatment Affects Protein-DNA Complexes in the hGR 1A Promoter/Exon

The complexity revealed by the deletion analyses was reminiscent of promoters that contain multiple, interacting, transcription factor binding sites in a glucocorticoid response unit, such as that found in the phosphoenolpyruvate carboxykinase promoter (27). Computer analysis and EMSA were performed in an attempt to identify the transcription factors that bind to the hGR 1A promoter/exon. Given the importance of FP7 (+191/+206), and FP11 and FP12 (+242/+269), we focused our analyses on these elements.

At least six protein-DNA complexes are seen when labeled FP7 is used to probe DNA-binding sequences in CEM-C7 cell nuclear extracts (Fig. 5A). Hormone treatment of CEM-C7 cells causes very subtle decreases in some of the protein complexes bound to FP7. In contrast to FP7, FP11 and FP12 showed very clear alterations in complexes formed upon DEX treatment (Fig. 5B). Also, different complexes appear to form on the FP11/12 oligo, which contains both FP11 and FP12, compared with those that appear using the separate FP11 and FP12 oligos. This suggests that the two footprints may be involved in the formation of new protein binding complexes when they are located adjacent to each other. That is, the recruitment of a
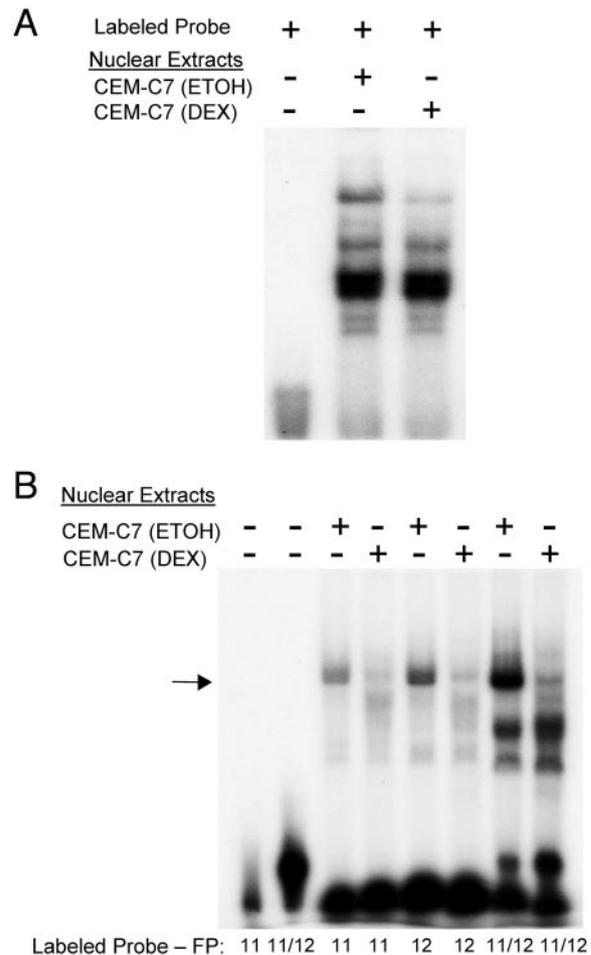

Fig. 5. EMSAs of FP7, FP11, FP12, and FP11/12 in the Presence and Absence of DEX

One microliter of crude, CEM-C7 cell nuclear extract (plus or minus DEX-treated CEM-C7 cells, protein concentration: 2.2 mg/ml) was incubated with 50 fmol ^{32}P end-labeled, oligonucleotide probes at room temperature. A, EMSA using the FP7 oligonucleotide. Three separate experiments were performed for the ethyl alcohol (ETOH)-treated nuclear extracts and four for the DEX-treated nuclear extracts. All of these gave similar, reproducible results. B, EMSA of the FP11, FP12, and FP11/12 oligonucleotides. The *arrow* on the *left* of the figure points to major band seen for FP11 and FP12 that is diminished upon hormone treatment. The somewhat more intense shifted bands (except the top band) seen for FP11/12 with the nuclear extracts from the DEX-treated cells were obtained in three separate experiments that were performed. This particular experiment was performed three separate times and all gave reproducible results similar to those shown here. When combining all experiments performed in this study (Figs. 5–7, and not shown), FP11 and FP12 gave the same pattern in eight separate gels, and FP11/12 gave the same pattern in 12 separate gels, when DEX-treated nuclear extracts were used.

