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Glucocorticoid Receptor Gene Mutations in Leukemic Cells Acquired *in Vitro* and *in Vivo*[1]

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ABSTRACT

Glucocorticoid resistance was investigated in human leukemic CCRF-CEM cells. A mutation (*L753F*), which renders the human glucocorticoid receptor (*hGR*) gene functionally hemizygous, was identified in all CEM-derived cell lines analyzed. Allele-specific PCR identified the same mutation in lymph node biopsy material from patient CEM cells. Given the correlation between hGR concentration and glucocorticoid sensitivity, this suggests that loss of functional heterozygosity may result in resistance to glucocorticoid-based chemotherapy. The *L753F* mutation was probably not responsible for the ontogeny of the disease because it did not appear to be present in all leukemic cells. Thus, it is unlikely that *hGR* mutations would be detected in leukemic patients at presentation, but they may occur, and be selected for, during treatment. Deletions and point mutations in the *hGR* gene of cells selected for steroid resistance *in vitro* were investigated by PCR-single strand conformation polymorphism analysis. Loss of hGR mRNA expression resulted from 5′-deletion of the *hGR* gene and nonsense mutations in exon 6. These results provide the first evidence for somatic mutation in the *hGR* gene of a patient with acute lymphoblastic leukemia, offer a potential *in vivo* mechanism for acquisition of steroid resistance in leukemia, and suggest that screening for additional *in vivo* mutations will require analysis of genomic DNA.

INTRODUCTION

Corticosteroids are commonly used in the treatment of leukemia and lymphoma. However, resistance to steroid therapy is a frequent phenomenon, and early studies showed that patients who relapse after single-agent induction of an initial remission are generally refractory to further steroid therapy (1–4). Subsequent studies identified a correlation between reduced GR[5] expression and a poor prognosis after relapse in patients with acute lymphocytic leukemia, suggesting that reduced GR expression could lead to clinical resistance (5–10). However, the mechanism(s) by which resistance arises, as well as the mechanism by which glucocorticoids induce lymphocytolysis, remains poorly understood.

The GR is capable of direct activation or repression of gene expression through interaction with positive or negative *cis*-acting regulatory elements in the promoters of hormonally responsive genes (11, 12). The GR can also indirectly regulate the expression of hormonally responsive genes through protein-protein interactions with other transcription factors (13–15). Recently, we showed that the glucocorticoid receptor mutant L753F, which is defective in transactivation but which retains the ability to repress AP-1-mediated activation of the collagenase promoter, is unable to mediate a lympholytic response in the human leukemic T cell line 6TG1.1 (16, 17). In addition, using mice in which the normal *GR* gene had been replaced by a mutant unable to form homodimers and bind to DNA, Reichardt *et al.* (18) showed that although glucocorticoid treatment resulted in repression of collagenase activity, there was no steroid-induced thymolysis. Thus, it appears that the ability of glucocorticoids to induce a lympholytic response is dependent upon the ability of the GR to mediate direct activation or repression of target genes. In addition, analysis of both human and mouse cell lines has shown that the principal mechanism for *in vitro* acquisition of glucocorticoid resistance is somatic mutation in the *GR* gene (16, 19–24). However, with the exception of alternatively spliced hGR transcripts, which have also been identified in normal individuals (25), functional or structural alterations in the GR in the cells of leukemic patients have not been identified (26). In part, this may be attributable to the fact that even a small percentage of resistant cells in an otherwise sensitive population would result in a poor clinical outcome, making it extremely difficult to identify aberrant GR structure or function in a background of normal receptor. Consequently, the value of *in vitro* model systems in providing insight into the mechanism of acquired *in vivo* drug resistance remains unclear.

CEM cells provide one of the most widely used model systems for investigating the mechanism of glucocorticoid-induced cell lymphocytolysis and the *in vitro* acquisition of steroid resistance in human cells. The original cell line CCRF-CEM was established from a patient with acute lymphoblastic leukemia (27). Subsequent analysis revealed that there was wide cell-to-cell variation in the degree of glucocorticoid sensitivity, leading to the establishment of clonal cell lines (28). Some of these clonal cell lines were extremely sensitive to steroid-induced lymphocytolysis, whereas others were completely resistant (28). Analysis of the *GR* genes in several of the glucocorticoid-sensitive clonal cell lines showed that although there is one normal *GR* gene, the second gene contains the mutation *L753F* (16, 20). Thus, the genotype of glucocorticoid-sensitive CEM cells is GR^{+}/GR^{753F}.

Analysis of clonal cell lines that were originally glucocorticoid-resistant demonstrated that resistance was the result of a defect downstream from the GR; steroid sensitivity could be restored by treatment with 5-azacytidine, and somatic hybrids between inherently steroid-resistant cells and cells lacking functional GR were steroid-sensitive (29, 30). However, Geley *et al.* (31) have reported that steroid-resistant CEM-C1 cells also contain one copy of the *L753F* mutation. This raised the possibility that the *L753F* mutation is present in all CCRF-CEM-derived cell lines, and perhaps even in the leukemic cells of the patient from whom CCRF-CEM was isolated. To test this possibility, we have used a variety of techniques to identify mutations in the *GR* gene from steroid-resistant cell lines isolated *in vitro*, as well as in archival biopsy material obtained from the patient from whom CCRF-CEM was isolated. Our results clearly show that, not only is it possible to readily identify mutations in the *GR* gene in resistant cells isolated *in vitro*, but that the *L753F* mutation present in both glucocorticoid-sensitive and -resistant cells *in vitro* is detectable

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[5] The abbreviations used are: GR, glucocorticoid receptor; hGR, human glucocorticoid receptor; LBD, ligand binding domain; SSCP, single-strand conformational polymorphism; UTR, untranslated region; RT-PCR, reverse transcription-PCR.

in cells obtained from the patient from whom the original cell line was isolated. These results provide the first evidence for somatic mutation in the *hGR* gene in leukemic cells *in vivo*, and suggest that clinical resistance may, in some cases, be a consequence of such somatic mutations.

MATERIALS AND METHODS

Cells and Cell Culture. The isolation and growth of the glucocorticoid-resistant T cell line, CEM-C1, and the glucocorticoid-sensitive CCRF-CEM-derived T cell line 6TG1.1 have been described previously (28, 32, 33). The derivation, from glucocorticoid-sensitive CEM cells, of the glucocorticoid-resistant mutant cell lines ICR27TK.3, 4R4, and BLMB1 have also been described (32–34). Cells were maintained in RPMI 1640 containing 10% fetal bovine serum and grown at 37°C in a humidified atmosphere containing 5% CO_2, as described previously (33). Cell number was determined with a Coulter Counter (model ZM; Coulter Electronics, Inc., Hialeah, CA).

Paraffin Blocks. Fixed and paraffin-embedded biopsy material from patient CEM (case number A64-307) was provided by Edmund C. Matczak (Department of Pathology, Children's Hospital, Boston, MA). During storage, many of the blocks had become fused, and the identifying tags had become dislodged. It was therefore not possible to correlate individual blocks with specific node biopsies.

Primers. Primer P1 (5′-CCAATTTGGAAGCCTGATC-3′), containing sequence from the middle of intron H, and primer P2 (5′-CGACTTTCTTTA-AGGCAACCATT-3′), containing sequence from the 3′-UTR of exon 9, were used to amplify the coding region of exon 9 for SSCP analysis. Primer P3 (5′-TTGCAGGTGGTTGAAAATCTCC-3′), containing sequence from the 3′-end of intron H, and primer P4 (5′-CCTCTACAGGACAAACTGATAG-3′), containing sequence from the 3′-UTR of exon 9, were used to amplify the region of the *hGR* LBD encoding codon 753 from genomic DNA. Primer P5 (5′-AGGAAAAGCCATTGTCAAGAGG-3′), containing exon 8 sequence, and primer P4 were used for amplification of the region of the hGR cDNA encoding residue 753. Primers P6 (5′-CTCATACCTTTATTTCTCTT-3′) and P7 (5′-GGGAAAATGACACACATACA-3′), containing sequences from introns E and F, respectively, were used to amplify exon 6 of the *hGR* from genomic DNA. Primers P (5′-CTTAACTATTGCTTCCAAACATT-3′), Q (5′-TCGACTTTCTTTAAGGCAACCA-3′), A (5′-ggggcgggcgCCCGAGAT-GTTA-3′), and B (5′-ggggcgggcgTGATGATTTCAGCA-3′) were used for allele-specific PCR (see below). Lowercase letters in primers A and B indicate the clamp sequence.

