C-MYB AND MEMBERS OF THE C-ETS FAMILY OF TRANSCRIPTION FACTORS ACT AS A MOLECULAR SWITCH TO MEDIATE OPPOSITE STEROID REGULATION OF THE HUMAN GLUCOCORTICOID RECEPTOR 1A PROMOTER*

Chuan-dong Geng and Wayne V. Vedeckis

From the Department of Biochemistry and Molecular Biology and Stanley S. Scott Cancer Center, Louisiana State University Health Sciences Center, New Orleans, Louisiana, 70112

Running title: c-Myb and c-Ets proteins modulate hGR 1A promoter expression.

Address correspondence to: Wayne V. Vedeckis, Ph.D., Department of Biochemistry and Molecular Biology, Louisiana State University Health Sciences Center, 533 Bolivar Street, New Orleans, Louisiana, 70112, Tel. 504 568-8175; Fax. 504 568-6997; E-mail: wvedec@lsuhsc.edu

Steroid auto-regulation of the human glucocorticoid receptor (hGR) 1A promoter in lymphoblast cells resides largely in two DNA elements (footprints 11 and 12). We show here that c-Myb and c-Ets family members (Ets-1/2, PU.1, and Spi-B) control hGR 1A promoter regulation in T- and B-lymphoblast cells. Two T-lymphoblast lines, CEM-C7 and Jurkat, contain high levels of c-Myb and low levels of PU.1, while the opposite is true in IM-9 B-lymphoblasts. In Jurkat cells, overexpression of c-Ets-1, c-Ets-2, or PU.1 effectively represses dexamethasone-mediated upregulation of an hGR 1A promoter-luciferase reporter gene, as do dominant negative c-Myb (c-Myb DNA binding domain) or Ets proteins (Ets-2 DNA binding domain). Overexpression of c-Myb in IM-9 cells confers hormone-dependent upregulation to the hGR 1A promoter reporter gene. Chromatin immunoprecipitation assays show that hormone treatment causes the recruitment of hGR and c-Myb to the hGR 1A promoter in CEM-C7 cells, while hGR and PU.1 are recruited to this promoter in IM-9 cells. These observations suggest that the specific transcription factor that binds to footprint 12, when hGR binds to the adjacent footprint 11, determines the direction of hGR 1A promoter auto-regulation. This leads to a “molecular switch” model for auto-regulation of the hGR 1A promoter.

Glucocorticoids (GCs) specifically induce apoptosis in several types of leukemia and lymphoma, and they are used routinely in treating T-cell Acute Lymphoblastic Leukemia (ALL) (1-6). The cellular function of GCs is mediated by the glucocorticoid receptor (GR) protein, which is widely expressed in most tissues and cell lines (7-10). The inactive, cytoplasmic GR binds the GC hormone to form an activated GR-ligand complex, which then translocates to the nucleus, recognizes and binds to specific DNA sequences in the gene promoter called Glucocorticoid Response Elements (GREs), and affects the expression of these genes (7,8,11,12). It is not yet clear how GCs cause apoptosis in certain lymphoblasts, but this fact is effectively used in chemotherapy regimens for certain types of leukemias and lymphomas (13-19).

There is a strong correlation between functional cellular GR level and the sensitivity of the cell to GCs, including the apoptotic response of lymphoblasts (3,20,21). Although the initial cellular GR level is not absolutely predictive, an auto-upregulation of GR to a certain threshold level is required for apoptosis in hormone-sensitive cells (22-28). Conversely, an auto-downregulation of GR levels is frequently observed in cells resistant to hormone-mediated apoptosis, such as the pre-B lymphoblast cell line, IM-9, and the lowered GR concentration actually dampens GC signaling (9,19,26). Thus, steroid auto-upregulation of GR gene expression in vivo could provide a sensitive indicator of hormonal sensitivity of hematologic malignancies.

Human GR gene expression is controlled by at least three promoters, 1A, 1B, and 1C (9,10,29). Promoters 1B and 1C are ubiquitously expressed, while the 1A promoter is selectively expressed in hematopoietic cells (9,10). The transcripts emanating from all three promoters are auto-upregulated in response to DEX treatment in hormone-sensitive, CEM-C7 cell, T-lymphoblasts,
and downregulated in IM-9 cells that are resistant to hormone-mediated apoptosis (9,26). Because no consensus GREs are present in any of these promoters, the molecular mechanism for auto-regulation of the 1A, 1B, and 1C promoters was unknown (9,10,30). Recently, we have identified the DNA sequence that mediates upregulation of 1A promoter activity (9,31). This DNA sequence contains a half GRE (Footprint 11; FP11) and a sequence (FP12) containing overlapping consensus binding sites for c-Myb or c-Ets proteins. Both FP 11 and FP 12 are required for full hormone responsiveness of the hGR 1A promoter.