certain protein may require that other proteins be bound to FP11 and FP12 in tandem, and this recruitment may not occur if only one of the DNA sequences is present in the absence of the other. The intensity of

the shifted bands for the FP11/12 oligo might indicate stronger, more stable protein-DNA complexes. As was true for the isolated FP11 and FP12 protein-DNA complexes, the protein complexes formed on the FP11/12 oligo also differed between the ethanol sample and DEX samples (Fig. 5B). The apparent loss of protein complexes for the FP7, FP11, FP12, and FP11/12 oligos after DEX treatment also raises the possibility that inhibitory complexes may be released from these sequences after hormone treatment, resulting in a derepression of the hGR 1A promoter. Taken together, the data suggest that FP11 and FP12 could primarily mediate the regulation of the 1A promoter by altering the protein complex(es) bound to these elements after the activation of cellular hGR protein.

hGR Binds to FP7, FP11, and FP11/12

Hormonal stimulation of the hGR 1A promoter/exon in T cells is a GR-dependent process (Fig. 1B). We next examined whether the GR proteins are directly involved in the response regulation mechanisms. When an anti-GRα antibody was used for a supershift analysis with an FP7 oligo, a decrease in intensity of three of these protein complexes (S, *arrows*) and the appearance of a faint supershifted band (SS) occurred, resulting in a doublet (Fig. 6A). Competition using an unlabeled consensus GRE resulted in a decreased intensity of all bands. The simplest explanation for these results is a direct binding of the GRα to FP7, with possible protein-protein interactions between other
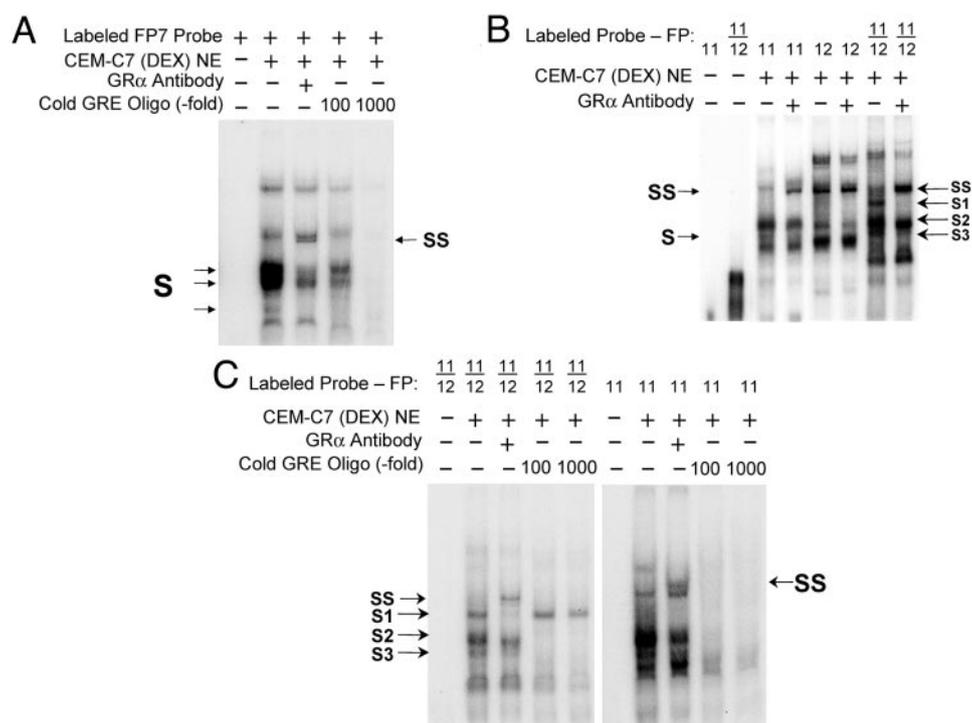