Isolation and Amplification of Genomic DNA. Genomic DNA was isolated from 10^7 cells as described (35). DNA was isolated from paraffin-embedded samples essentially as described (36, 37). Tissue scraped from paraffin blocks was incubated in 200 μl of digestion buffer A [10 mM Tris-HCl (pH 8.0), containing 100 mM NaCl, 25 mM EDTA, 0.5% SDS, and 0.1 mg/ml proteinase K] at 37°C for 5 days. After centrifugation, at 15,800 $\times$ *g* for 5 min, the supernatant was extracted once with phenol:chloroform:isoamyl alcohol (25:24:1), once with chloroform, and precipitated at −20°C with 100% ethanol in the presence of ammonium acetate. Nucleic acid recovered after centrifugation was washed with 70% ethanol, resuspended in 50 μl of H_2O, and stored at 4°C. Alternatively, tissue sections were incubated in 100 μl of digestion buffer B [10 mM Tris-HCl (pH 8.0), containing 100 mM NaCl, 25 mM EDTA, 0.1% Tween 20, and 0.1 mg/ml proteinase K] at 55°C for 3 h. After centrifugation at 15,800 $\times$ *g*, the supernatant was incubated at 95°C for 8 min, and 1 μl was directly used for PCR. Genomic DNA isolated from tissue culture cells was amplified in a 100 μl reaction containing 10 μl of 10$\times$ PCR buffer [100 mM Tris-HCl, 500 mM KCl (pH 8.3)], 2.5 mM $MgCl_2$, 250 nM of each deoxynucleotide triphosphate, 20 pmol of each primer, and 1 μg of DNA. After denaturation at 95°C for 2.5 min, the reaction was initiated by addition of 2.5 units of AmpliTaq DNA polymerase (PE Biosystems, Foster City, CA). Amplification of exon 9 was accomplished using 25–30 cycles of incubation at 95°C for 30 s, 48°C for 30 s, and 72°C for 1 min, followed by a final extension at 72°C for 4 min. Amplification of exon 6 was performed under the same conditions, except that primers were annealed at 58°C. Amplification of the region of the LBD containing codon 753 with primers P3 and P4 was performed under the same conditions, except that primers were annealed at 55°C. Amplified samples were extracted with phenol:chloroform:isoamyl alcohol (25:24:1) and precipitated in ethanol or purified on Ultrafree-MC spin filters (30,000 NWML; Millipore Corp., Bedford, MA). Alternatively, PCR products were purified using the QIAquick Spin PCR Purification kit (Qiagen, Inc., Valencia, CA) according to the manufacturer's instructions.

Allele-specific PCR. Allele-specific PCR was performed as described by Liu *et al.* (38). DNA extracted from archival samples (0.9–1.5 μg) was amplified in 100 μl of 10 mM Tris-HCl buffer (pH 8.3) containing 50 mM KCl, 2.5 mM $MgCl_2$, 0.25 mM each deoxynucleotide triphosphate, and 2.5 units of AmpliTaq DNA polymerase. Primers P, Q, wild-type forward primer A, and mutant reverse primer B were used in varying concentrations to optimize the yield of fragments PB (73 bp) and AQ (122 bp). After an initial incubation at 95°C for 3.5 min, amplification was performed by 60 cycles of incubation at 95°C for 30 s, 55°C for 1 min, and 72°C for 2 min, followed by a final extension at 72°C for 7 min. Amplified products were purified using the QIAquick Spin PCR purification kit.

RT-PCR. Total cell RNA was isolated from 10^7 cells using RNAzol B (Tel-Test, Inc., Pearland, TX) according to the manufacturer's instructions. Final pellets were suspended in H_2O at a concentration of 1 mg/ml, and 1.5 μg were reverse transcribed in 30 μl of 50 mM Tris-HCl (pH 8.8) containing 50 mM KCl, 2.0 mM $MgCl_2$, 1.33 nM random hexamers, and 50 units of Moloney murine leukemia virus reverse transcriptase (Life Technologies, Inc., Rockville, MD) at 37°C for 1 h. A single Ampliwax PCR Gem 100 wax bead (PE Biosystems) was added to each reaction mixture and melted, and the mixture was brought to room temperature. Each reaction was overlayed with 45 μl of Tris-HCl (pH 8.8) containing 50 mM KCl, 20 pmol of each primer, and 2.5 units of AmpliTaq DNA polymerase. Amplification was performed by 40 cycles of incubation at 95°C for 30 s, 55°C for 30 s, and 72°C for 1 min, followed by a final extension at 72°C for 4 min. Amplified products were extracted with phenol:chloroform:isoamyl alcohol (25:24:1), precipitated in ethanol, and fractionated by electrophoresis in 5% NuSieve agarose gels (FMC Bioproducts, Rockland, ME) either before, or after, digestion with *Alu*I. Fragments were stained with ethidium bromide.

SSCP. SSCP was performed essentially as described by Orita *et al.* (39). A single primer was ^{32}P-end-labeled with [γ-^{32}P]ATP (Amersham Pharmacia Biotech, Inc., Piscataway, NJ) using T4 polynucleotide kinase. One labeled primer (P2 for exon 9 and P6 for exon 6) and one unlabeled primer (P1 for exon 9 and P7 for exon 6) were then used to amplify genomic DNA as described above. Amplified DNA (10 μl) was added to 90 μl of 10 mM EDTA containing 0.1% SDS, and 2 μl of each sample were mixed with an equal volume of sample buffer (95% formamide containing 20 mM EDTA, 0.05% xylene cyanol, and 0.05% bromphenol blue), heated at 80°C, and loaded onto 16 $\times$ 16 $\times$ 0.1-cm nondenaturing 6% polyacrylamide gels (acrylamide: bisacrylamide, 29:1) containing 10% glycerol. Electrophoresis was performed at constant power (30 W) at 20°C (exon 6) or 30°C (exon 9). After electrophoresis, gels were transferred to 3MM paper and dried under vacuum at 80°C; ^{32}P-labeled DNA was visualized by autoradiography.

Analysis of *Bcl*I and *Alu*I Polymorphisms. Genomic DNA was digested with *Bcl*I and analyzed by Southern blotting using a probe specific for the 5′-portion of the *hGR* gene as described previously (40). Alternatively, genomic DNA amplified using primers P3 and P4 was digested with *Alu*I, fractionated by agarose gel electrophoresis, and stained with ethidium bromide. In some cases, PCR of exon 9 was performed using ^{32}P-end-labeled primer P3. In these cases, DNA was visualized by autoradiography after agarose gel electrophoresis.

Cloning and Sequencing. Amplified DNA was cloned into pCRII (Invitrogen, Carlsbad, CA) as described by the manufacturer. DNA was sequenced manually as described previously (16). Alternatively, allele-specific PCR products were cloned into PCR2.1 (Invitrogen), and 300–500 ng of purified plasmid were cycle sequenced using T7 forward or M13 reverse sequencing primers, the Big Dye Cycle Sequencing System (PE Biosystems), and an ABI Prism 377 DNA sequencer (PE Biosystems).