Fig. 6. EMSA, Supershift, and Competition Analyses of FP7, FP11, FP12, and FP11/12

A, Gel shift, and supershift assay of FP7 oligos. S *arrows*, Protein-DNA complexes that were diminished upon addition of the GRα antibody. SS *arrow*, Supershifted band that appeared after addition of the hGRα antibody. The supershifting experiment was performed four separate times with similar, reproducible results. An unlabeled (cold) GRE consensus oligonucleotide was added at 100× and 1000× molar excess over the labeled probe for the competition binding assay. The competition experiments were repeated three separate times with similar, reproducible results. B, EMSA and supershift assay of FP11, FP12 and FP11/12 oligonucleotides. The S *arrow* on the *left* indicates a faint band in FP11 (lane 3) that resides between the two dark bands and which is diminished after adding the GRα antibody. The SS *arrow* on the *left* points to a new, supershifted band seen for FP11 after the addition of the GRα antibody (lane 4). S1, S2, and S3 on the *right* indicate three shifted bands seen for the FP11/12 oligonucleotide that are lost when the GRα antibody is added (S2 is the *top band* of a doublet seen in lane 7). SS on the *right* is the supershifted band seen for the FP11/12 oligonucleotide. C, Supershift and competition analyses of the FP11 and FP11/12 oligonucleotides. S1, S2, and S3 are the same specific protein-DNA as in Fig. 6B. The SS *arrow* on the *left* is the GRα antibody-supershifted complex seen for FP11/12, whereas the SS on the *right* points to the FP11 supershifted complex. The unlabeled (cold) consensus GRE oligonucleotide was added at the indicated molar excess over the labeled probe in the competition studies. The gel in 6C was run for a longer time than that in Fig. 6B to better resolve the complexes formed. The specific experiments seen in panels B and C were performed three separate times and all gave reproducible results similar to those shown here. When combining all experiments performed in this study (Figs. 5–7 and data not shown), FP11 and FP12 gave the same supershifting pattern in eight separate gels, and FP11/12 gave the same supershifting pattern in 12 separate gels, when DEX-treated nuclear extracts and the GRα antibody were used.

proteins and the GR giving rise to the multiple protein complexes that are seen. Alternatively, direct binding of the unlabelled consensus GRE to the GR could disrupt GR interactions with a different protein bound to FP7. This could occur, for example, if the GRE interacts with the same amino acid residues needed for GR protein-protein interactions with the *bona fide* FP7 DNA-binding protein, or if a conformational change occurred in the GR upon binding to the GRE that prevented protein-protein interactions from occurring between the GR and a protein bound at FP7. Some of these protein-protein interactions may also block the epitope on the GR, thus explaining why all of the GR complexes are not supershifted. Nonetheless, it is clear that the GR is involved in a complex at FP7.

FP11 and FP12 are critical for the hormonal response of the hGR 1A promoter/exon (Figs. 3B and 4, A and B). Thus, we determined whether the GR can directly or indirectly bind to these DNA sequences or not. Besides directly binding to DNA, the GR could form protein-protein complexes with other DNA-bound proteins and still affect gene transcription. When an hGRα-specific antibody was added, both decreases in protein-DNA complexes, and super-shifted bands were observed for FP11 and FP11/12 (Fig. 6, B and C), whereas no alteration in the protein-DNA complexes was noted when the FP12 oligo alone was used (Fig. 6B). This indicates that the GR is physically included in the complexes formed on FP11 and FP11/12, but not on FP12 alone. Competition experiments were performed with an unlabeled consensus GRE (Fig. 6, A and C). The bands for FP7 and FP11 can all be competed out by 1000 times of excessive cold GRE, and all but one band (S1, Fig. 6C) is lost for FP11/12. Using a lower concentration of unlabeled GRE (100-fold), only some bands were lost [the lowest band for FP7 (Fig. 6A); S2 and S3 for FP11/12 (Fig. 6C)]. The multiple bands and competition results imply that GR proteins are present in the FP7, FP11, and 11/12 complexes and may be interacting with other proteins binding to the GR protein and/or the same oligo probes.

FP12 Is a Functional Binding Site for c-Myb and c-Ets

Computer analysis of the FP12 sequence revealed that it might be recognized by the c-Myb and the c-Ets DNA binding proteins because the FP12 sequence is a perfect match for the consensus DNA-binding sites of both proteins (Fig. 7A). Interestingly, the c-Myb and c-Ets recognition sites overlap, suggesting that c-Myb and c-Ets might compete to bind at this footprint for transcription regulatory control of the promoter and might affect GR binding at the adjacent FP11 sequences. Figure 7B shows that both c-Myb and c-Ets proteins can bind at FP12 as indicated by the same gel shift pattern seen when antibodies that are specific for either c-Myb or c-Ets are used (a decrease in S4 and the appearance of a supershifted band, SS). Because

previous studies (Fig. 6) suggested that the interaction of proteins binding to FP11 and FP12 resulted in specific complexes seen in FP11/12, we analyzed the supershift patterns using c-Myb-, c-Ets-, and GRα-specific antibodies to determine whether GR, c-Myb, and/or c-Ets are included in the same DNA binding complex on FP11/12 or not. Figure 7B shows that some complexes formed on FP11/12 include GRα protein, c-Myb, and/or Ets because the same bands were supershifted to the same position (SS) by all three protein-specific antibodies. This was specific because the use of preimmune rabbit or goat antisera, or antibodies against actin or tubulin, did not give the same supershift pattern as with the GRα, c-Myb, or c-Ets antibodies. Rather, the same pattern was obtained as for the lane in which no antibody was added at all (data not shown).

DISCUSSION

Although corticosteroid-induced T lymphoblast death has been observed for decades, the molecular mechanism of this process is unknown. Many studies suggest a critical role for the intracellular GR levels in T-ALL and lymphoid tumors, and hormone treatment causes cell growth inhibition and subsequent apoptosis (28–31). However, the absolute numbers of GR in human leukemia cell lines do not completely reflect its sensitivity to the steroid (32). In addition, a characteristic auto-up-regulation of hGR by its hormone ligand in sensitive, hormone-responsive T cells seems to be required for hormone-induced T-lymphoblast death (18, 20, 21, 33). It is likely that the GR protein levels must be induced above a certain threshold level to stimulate the cascade of cellular events that ultimately lead to apoptosis. Thus, steroid-mediated up-regulation of hGR gene expression appears to be essential for the therapeutic response.

The most likely mechanism for steroid-mediated up-regulation of hGR gene expression is at the promoter level. Recent studies have added to the complexity of this mechanism because three different hGR promoters (1A, 1B, and 1C) have been identified (22, 23, 34, 35). Initial studies indicated that transcripts emanating from hGR promoter 1A were selectively expressed and hormone-induced (22). In addition, luciferase reporter genes containing the hGR 1A promoter/exon are stimulated by DEX in T-lymphoblasts (Ref. 22 and this paper), whereas those containing a 1B and 1C promoter sequence together are not (our unpublished data). In addition, more recent studies (36) show that expression of all GR transcripts containing different first exons and deriving from all three promoters (1A, 1B, 1C) are up-regulated by hormone treatment, although the induction is more robust for 1A-containing transcripts. Thus, we focused on the hGR 1A promoter/exon in a search for glucocorticoid-responsive sequences.