RESULTS

Identification and Expression of the *L753F* Mutation in the *hGR* Gene in Glucocorticoid-resistant Cells. We have shown previously that the glucocorticoid-sensitive CEM cell line 6TG1.1 contains one normal *hGR* gene and one mutant gene (*L753F*) that encodes

a protein unable to induce transcription from a GRE-containing promoter and that cannot mediate an apoptotic response (16, 17, 41). The presence of this mutation in cells grown in culture can be readily detected by PCR-SSCP analysis of *hGR* exon 9. DNA isolated from IM-9 cells, homozygous for the wild-type *hGR* gene, yields a single electrophoretic species, whereas DNA isolated from 6TG1.1 cells, containing both mutant and wild-type genes, yields two electrophoretic species (Fig. 1*B*). The more rapidly migrating of these species comigrates with DNA amplified from ICR27TK.3 cells, which contain only the mutant *L753F* hGR gene (16, 40), demonstrating that the more slowly migrating band is derived from the wild-type gene and that the more rapidly migrating band is derived from the mutant *L753F* gene. PCR-SSCP also identified the presence of a mutant *hGR* gene in CEM-C1 cells, confirming the results of Geley *et al.* (31), who showed that, like glucocorticoid-sensitive 6TG1.1 cells, these cells also contain one normal and one mutant *hGR* gene. Surprisingly, PCR-SSCP analysis also indicated the presence of both normal and mutant *hGR* exon 9 sequences in the glucocorticoid-resistant cell line 4R4 (Fig. 1*B, Lane 5*). Analysis in this cell line of a *Bcl*I RFLP in the 5′ region of the *hGR* gene indicated that the 5′-portion of the gene is deleted (Ref. 20; data not shown). Thus, steroid resistance in this cell line appears to be the result of a partial gene deletion.

In addition to generating a conformation detectable by SSCP analysis, the *L753F* mutation results in the loss of an *Alu*I site in exon 9, creating a fortuitous RFLP that can also be used to identify the presence and expression of the mutant *hGR* gene. To confirm the results of the PCR-SSCP analysis, DNA isolated from glucocorticoid-sensitive and -resistant cell lines was therefore examined for the presence of this polymorphism. *Alu*I digestion of DNA amplified from IM-9 cells generated the three fragments (37, 85, and 119 bp) characteristic of the wild-type gene, whereas digestion of DNA isolated from ICR27TK.3 cells yielded only two fragments of 37 and 204 bp (Fig. 2*B*). As expected, DNA isolated from glucocorticoid-sensitive

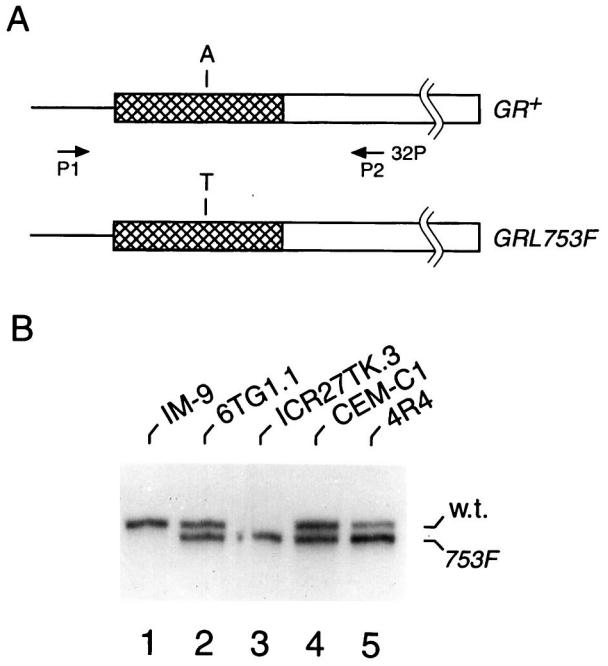

Fig. 1. SSCP analysis of *hGR* exon 9. *A,* schematic representation of amplification of exon 9. *P1* and *P2,* positions of the unlabeled and ^{32}P-labeled primers; *thin line,* intron H sequence; *cross-hatched region,* coding region of exon 9; *open area,* 3′-UTR. *B,* genomic DNA isolated from various cell lines was amplified and resolved by SSCP analysis as described in "Materials and Methods" and visualized by autoradiography. *w.t.,* wild type.

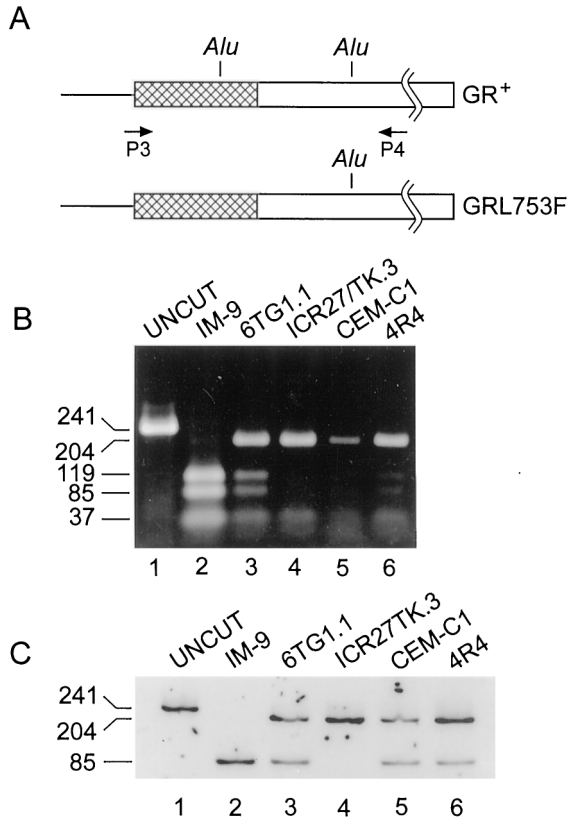

Fig. 2. *Alu*I polymorphism in wild-type and mutant exon 9 genomic DNA. *A,* schematic representation of amplification of region of exon 9 containing codon 753 from normal and mutant DNA. *P3* and *P4,* positions of the primers; *thin line,* intron H sequence; *cross-hatched region,* coding region of exon 9; *open area,* 3′-UTR. *B,* genomic DNA isolated from a variety of cell lines was amplified, digested with *Alu*I, resolved by agarose gel electrophoresis, and stained with ethidium bromide. *Lane 1* contains uncut DNA amplified from IM-9 cells. *Left,* sizes of the various digestion products. *C,* genomic DNA was amplified using ^{32}P-labeled primer P3, digested with *Alu*I, resolved by agarose gel electrophoresis, and visualized by autoradiography.

6TG1.1 cells and glucocorticoid-resistant CEM-C1 cells yielded a combination of the two patterns, indicating the presence of both the wild-type and mutant genes and confirming the results of the SSCP analysis. A combination of both patterns was also obtained after *Alu*I digestion of DNA isolated from 4R4 cells, providing additional evidence that the 3′ region of the wild-type *hGR* gene is present in these cells. This is more clearly seen in Fig. 2*C,* where digestion of DNA amplified from 4R4 cells using ^{32}P-labeled primer P3 yielded both the labeled 85-bp fragment characteristic of the normal gene and a labeled 204-bp fragment characteristic of the *L753F* gene. It therefore appears that steroid resistance in 4R4 cells is the result of a partial gene deletion.

Deletion of the 5′ portion of the wild-type gene could result in loss of hGR expression. Alternatively, it could result in fusion of the remaining portion of the hGR gene to an upstream gene and synthesis of a chimeric protein containing the COOH-terminal portion of the hGR under the control of a heterologous promoter. To distinguish these possibilities, RNA isolated from 4R4 cells was subjected to RT-PCR, and the amplified product was digested with *Alu*I to identify the presence of transcripts containing wild-type and mutant hGR exon 9 sequences. In these experiments, the 5′ primer for amplification of the cDNA corresponds to sequence in exon 8 of the *hGR* gene (42, 43), thereby eliminating the possibility of amplification of contaminating genomic DNA. *Alu*I digestion of RT-PCR products obtained from 4R4 cell mRNA yielded, exclusively, the 289- and 37-bp products predicted for the transcripts containing the mutant sequence (Fig.