Fig. 7. Sequence Comparison, EMSA, and Supershift Analysis of FP12 and FP11/12 using c-Myb, c-Ets, and GRα Antibodies
   A, The sequence of FP12 is compared with the consensus DNA-binding sequences of c-Myb and c-Ets. *Letters under the horizontal lines* in the consensus sequences of c-Myb and c-Ets indicate that the consensus sequence contains either this base or the one above the line at approximately equal frequencies. There is a perfect match for the two consensus sequences in FP12, and they overlap each other. B, EMSA and supershift analyses using c-Myb, Ets1/2 and hGRα-specific antibodies. *Left*, The S4 band on FP12 is diminished upon adding either the c-Myb or c-Ets antibody, and the SS *arrow* points to a new, identical, supershifted band seen when either antibody is used. *Right*, S1, S2, and S3 are the same shifted bands seen in Fig. 6, B and C, for FP11/12. The SS *arrow* points to the same supershifted complex seen in Fig. 6, B and C, and it is obtained for all three antibodies, GRα, c-Myb, and c-Ets. This particular experiment was performed four separate times and all gave reproducible results similar to those shown here.

Table 1. Identification of DNase I Footprints in the hGR 1A Promoter/Exon

	Protected Sequences in 1A Promoter/Exon Region	Possible Binding Factors	Identified by
FP1	5'-TATGTTAATATTTTATTCA-3'	?	–
FP2	5'-TCCAAATTAACTATAAATTGT-3'	?	–
FP3	5'-TGAGAAATTAGGAAAATCCA-3'	NFκB (p65)	Sequence analysis, gel shift
FP4	5'-CTTGATGACACAGACTAATAACCAATGA-3'	?	–
FP5	5'-AGAGGCGAATCACTTTCACTTCTG-3'	IRF-1/2	Sequence analysis, gel shift
FP6	5'-GGAGAAAACTTAGATCTTCTGAT-3'	GR	Sequence analysis, gel shift
FP7	5'-TACCAAATCACTGGACCT-3'	GR	Gel shift
FP8	5'-GAAGGTCAGAAATCTTTC-3'	COUPTF/Nur77/GR	Sequence analysis, gel shift
FP9	5'-AGCCCTG-3'	?	–
FP10	5'-GGA-3'	?	–
FP11	5'-GTAAAATGCGC-3'	GR	Gel shift
FP12	5'-TGTCCAACGGAAGC-3'	c-Myb/Ets	Sequence analysis, gel shift

?, Unknown; –, not determined.

To investigate the factors that may influence hGR 1A promoter/exon expression, we identified several protein-binding, DNase I footprints using hormone-treated CEM-C7 nuclear extracts (Table 1). This analysis, and the high density of DNA-bound proteins, suggests that multiple signaling pathways may mod-

ulate 1A promoter activity. However, no positive, consensus GREs were found in this promoter. Nonetheless, our studies clearly show that the hGR 1A promoter/exon confers positive hormonal regulation in T cells. In some specific genes the GR can bind to other, nonconsensus, GREs (or half GREs) as a mono-

mer, dimer, or even trimer (25, 37–39). These GRE-like elements, unlike the classical consensus GRE, are very weak in binding the GR and in mediating the hormonal response when they are separated from the specific gene promoters and used with a heterologous promoter. Importantly, in most of these cases, the function of the GR (transactivation or repression) depends largely upon the cooperation of cofactors binding at DNA elements that are adjacent to these GRE-like sequences. For example, Ets2, *Xenopus* glucocorticoid receptor accessory factor (gAF1–3), chicken ovalbumin upstream promoter-transcription factor (COUP-TF), and hepatic nuclear factor-4 physically interact with GR proteins in the complexes that are formed upon hormone induction (38–43). These accessory proteins can facilitate a stronger binding of the GR protein to the weak GRE element, create a binding site for other cofactors that do not directly interact with GR, and/or bind to a weak DNA response element when the GR is simultaneously bound to its weak GRE-like element. Thus, the context of the promoter can contribute to both the binding of the GR and to its functional role in transcription regulation.