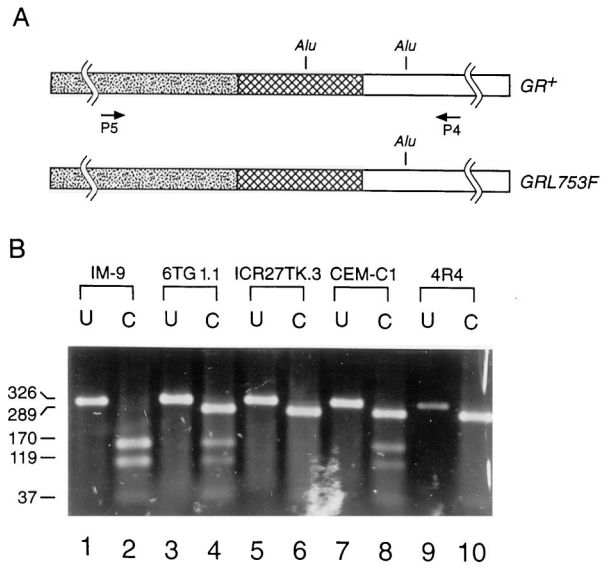

Fig. 3. RT-PCR of wild-type and mutant hGR mRNA. *A*, schematic representation of RT-PCR of region of hGR mRNA containing normal and mutant codon 753. *Stippled region*, exon 8 sequence; *cross-hatched region*, coding sequence of exon 9; *open region*, 3′-UTR of exon 9. *B*, mRNA isolated from various cell lines was reverse transcribed and amplified as described in "Materials and Methods." Amplified products were resolved by agarose gel electrophoresis before (*U*) or after (*C*) digestion with *Alu*I. *Left*, sizes of the undigested and digested fragments.

3*B*). The same pattern was seen after digestion of RT-PCR product prepared from ICR27TK.3 cells in which the entire wild-type *hGR* gene is deleted as the result of an unbalanced translocation involving chromosomes 5 and 15 (40). Fragments of 119 and 170 bp, characteristic of digestion of material amplified from wild-type mRNA, were not detected after digestion of material amplified from 4R4 cell mRNA, indicating that transcripts containing wild-type sequence are not expressed in 4R4 cells. These fragments were easily detected after digestion of material prepared from IM-9 cells, which contain two copies of the wild-type gene. In addition, consistent with the presence of both normal and mutant genes in 6TG1.1 and CEM-C1 cells, digestion of material obtained after RT-PCR of mRNA from these cells yielded all four fragments. Thus, deletion of the 5′ portion of the *hGR* in 4R4 cells does not result in the synthesis of a chimeric transcript containing sequence encoding the LBD of the hGR.

The *L753F* Mutation Was Present in the Patient from Whom CEM Cells Were Established. The results presented above and those from other laboratories suggest that the *L753F* mutation is present in all cell lines derived from CCRF-CEM cells. This raises the question of whether this mutation was also present in the leukemic cells of the individual from whom this cell line was isolated. To address this question, DNA was isolated from paraffin blocks containing biopsy material obtained from patient CEM during the course of her therapy (27). Unfortunately, DNA isolated from paraffin blocks was badly degraded and proved a poor substrate for PCR amplification of fragments of the size used for SSCP or *Alu*I RFLP analysis. Allele-specific PCR of shorter fragments, using one forward primer (primer A) containing wild-type sequence and one reverse primer (primer B) terminating in the mutant sequence, was therefore used to examine archival DNA for the presence of the *L753F* mutation (Fig. 4*A*). As expected, allele-specific PCR of DNA isolated from IM-9 cells yielded wild-type fragment AQ, whereas allele-specific PCR of DNA isolated from ICR27TK.3 cells yielded only mutant fragment PB (Fig. 4*B*). In contrast, DNA isolated from the GR^{+}/GR^{753F} cell line 6TG1.1 yielded both fragments. When archival DNA was examined using conditions optimized for amplification of mutant fragment PB, the 73-bp fragment containing the *L753F* mutation was identified in four of seven samples examined (Fig. 4*C*, *Lanes 6*, *8*, and *10* and data not shown). Archival material also contained the wild-type *hGR* gene, because fragment AQ was detected after amplification of the same samples using conditions optimized for amplification of this fragment (Fig. 4*C*, *Lanes 7* and *9* and data not shown). It is highly improbable that these results reflect contamination with exogenous DNA, because reactions from which template was omitted yielded no identifiable fragments, and no mutant band was ever detected after amplification of IM-9 cell DNA.

To confirm the presence of the *L753F* mutation in archival material, DNA isolated from two different samples was amplified, cloned, and sequenced. Table 1 shows the frequency with which the *L753F*

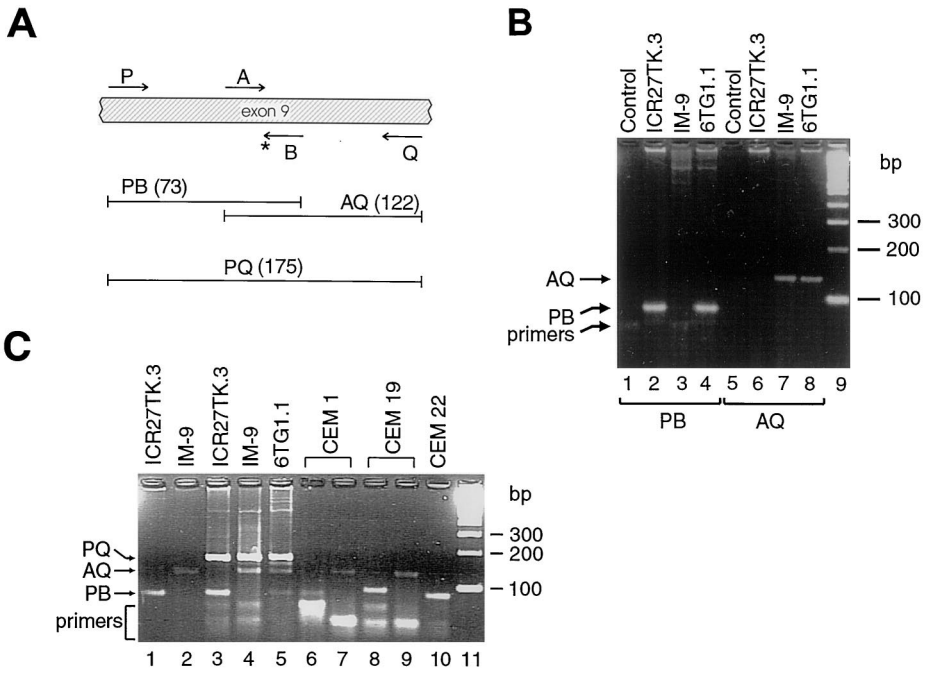

Fig. 4. Allele-specific PCR of *L753F* mutation. *A*, schematic representation of allele-specific PCR showing external primers P and Q, forward wild-type primer A, and reverse mutant primer B. *, mutant nucleotide at the 3′-end of primer B. *B*, DNA isolated from IM-9 (GR^{+}/GR^{+}), 6TG1.1 (GR^{+}/GR^{753F}), and ICR27TK.3 ($\Delta GR/GR^{753F}$) cells was amplified with primers P and B, or A and Q, and the products were resolved by agarose gel electrophoresis. Control lanes show the results of reactions in which template was omitted. *Lane 9* contains 100-bp ladder. *C*, DNA isolated from ICR27TK.3 (*Lanes 1* and *3*), IM-9 (*Lanes 2* and *4*), or 6TG1.1 (*Lane 5*) cells or from different paraffin blocks (*Lanes 6–10*) was amplified with primers P and B (*Lanes 1* and *6*), A and Q (*Lanes 2* and *7*), or all four primers (*Lanes 3–5* and *8–10*), under conditions favoring the amplification of mutant fragment PB (0.1 μM all primers; *Lanes 3–5*, *8*, and *10*) or conditions favoring the amplification of wild-type fragment AQ (0.1 μM primers P and B, and 0.2 μM primers A and Q; *Lane 9*).