The steroid response elements in the hGR 1A promoter resemble this type of GRE, and there is redundancy built into the system as well. Of the 12 footprints that have been identified in the hGR 1A promoter/exon extending from −218 to +269 (Ref. 22 and this paper; see Table 1), the GR is present in the complexes formed on four of these (FP6, FP7, FP8, FP11). However, no hormonal effect has been discovered to date for FP6 or FP8. Perhaps these function (either negatively or positively) in cell types or under specific physiological conditions that have not yet been studied. The redundancy and complexity of the steroid-responsiveness of the hGR 1A promoter/exon was clearly seen when different constructs were used (Figs. 3 and 4). In the longer construct (+41/+269), FP7 was dispensable because its deletion had no effect on the stimulation of the reporter gene by DEX, whereas its deletion from the shorter construct (+191/+269) resulted in the complete loss of DEX stimulation as well as basal promoter activity. This suggests that additional sequences between +41 and +191 are required for the hormonal response mediated by FP7. Because basal promoter activity is lost in the construct lacking FP5, FP6, and FP7, it is difficult to determine whether the role of FP7 is primarily to provide enough basal promoter activity to allow FP11 plus FP12 to exhibit its GRE activity or whether FP7, by virtue of its potential ability to bind the GR, is a *bona fide* GRE itself. On the other hand, FP11 and FP12 are indispensable for the hormonal response in either construct, and (as opposed to FP7) the hormonal responsiveness is independent of sequences between +41 and +191.

Because of the crucial nature of FP11 and FP12, we focused on these sequences. For reasons described in the *Results* for FP7, it is not possible to unequivocally determine whether binding of the GR to FP11 is direct or indirect. If FP11 binding by the GR is direct,

and FP12 contains binding sites for both c-Myb and c-Ets, an attractive model can be proposed. Supershift analysis (Fig. 7B) suggests that the GR, c-Myb and/or c-Ets can exist in a common complex, although it is not likely that c-Myb and c-Ets can bind to FP12 simultaneously, as their DNA-binding sites overlap. Thus, it is tempting to speculate that GR binding to FP11 requires simultaneous binding of either c-Myb or c-Ets to FP12 to stabilize GR binding to its weak GRE-like element in FP11. A comparison of the complexes formed on FP11 alone or on FP11/12 is of particular interest. One band (S1 for FP11/12 in Fig. 6C) is apparently supershifted by the GRα antibody, but it is not lost even with a 1000-fold excess of unlabeled GRE competitor. No such similar situation is seen for FP11 alone, and all bands are lost even at 100-fold excess unlabeled GRE competitor (Fig. 6C). This suggests that S1 seen for FP11/12 is a complex containing the GR that interacts with another DNA-binding protein that binds to the FP12 portion of FP11/12. Thus, it is possible that the GR protein physically interacts with c-Myb or c-Ets proteins binding at FP12 during hormone stimulation of the hGR 1A promoter in CEM-C7 cells.

c-Myb is expressed in immature hematopoietic cells of various lineages (44, 45). It is crucial in the early development of the T cell lineage, and, under normal conditions, it is expressed in T but not B cells. c-Ets-1 is expressed primarily in B, T, and NK cells, and it is essential for their survival and maturation (reviewed in Ref. 46). Its absence causes apoptosis in T cells (47, 48). Other members of this family play a crucial role in lymphoid cells, most notably PU.1 and Spi-B. PU.1 is expressed in B cells but not in T cells (49), whereas Spi-B is expressed only in lymphoid lineages. Interestingly, binding of the GR to a cryptic GRE in the rat cytochrome P-450c27 promoter stabilizes c-Ets-2 binding to an adjacent weak Ets-like DNA response element, and this involves protein-protein interactions between the DNA binding domain of the GR and c-Ets-2 (39). Thus, GR/c-Ets cross-talk and their physical interaction have already been observed. Preliminary experiments show that the transfection of plasmids for c-Myb, c-Ets, and the dominant-negative DNA-binding domain fragments of these proteins affect both basal transcription from the hGR 1A promoter and the response of the promoter to hormone (our unpublished data). Cotransfection of hGR and c-Myb expression vectors into the IM-9 B-lymphoblast cell line, which down-regulates the hGR and contains no endogenous c-Myb, results in the up-regulation of an hGR 1A promoter-luciferase reporter gene in a c-Myb dose-dependent manner (data not shown). Finally, preliminary chromatin immunoprecipitation assays show that the GR and c-Myb are not bound to the hGR 1A promoter in the absence of DEX but that both are recruited to the promoter, after hormone treatment (data not shown). Because hGR transcripts are either negatively or positively regulated by the hormone-GR complex in a cell type-specific manner, it is possible

that the interaction of the GR with a cell type-specific transcription factor bound to an adjacent DNA sequence (*e.g.* c-Myb or c-Ets) might determine the direction of transcript regulation (up or down) in the cell. The level and control of these accessory transcription factors may also be important in determining the apoptotic response of T cell ALL to corticosteroid hormones. Studies to explore this possibility are being pursued.

MATERIALS AND METHODS

Cell Culture

Human Jurkat T-ALL cells (ATCC, Manassas, VA) were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum (Life Technologies, Rockville, MD). Human CEM-C7 cells were a kind gift from Dr. E. B. Thompson (University of Texas Medical Branch, Galveston, TX) and were grown in RPMI 1640 with 10% dialyzed fetal bovine serum (Life Technologies). DEX (Sigma, St. Louis, MO) in ethanol was the glucocorticoid used to treat cells. Controls were treated with 0.01% ethanol alone.

DNA Constructs

pXP-1 −964/+269 1A promoter/exon and pXP-1 +179/+269 1A promoter/exon constructs were as described previously (22). 3′ and 5′ Serial deletion constructs were generated via PCR using a fixed 5′ or 3′ PCR primer (fixed 5′ deletion primer, 5′-GCAAAGCTTCCGTTGGACACATGCG-CAT-3′; fixed 3′ deletion primer, 5′-ACAGGATCCTCTTCCTCT-TCTCTTGCTCCC-3′) and various reverse primers. The 3′ deletion primers were: 5′-ACAGGATCCTCTTCCTCTTCTCTT-GCTCCC-3′ (1A +41/+269); 5′-CACAAGCTTTACGGTCCTG-CAGGGCTTGAA-3′ (1A +41/+243); 5′-CGCAAGCTTCTGAC-CTTCTAAGGTCCAGTGA-3′ (1A +41/+222); and, 5′-CGCA-AGCTTTGGTATCAGAAGATCTAAGTT-3′ (1A +41/+195). The 5′ deletion primer was 5′-TCTGGATCCAAATCACTGGACCT-TAGAAGGT-3′ (1A +191/+269). The resultant PCR products were digested with *Hin*dIII and *Bam*HI and ligated into phosphatase-treated pXP-1 that was digested with the same enzymes.

PCR Introduced *in Vitro* Site-Directed Mutagenesis

Three deletion constructs that removed DNase I footprinted regions that existed at the ends of already available constructs were generated by using primers just adjacent to these regions. pXP-1 +41/+269 ΔFP12 (pXP-1 +41/+253) was obtained via PCR amplification using the pXP-1 +41/+269 1A construct as template with the fixed 5′ PCR primer described above and the reverse primer 5′-GAGAAGCTT-GCGCATTTTACGGTCCTG-3′, which is just upstream of FP12 and results in its deletion. pXP-1 +191/+269ΔFP12 (pXP-1 +191/+253) was made in the same way with the same 3′ reverse primer and 5′ forward primer used to construct pXP-1 1A +193/+269 described above. pXP-1 +191/+269 ΔFP7 (pXP-1 +206/+269) was made using the 5′ primer 5′-ACGGATCCTTAGAAGGTCAGAAATCTTTCAA-CTAG-3′, which is just downstream of FP7 and the fixed 3′ PCR primer described above. The PCR products were digested with the appropriate restriction enzymes and cloned into pXP-1. The insertion of the proper PCR products into the reporter constructs was confirmed by DNA sequencing.