Table 1 *Frequency of mutant L753 gene in archival DNA*[a]

	No. of mutant clones	No. of wild-type clones	Frequency
Sample 1	3	19	0.18
Sample 2	1	25	0.04

[a] Fragment PQ was amplified from two different archival samples, cloned into PCR2.1, and sequenced as described in "Materials and Methods." The numbers of genomic clones containing mutant (*L753F*) and wild-type sequences are indicated.

mutation was detected. Four of 44 clones examined contained the *L753F* mutation, establishing the presence of this mutation in at least some of the leukemic cells of patient CEM. However, the frequency was substantially lower than the 0.5 expected if every cell contained one copy of the mutant gene, suggesting that not every leukemic cell contained this mutation or that the biopsy material was contaminated with normal cells. In either case, however, these results establish, for the first time, the presence of an *hGR* mutation in a patient with acute lymphoblastic leukemia.

Nonsense Mutations Result in Loss of hGR mRNA Expression. Although allele-specific PCR is well suited to the identification of known mutations, it cannot be used to identify unknown mutations. To identify other mutations in the *hGR* gene responsible for steroid resistance, PCR-SSCP was used to scan for mutations in exons 3–9 of the *hGR* gene in glucocorticoid-resistant cell lines derived from glucocorticoid-sensitive 6TG1.1 cells after mutagenesis with bleomycin (40). The results for PCR-SSCP of exon 6 of the *hGR* genes in glucocorticoid-resistant BLMB1 cells are presented in Fig. 5*A*. In addition to the normal electrophoretic species that comigrates with material amplified from IM-9 and various CEM-derived cell lines, an additional band with slightly greater mobility was detected. Sequencing of genomic clones derived after amplification of exon 6 revealed the presence of clones with normal exon 6 sequence, derived from amplification of the gene containing the *L753F* mutation, and clones containing a single C→T mutation in the first nucleotide of codon 615, resulting in the creation of a premature stop codon in the hGR mRNA (Fig. 5*B*). The genotype of BLMB1 cells is therefore $GR^{Q615Stop}/GR^{753F}$.

In some genes, the occurrence of a nonsense mutation in an exon upstream from the terminal coding exon results in loss of expression of mature mRNA (44, 45). To determine whether the *hGR* belongs to this class or whether upstream nonsense mutations could potentially result in the synthesis of truncated receptor proteins lacking portions of the LBD, RT-PCR of exon 9 was used to assess mRNA expression from the mutant $GR^{Q615Stop}$ gene, as well as other bleomycin-induced mutant *hGR* genes. *Alu*I digestion of material amplified from all cell lines yielded the 289- and 37-bp fragments characteristic of mRNA synthesized from the mutant *L753F* gene. However, the 119- and 170-bp bands, characteristic of mRNA containing normal exon 9 sequence, were absent after digestion of material amplified from BLMB1 cell mRNA (Fig. 5*C*, *Lane 6*), indicating that these cells contain little if any mRNA from the mutant *Q615Stop* gene. A comparable result was seen for mRNA isolated from BLMA4 cells in which a different nonsense mutation ($GR^{L587Stop}$) is present in exon 6,[6] but not for mRNA isolated from glucocorticoid-resistant BLMA2 cells, in which there is an *R611I* missense mutation in exon 6, or from mRNA isolated from hGR mutants BLMA2 and BLMA5, which contain nonsense mutations in codon 772 of exon 9.[6] Thus, nonsense mutations in upstream exons of the *hGR* gene result in loss of mRNA expression.

DISCUSSION

The presence of the *L753F* mutation, routinely found in one of the *hGR* genes of glucocorticoid-sensitive and glucocorticoid-resistant CCRF-CEM cell lines (16, 20, 23, 31), was identified in biopsy material from the patient (CEM) from whom the cell line CCRF-CEM was derived (27). Polymorphisms have been identified in the coding and noncoding regions of the *hGR* gene (46–49), some of which have been associated with accumulation of abdominal fat and obesity (50, 51), hyperinsulinemia (52), or increased corticosteroid sensitivity (53). Inherited germ-line mutations have been identified in patients with generalized inherited glucocorticoid resistance (43, 54, 55), and a *de novo* dominant-negative germ line mutation has been identified in a patient with sporadic generalized glucocorticoid resistance (56). In addition, a somatic mutation in exon 2 has been identified in a patient with Nelson's syndrome (57). However, despite the widespread use of glucocorticoids in the treatment of leukemias, lymphomas, and multiple myeloma, somatic *hGR* mutations responsible for the acquisition of glucocorticoid resistance in malignant human cells has only been demonstrated in cultured cells (16, 20–24, 31, 58).

The frequency of genomic clones containing the *L753F* mutation was substantially <50%, indicating that only a fraction of the leukemic cells

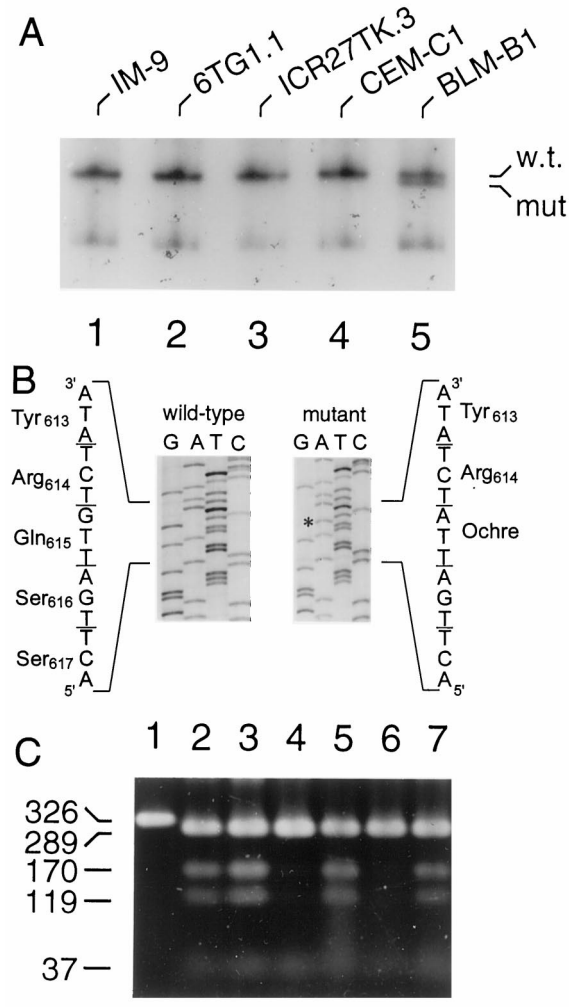

Fig. 5. Identification, cloning, and RT-PCR analysis of nonsense mutation in exon 6. *A*, DNA isolated from various cell lines was analyzed by PCR-SSCP of exon 6 as described in "Materials and Methods." *B*, DNA isolated from BLMB1 cells was amplified, cloned, and sequenced as described in "Materials and Methods." Shown are the antisense sequences from one wild-type (*left-hand panel*) and one mutant (*right-hand panel*) clone. *, position of the mutant nucleotide. *C*, mRNA isolated from glucocorticoid-sensitive 6TG1.1 cells (GR^{+}/GR^{753F}; *Lanes 1* and *2*) and glucocorticoid-resistant BLMA2 (GR^{R611I}/GR^{753F}; *Lane 3*), BLMA4 ($GR^{L587Stop}/GR^{753F}$; *Lane 4*), BLMA5 ($GR^{L772Stop}/GR^{753F}$; *Lane 5*), BLMB1 ($GR^{Q615Stop}/GR^{753F}$; *Lane 6*), and BLMB2 ($GR^{L772Stop}/GR^{753F}$; *Lane 7*) cells was analyzed by RT-PCR either before (*Lane 1*) or after (*Lanes 2–7*) digestion with *Alu*I. *Left*, sizes of the undigested and digested fragments.

[6] A. G. Hillmann, K. Multanen, J. Ramdas, and J. M. Harmon, manuscript in preparation.

contained the *L753F* mutation, and suggesting that this mutation occurred during progression of the disease. Although it is not possible to state with certainty at what point in the progression of the disease the biopsy material was obtained, it appears that several rounds of corticosteroid therapy preceded the collection of material (27). It is therefore unlikely that this mutation contributed to the development of the disease. However, numerous *in vitro* studies have established a direct correlation between receptor concentration and the ability of corticosteroids to induce a lytic response in sensitive T cells (59–61). Indeed, we showed recently that a 2-fold increase in hGR concentration is sufficient to render otherwise steroid-resistant CEM cells sensitive to the growth-inhibitory and cytotoxic effects of dexamethasone (62). In addition, several studies of leukemic blasts have found a direct correlation between hGR concentration and clinical prognosis (5–7, 63–65), and loss of heterozygosity at the *hGR* locus has been identified in a substantial proportion of patients with adrenocorticotropic hormone-secreting pituitary adenomas in which negative feedback of adrenocorticotropic hormone production is attenuated (66). Thus, loss of one functional copy of the *hGR* gene may result in decreased glucocorticoid sensitivity and/or partial glucocorticoid resistance.

The *L753F* mutation could have arisen spontaneously and been selected during multiple rounds of chemotherapy. Alternatively, one of the other drugs in the combination regimens used for treatment could have induced this mutation in the *hGR* gene. Indeed, we have shown previously that several common chemotherapeutic agents are capable of *in vitro* induction of mutations in the *hGR* gene that result in steroid resistance. Using the fact that CEM cells are heterozygous for the *Bcl*I RFLP in the 5′ portion of the *hGR* gene, it was shown previously that the *hGR* gene is deleted in two cell lines derived after chemical mutagenesis or treatment with the radiomimetic agent bleomycin (40). Using this same polymorphism, Ashraf and Thompson showed that the 5′-end of the *hGR* gene was deleted in the spontaneously derived glucocorticoid-resistant cell line 4R4 (20). To assess the integrity of the 3′-end of the *hGR* gene, the *L753F* polymorphism in exon 9 was examined. Surprisingly, the 3′-end of the wild-type gene was intact in 4R4 cells, demonstrating that, at least *in vitro*, glucocorticoid resistance can result from partial deletion of the *hGR* gene. The presence of a microdeletion at the boundary of exon 6 and intron F of the *hGR* responsible for glucocorticoid resistance in a family with familial glucocorticoid resistance demonstrates that *in vivo* deletions are also possible (67). Thus, although the *L753F* mutation is unlikely to be present in other leukemic patients, a sufficient number of naturally occurring polymorphisms in the 5′- and 3′-ends of the *hGR* gene have been identified to allow screening for full or partial deletion of the *hGR* gene in clinical samples. Identification of additional polymorphisms would facilitate the identification and mapping of even smaller deletions.

The L753F mutation causes an increased rate of ligand dissociation from both the unactivated and activated hGR (34). However, this is accompanied by an increased rate of association of ligand with the unactivated receptor, resulting in an apparently normal equilibrium dissociation constant (34). Consequently, assays that measure the concentration of unactivated hGR would fail to detect any abnormality in receptor number or affinity. This is probably true of other mutations in the LBD, as well as in other regions of the receptor. In addition, as the data presented here suggest, not every cell in the leukemic population must necessarily contain a receptor mutant for therapy to fail, thereby limiting the sensitivity of assays that measure receptor concentration in a heterogeneous population of cells. Thus, simple measurement of receptor concentration, or affinity, is almost certainly inadequate to identify subtle receptor defects contributing to glucocorticoid resistance.

SSCP-PCR identified a point mutation creating a premature stop codon in exon 6 of the *hGR*. Interestingly, no mRNA from this mutant gene was detected. This is comparable to the lack of mRNA expression seen from the Δ4 allele in individuals with familial glucocorticoid resistance (43) and suggests that the *hGR* belongs to that family of genes the transcripts of which are degraded if mutation introduces a premature stop codon (44, 45). Indeed, a nonsense mutation in any region of the *hGR* from the distal portion of exon 2 through exon 8 appears to result in loss of mRNA expression.[6] Thus, although spontaneous or induced nonsense mutations could potentially occur with high frequency in the *hGR* gene, it is unlikely that they would result in the synthesis of a truncated protein with aberrant or constitutive function. The *hGR*, therefore, appears similar to the human androgen and vitamin D receptors, in which nonsense mutations in upstream exons result in substantial loss of mRNA and protein expression (68, 69). These results also suggest that the protein truncation test, widely used to detect nonsense mutations, or analysis of cDNA clones prepared from leukemic cell mRNA would fail to detect a significant percentage of *hGR* mutations resulting in functional loss of heterozygosity. Similarly, analysis of cDNAs by SSCP or other methods for detection of *hGR* coding region mutations would fail to detect nonsense mutations in exons 2–8, perhaps accounting for the failure of Soufi *et al.* (26) to find mutations in the leukemic cells of patients with chronic lymphocytic leukemia.

The presence of the *L753F* mutation was demonstrated in patient CEM, from whom the cell line CCRF-CEM was derived. The results presented here suggest that this mutation was not responsible for the ontogeny of the leukemia. However, it may have contributed to emergence of a glucocorticoid-resistant cell population. The determination of whether somatic mutation in the *hGR* gene is a significant cause of glucocorticoid resistance will require detailed analysis of the *hGR* gene in a variety of leukemias, lymphomas, and multiple myeloma.

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REFERENCES

1. Vietti, T. J., Sullivan, M. P., Berry, B. H., Hardy, T., Haggard, M., and Blattner, R. The response of acute childhood leukemia to an initial and second course of prednisone. J. Pediatr., *66:* 18–26, 1965.
2. Wolff, J. A., Brubaker, C. A., Murphy, M. L., Pierce, M. I., and Severo, N. Prednisone therapy of childhood acute leukemia: prognosis and duration of response in 330 treated patients. J. Pediatr., *70:* 626–631, 1967.
3. Shanbrom, E., and Miller, S. Critical evaluation of massive steroid therapy of acute leukemia. N. Engl. J. Med., *266:* 1354–1358, 1962.
4. Hyman, C., Borda, E., Brubaker, C., Hammond, D., and Sturgeon, P. Prednisone in childhood leukemia: comparison of interrupted with continuous therapy. Pediatrics, *24:* 1005–1008, 1959.
5. Bloomfield, C. D., Smith, K. A., Peterson, B. A., and Munck, A. Glucocorticoid receptors in adult acute lymphoblastic leukemia. Cancer Res., *41:* 4857–4860, 1981.
6. Pui, C. H., Dahl, G. V., Rivera, G., Murphy, S. B., and Costlow, M. E. The relationship of blast cell glucocorticoid receptor levels to response to single-agent steroid trial and remission response in children with acute lymphoblastic leukemia. Leuk. Res., *8:* 579–585, 1984.
7. Pui, C. H., and Costlow, M. E. Sequential studies of lymphoblast glucocorticoid receptor levels at diagnosis and relapse in childhood leukemia: an update. Leuk. Res., *10:* 227–229, 1986.
8. Wells, R. J., Mascaro, K., Young, P. C., and Cleary, R. E. Glucocorticoid receptors in the lymphoblasts of patients with glucocorticoid-resistant childhood acute lymphocytic leukemia. Am. J. Pediatr. Hematol. Oncol., *3:* 259–264, 1981.
9. Lippman, M. E. Clinical implications of glucocorticoid receptors in human leukemia. Am. J. Physiol., *243:* E103–E108, 1982.
10. Pui, C. H., Costlow, M. E., Dahl, G. V., Rivera, G., and Murphy, S. B. Response of recurrent acute lymphoblastic leukemia to glucocorticoids: serial studies of receptor content, *in vivo* cytokinetic changes and clinical responses. Leuk. Res., *7:* 747–753, 1983.
11. Beato, M., Chavez, S., and Truss, M. Transcriptional regulation by steroid hormones. Steroids, *61:* 240–251, 1996.
12. Reichardt, H. M., and Schutz, G. Glucocorticoid signalling–multiple variations of a common theme. Mol. Cell. Endocrinol., *146:* 1–6, 1998.
13. McEwan, I. J., Wright, A. P. H., and Gustafsson, J-Å. Mechanism of gene expression by the glucocorticoid receptor: role of protein-protein interactions. Bioessays, *19:* 153–160, 1997.