Internal deletions of the DNase I footprinted regions in the pXP-1 −964/+269, pXP-1 +41/+269, and pXP-1 +191/+269 1A constructs were performed using PCR-mediated deletion. The deletion primers (only one set of the complementary pair are shown) used were: FP11 deletion, 5′-CAAGCCCTGCAGGACGTGTCCAACGGAAGC-3′; FP8 deletion, 5′-CAAATCACTGGACCTTGAAATCTTTCAAGC-3′; FP7 deletion (+41/+269), 5′-AAGGAGAAAACTTAGATCTTTCTG-GAAGGTCAGAAATCTTTCAAGC-3′; and, FP6 deletion, 5′-CAGTGATTTGGTATCTCTAAGCCTTCTCTCTGCC-3′. Plasmids used for templates in these deletions were: pXP-1 −964/+269 1A, pXP-1 +41/+269 1A, and pXP-1 +191/+269 1A. The resultant PCR amplified plasmid products were completely digested by *Dpn*I and transformed into bacterial cells DH5α for selection. All the deletion constructs were confirmed by DNA sequencing.

Transient Transfections

Jurkat cells were seeded at 8×10^5 cells/ml in six-well cell culture dishes. Typically, 3.5 μg of total DNA (1.5 μg reporter construct, 1.0 μg pCYGR and 1.0 μg pCMV/β-galactosidase plasmid) were used in each transient transfection. Transfection was performed with the Superfect transfection kit according to the manufacturer's instructions (QIAGEN, Santa Clarita, CA). DEX or ethanol was added after 24 h. After another 24 h, cells were collected, lysed, and the lysate was assayed for luciferase and β-galactosidase activities in a Labsystem Luminoskan Ascent luminometer as previously described (22, 35).

DNase I Footprint Analysis

DNase I footprinting was performed as previously described (22) with minor modifications. ^{32}P-5′ end-labeled primers (5′-CTATTCCTTCCCCACTCATGCCC-3′ and 5′-CTCTTAC-CCTCTTTCTGTTTCTA-3′) were used to generate single end-labeled DNA fragments (+167/+296 1A and +127/+316 1A) by PCR. The nuclear extracts were prepared from DEX- or ethanol-treated (24 h) CEM-C7 cells using NE-PER Nuclear and Cytoplasmic Extraction Regents (Pierce, Rockford, IL) according to the manufacturer's directions. The protein concentration was determined using the Dc protein assay kit (Bio-Rad, Hercules, CA).

EMSAs

EMSAs were performed as previously described (34), using the same nuclear extracts as for DNase I footprinting. For the supershift assays, antibodies to hGRα (PA1–516) or hGRβ (PA3–514) were from Affinity BioReagents (Golden, CO), whereas the Nur77 (sc-7014X), COUP TFI (sc-6575X), COUP TFII (sc-6576X), c-Myb (sc-7874X), and Ets1/2 (sc-112X) antibodies were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Antibodies (2 μg) were incubated with the binding reaction mixture 45 min at room temperature before adding the labeled oligonucleotides. The reactions were then incubated for an additional 15 min before loading onto a 5% nondenaturing PAGE gel. The consensus GRE oligonucleotide was purchased from Santa Cruz Biotechnology (catalog no. 2545). Oligonucleotides (including the complementary strand; not shown) were as follows: FP7, 5′-TCTGATAC-CAAATCACTGGACCTTA-3′; FP8, 5′-TAGAAGGTCAGAAA-TCTTTCAA-3′; FP11, 5′-GACCGTAAAATGCGCATG-3′; FP12, 5′-ATGTGTCCAACGGAAGCACT-3′; and, FP11/12, 5′-CCGTAAAATGCGCATGTGTCCAACGGAAGC-3′.

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