14. Dumont, A., Hehner, S. P., Schmitz, M. L., Gustafsson, J-Å., Liden, J., Okret, S., van der Saag, P. T., Wissink, S., van der Burg, B., Herrlich, P., Haegeman, G., De Bosscher, K., and Fiers, W. Cross-talk between steroids and NF-κ: what language? Trends Biochem. Sci., *23:* 233–235, 1998.
15. Gottlicher, M., Heck, S., and Herrlich, P. Transcriptional cross-talk, the second mode of steroid hormone receptor action. J. Mol. Med., *76:* 480–489, 1998.
16. Powers, J. H., Hillmann, A. G., Tang, D. C., and Harmon, J. M. Cloning and expression of mutant glucocorticoid receptors from glucocorticoid-sensitive and glucocorticoid-resistant human leukemic cells. Cancer Res., *53:* 4059–4065, 1993.
17. Liu, W., Hillmann, A. G., and Harmon, J. M. Hormone-independent repression of AP-1-inducible collagenase promoter activity by glucocorticoid receptors. Mol. Cell. Biol., *15:* 1005–1013, 1995.
18. Reichardt, H. M., Kaestner, K. H., Tuckermann, J., Kretz, O., Wessely, O., Bock, R., Gass, P., Schmid, W., Herrlich, P., Angel, P., and Schutz, G. DNA binding of the glucocorticoid receptor is not essential for survival. Cell, *93:* 531–541, 1998.
19. Danielsen, M., Northrop, J. P., and Ringold, G. M. The mouse glucocorticoid receptor: mapping of functional domains by cloning, sequencing and expression of wild-type and mutant receptor proteins. EMBO J., *5:* 2513–2522, 1986.
20. Ashraf, J., and Thompson, E. B. Identification of the activation-labile gene: a single point mutation in the human glucocorticoid receptor presents as two distinct receptor phenotypes. Mol. Endocrinol., *7:* 631–642, 1993.
21. Lee, S., Duncan, A., Chou, H., Chen, D., Kohli, K., Huang, C. F., and Stallcup, M. R. A somatic cell genetic method for identification of untargeted mutations in the glucocorticoid receptor that cause hormone binding deficiencies. Mol. Endocrinol., *9:* 826–837, 1995.
22. Strasser-Wozak, E. M. C., Hattmannstorfer, R., Hala, M., Hartmann, B. L., Fiegl, M., Geley, S., and Kofler, R. Splice site mutation in the glucocorticoid receptor gene causes resistance to glucocorticoid-induced apoptosis in a human acute leukemic cell line. Cancer Res., *55:* 348–353, 1995.
23. Hala, M., Hartmann, B. L., Bock, G., Geley, S., and Kofler, R. Glucocorticoid-receptor-gene defects and resistance to glucocorticoid-induced apoptosis in human leukemic cell lines. Int. J. Cancer, *68:* 663–668, 1996.
24. Karkera, J. D., Taymans, S. E., Turner, G., Yoshikawa, T., Detera-Wadleigh, S. D., and Wadleigh, R. G. Deletion of a consensus oestrogen response element half-site in the glucocorticoid receptor of human multiple myeloma. Br. J. Haematol., *99:* 372–374, 1997.
25. Krett, N. L., Pillay, S., Moalli, P. A., Greipp, P. R., and Rosen, S. T. A variant glucocorticoid receptor messenger RNA is expressed in multiple myeloma patients. Cancer Res., *55:* 2727–2729, 1995.
26. Soufi, M., Kaiser, U., Schneider, A., Beato, M., and Westphal, H. M. The DNA and steroid binding domains of the glucocorticoid receptor are not altered in mononuclear cells of treated CLL patients. Exp. Clin. Endocrinol. Diabetes, *103:* 175–183, 1995.
27. Foley, G. E., Lazarus, H., Farber, S., Uzman, B. G., Boone, B. A., and McCarthy, R. E. Continuous culture of human lymphoblasts from peripheral blood of a child with acute leukemia. Cancer (Phila.), *18:* 522–529, 1965.
28. Norman, M. R., and Thompson, E. B. Characterization of a glucocorticoid-sensitive human lymphoid cell line. Cancer Res., *37:* 3785–3791, 1977.
29. Thompson, E. B., Smith, J. R., Bourgeois, S., and Harmon, J. M. Glucocorticoid receptors in human leukemias and related diseases. Klin. Wochenschr., *63:* 689–698, 1985.
30. Yuh, Y-S., and Thompson, E. B. Complementation between glucocorticoid receptor and lymphocytolysis in somatic cell hybrids of two glucocorticoid-resistant human leukemic clonal cell lines. Somat. Cell Mol. Genet., *13:* 33–46, 1987.
31. Geley, S., Hartmann, B. L., Hala, M., Strasser-Wozak, E. M. C., Kapelari, K., and Kofler, R. Resistance to glucocorticoid-induced apoptosis in human T-cell acute lymphoblastic leukemia CEM-C1 cells is due to insufficient glucocorticoid receptor expression. Cancer Res., *56:* 5033–5038, 1996.
32. Harmon, J. M., Thompson, E. B., and Baione, K. A. Analysis of glucocorticoid-resistant human leukemic cells by somatic cell hybridization. Cancer Res., *45:* 1587–1593, 1985.
33. Harmon, J. M., and Thompson, E. B. Isolation and characterization of dexamethasone-resistant mutants from human lymphoid cell line CEM-C7. Mol. Cell. Biol., *1:* 512–521, 1981.
34. Palmer, L. A., and Harmon, J. M. Biochemical evidence that glucocorticoid-sensitive cell lines derived from the human leukemic cell line CCRF-CEM express a normal and a mutant glucocorticoid receptor gene. Cancer Res., *51:* 5224–5231, 1991.
35. Strauss, W. M. Preparation of genomic DNA from mammalian tissue. *In:* Ausbel, M. E., Brent, R., Kingston, R. E., Moore, D. E., Seidman, J. G., Smith, J. A., and Strehl, K. (eds.), Current Protocols in Molecular Biology, pp. 2.2.1–2.2.3. New York: Greene Publishing Associates and Wiley-Interscience, 1998.
36. Jackson, D. P., Hayden, J. D., and Quirke, P. Extraction of nucleic acids from fresh and archival material. *In:* M. J. McPherson, P. Quirke, and G. R. Taylor (eds.), PCR: A Practical Approach, Vol. 1, pp. 29–50. Oxford: Oxford University Press, 1991.
37. Greer, E. G., Wheeler, C. M., and Manos, M. M. PCR amplification from paraffin-embedded tissues, sample preparation and the effects of fixation. *In:* C. W. Dieffenbach and G. S. Dveksler (eds.), PCR Primer: A Laboratory Manual, pp. 99–112. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory, 1995.
38. Liu, Q., Thorland, E. C., Heit, J. A., and Sommer, S. S. Overlapping PCR for bidirectional PCR amplification of specific alleles: a rapid one-tube method for simultaneously differentiating homozygotes and heterozygotes. Genome Res., *7:* 389–398, 1997.
39. Orita, M., Iwahana, H., Kanazawa, H., Hayashi, K., and Sekiya, T. Detection of polymorphisms of human DNA by gel electrophoresis as single-strand conformation polymorphisms. Proc. Natl. Acad. Sci. USA, *86:* 2766–2770, 1989.
40. Palmer, L. A., Hukku, B., and Harmon, J. M. Human glucocorticoid receptor gene deletion following exposure to cancer chemotherapeutic drugs and chemical mutagens. Cancer Res., *52:* 6612–6618, 1992.
41. Ramdas, J., and Harmon, J. M. Glucocorticoid-induced apoptosis and regulation of NF-κ activity in human leukemic T-cells. Endocrinology, *139:* 3813–3821, 1998.
42. Encio, I. J., and Detera-Wadleigh, S. D. The genomic structure of the human glucocorticoid receptor. J. Biol. Chem., *266:* 7182–7188, 1991.
43. Karl, M., Lamberts, S. W. J., Detera-Wadleigh, S. D., Encio, I. J., Stratakis, C. A., Hurley, D. M., Accili, D., and Chrousos, G. P. Familial glucocorticoid resistance caused by a splice site deletion in the human glucocorticoid receptor gene. J. Clin. Endocrinol. Metab., *76:* 683–689, 1993.
44. Hentze, M. W., and Kulozik, A. E. A perfect message: RNA surveillance and nonsense-mediated decay. Cell, *96:* 307–310, 1999.
45. Maquat, L. E. When cells stop making sense: effects of nonsense codons on RNA metabolism in vertebrate cells. RNA, *1:* 453–465, 1995.
46. Murray, J. C., Ardinger, H. A., and Weinberger, C. RFLP for the glucocorticoid receptor (GRL) located at 5q11–5q13. Nucleic Acids Res., *15:* 6765, 1987.
47. Detera-Wadleigh, S. D., Encio, I. J., Rollins, D. Y., Coffman, D., and Wiesch, D. A TthIII1 polymorphism on the 5′ flanking region of the glucocorticoid receptor gene (*GRL*). Nucleic Acids Res., *19:* 1960, 1991.
48. Koper, J. W., Stolk, R. P., Delange, P., Huizenga, N. A. T. M., Molijn, G. J., Pols, H. A. P., Grobbee, D. E., Karl, M., Dejong, F. H., Brinkmann, A. O., and Lamberts, S. W. J. Lack of association between five polymorphisms in the human glucocorticoid receptor gene and glucocorticoid resistance. Hum. Genet., *99:* 663–668, 1997.
49. Witchel, S. F., and Smith, R. R. Glucocorticoid resistance in premature pubarche and adolescent hyperandrogenism. Mol. Genet. Metab., *66:* 137–141, 1999.
50. Buemann, B., Vohl, M. C., Chagnon, M., Chagnon, Y. C., Gagnon, J., Perusse, L., Dionne, F., Despres, J. P., Tremblay, A., Nadeau, A., and Bouchard, C. Abdominal visceral fat is associated with a *Bcl*I restriction fragment length polymorphism at the glucocorticoid receptor gene locus. Obes. Res., *5:* 186–192, 1997.
51. Lin, R. C., Wang, W. Y., and Morris, B. J. High penetrance, overweight, and glucocorticoid receptor variant: case-control study. Br. Med. J., *319:* 1337–1338, 1999.
52. Weaver, J. U., Hitman, G. A., and Kopelman, P. G. An association between a *Bc1*I restriction fragment length polymorphism of the glucocorticoid receptor locus and hyperinsulinaemia in obese women. J. Mol. Endocrinol., *9:* 295–300, 1992.
53. Huizenga, N. A., Koper, J. W., De Lange, P., Pols, H. A., Stolk, R. P., Burger, H., Grobbee, D. E., Brinkmann, A. O., De Jong, F. H., and Lamberts, S. W. A polymorphism in the glucocorticoid receptor gene may be associated with and increased sensitivity to glucocorticoids in vivo. J. Clin. Endocrinol. Metab., *83:* 144–151, 1998.
54. Hurley, D. M., Accili, D., Stratakis, C. A., Karl, M., Vamvakopoulos, N., Rorer, E., Constantine, K., Taylor, S. I., and Chrousos, G. P. Point mutation causing a single amino acid substitution in the hormone binding domain of the glucocorticoid receptor in familial glucocorticoid resistance. J. Clin. Investig., *87:* 680–686, 1991.
55. Malchoff, D. M., Brufsky, A., Reardon, G., McDermott, P., Javier, E. C., Bergh, C. H., Rowe, D., and Malchoff, C. D. A mutation of the glucocorticoid receptor in primary cortisol resistance. J. Clin. Investig., *91:* 1918–1925, 1993.
56. Karl, M., Lamberts, S. W., Koper, J. W., Katz, D. A., Huizenga, N. E., Kino, T., Haddad, B. R., Hughes, M. R., and Chrousos, G. P. Cushing's disease preceded by generalized glucocorticoid resistance: clinical consequences of a novel, dominant-negative glucocorticoid receptor mutation. Proc. Assoc. Am. Physicians, *108:* 296–307, 1996.
57. Karl, M., Von Wichert, G., Kempter, E., Katz, D. A., Reincke, M., Monig, H., Ali, I. U., Stratakis, C. A., Oldfield, E. H., Chrousos, G. P., and Schulte, H. M. Nelson's syndrome associated with a somatic frame shift mutation in the glucocorticoid receptor gene. J. Clin. Endocrinol. Metab., *81:* 124–129, 1996.
58. Moalli, P. A., Pillay, S., Weiner, D., Leikin, R., and Rosen, S. T. A mechanism of resistance to glucocorticoids in multiple myeloma: transient expression of a truncated glucocorticoid receptor messenger RNA. Blood, *79:* 213–222, 1992.
59. Gehring, U., Mugele, K., and Ulrich, J. Cellular receptor levels and glucocorticoid responsiveness of lymphoma cells. Mol. Cell. Endocrinol., *36:* 107–113, 1984.
60. Bourgeois, S., and Newby, R. F. Diploid and haploid states of the glucocorticoid receptor gene of mouse lymphoid cell lines. Cell, *11:* 423–430, 1977.
61. Chapman, M. S., Askew, D. J., Kuscuoglu, U., and Miesfeld, R. L. Transcriptional control of steroid-regulated apoptosis in murine thymoma cells. Mol. Endocrinol., *10:* 967–978, 1996.
62. Ramdas, J., Liu, W., and Harmon, J. M. Glucocorticoid-induced cell death requires autoinduction of glucocorticoid receptor expression in human leukemic T cells. Cancer Res., *59:* 1378–1385, 1999.
63. Kato, G. J., Quddus, F. F., Shuster, J. J., Boyett, J., Pullen, J. D., Borowitz, M. J., Whitehead, V. M., Crist, W. M., and Leventhal, B. G. High glucocorticoid receptor content of leukemic blasts is a favorable prognostic factor in childhood acute lymphoblastic leukemia. Blood, *82:* 2304–2309, 1993.
64. Iacobelli, S., Marchetti, P., De Rossi, G., Mandelli, F., and Gentiloni, N. Glucocorticoid receptors predict response to combination chemotherapy in patients with acute lymphoblastic leukemia. Oncology, *44:* 13–16, 1987.
65. Pui, C. H., Ochs, J., Kalwinsky, D. K., and Costlow, M. E. Impact of treatment efficacy on the prognostic value of glucocorticoid receptor levels in childhood acute lymphoblastic leukemia. Leuk. Res., *8:* 345–350, 1984.
66. Huizenga, N. A., de Lange, P., Koper, J. W., Clayton, R. N., Farrell, W. E., van der Lely, A. J., Brinkmann, A. O., de Jong, F. H., and Lamberts, S. W. Human adrenocorticotropin-secreting pituitary adenomas show frequent loss of heterozygosity at the glucocorticoid receptor gene locus. J. Clin. Endocrinol. Metab., *83:* 917–921, 1998.
67. Chrousos, G. P., Detera-Wadleigh, S. D., and Karl, M. Syndromes of glucocorticoid resistance. Ann. Int. Med., *119:* 1113–1124, 1993.
68. Wilson, C. M., Griffin, J. E., Wilson, J. D., Marcelli, M., Zoppi, S., and Mcphaul, M. J. Immunoreactive androgen receptor expression in subjects with androgen resistance. J. Clin. Endocrinol. Metab., *75:* 1474–1478, 1992.
69. Malloy, P. J., Hochberg, Z., Tiosano, D., Pike, J. W., Hughes, M. R., and Feldman, D. The molecular basis of hereditary 1,25-dihydroxyvitamin-D3 resistant rickets in 7 related families. J. Clin. Investig., *86:* 2071–2079, 1990.