In the present study we have further investigated the roles of c-Myb and c-Ets protein members in steroid-mediated auto-regulation of the hGR 1A promoter. Our data show that c-Myb and Ets family members (Ets-1, Ets-2, PU.1, and Spi-B) can affect hGR 1A promoter activity. Western blot and functional studies indicate that c-Myb and PU.1 are the most likely candidates in mediating the opposite hormonal response of the hGR 1A promoter in T- and B-lymphoblasts. Chromatin Immunoprecipitation analyses show that, in response to DEX treatment, GR and c-Myb are recruited by the hGR 1A promoter in CEM-C7 T-cells, while GR and PU.1 are recruited in IM-9 B-cells. These results suggest a “molecular switch” model for hGR 1A promoter regulation, in which GR binding to the hGR 1A promoter recruits cell type-specific transcription factors to an adjacent DNA sequence. c-Myb is recruited in T-lymphoblasts, and this results in GR upregulation and apoptosis. PU.1 is recruited in B-cells that do not undergo hormone-mediated apoptosis, and the GR is downregulated in these cells. These findings may lead to novel clinical therapies that could increase the response rate and the magnitude of the response to steroid treatment in certain hematologic malignancies.

Methods and Materials

Cell Culture - The human Jurkat (T-ALL) and IM-9 B-lymphoblastic cell lines (both from the American Type Culture Collection, Manassas, VA) were grown in RPMI 1640 plus 10% fetal bovine serum (FBS; Life Technologies, Rockville, MD). The human, CEM-C7, acute lymphoblastic leukemia cell line was a kind gift from Dr. E Brad Thompson (University of Texas Medical Branch, Galveston, TX), and it was maintained in RPMI 1640 supplemented with 10% dialyzed FBS (Life Technologies). All cells were grown in 5% CO₂ at 37 °C. To treat the cells, 1 µM dexamethasone (Sigma, St. Louis, MO) in ethanol was added to the culture medium, and the same final concentration of ethanol vehicle (0.01%) was used in the controls.

DNA Constructs - The human 1A GR promoter - luciferase reporter constructs, pXP1-1A -964/+269 and pXP1-1A +41/+269-Luc (and its FP11/12 deletions), were described previously (9,31). PCR directed mutagenesis was performed to make the FP11 deletion in promoter 1A -964/+269, as previously described (9,31). The primers used for this deletion were: forward, 5'-CAAGCCCTGAGAGAGTGATGTCAGGAACGAG-3' and reverse: 5'-GCTTCCGTGGGACACGTCCTGCAAGGCTTG-3'. For the FP12 and FP11/12 deletions in pXP1-1A -964/+269, PCR-amplified fragments -964/+253 (FP12 deleted) and -964/+243 (FP11/12 deleted) containing engineered BamHI (5') and HindIII (3') restriction sites were inserted into the pXP1 vector digested with the same restriction enzymes. The primers used to construct these deletions were: forward primer, 5'-CCAAACTCATCAATGTATCT-3'; FP12del reverse (FP12 deletion), 5'-GAGAAGCTTGCGCATTTTACGGTCCTG-3'; and, FP11/12del reverse (FP11/12 deletion), 5'-CACAAGCTTTACGGTCCTGCAAGGGCTTGAA-3'. All constructs were confirmed by DNA sequencing.

The pCYGR construct was provided by Dr. John A. Cidlowski (NIEHS, Research Triangle Park, NC). The human c-Myb expression construct, pcDNA3-c-MybHA, and the c-Myb DNA Binding Domain (DBD) expression construct, pcDNA3-c-Myb DBD, were provided by Dr. Giuseppe Raschellà (Ente Nuove Tecnologie Energia Ambiente (ENEA), Rome, Italy) (32). The mouse c-Myb expression plasmid, pc75, and the C-terminal negative regulatory domain truncated c-Myb expression plasmid, pCT, were gifts from Dr. E Premkumar Reddy and Dr. Ramana V. Tantravahi (Temple Univ., Philadelphia, PA) (33). pFN Ets-1, pFN Ets-2 and pFN Ets-2 DBD were supplied by Dr. Craig A. Hauser (The Burnham Institute, La Jolla, CA).
The PU.1 and Spi-B expression constructs were described previously (35).

**Transient Transfection and Luciferase Reporter Gene Assays** - Superfect transfection reagent (Qiagen, Los Angeles, CA) was used to transfect Jurkat cells, according to the manufacturer’s directions. Cells were treated (with ETOH or DEX) 24 hours after transfection and were collected for analysis after an additional 24 hours of incubation. The collected cells were lysed and measured for firefly luciferase and β-galactosidase activity on an Ascent Luminoskan (Labsystems, Franklin, MA) as previously described (9,31).

**Electroporation** - IM-9 cells were electroporated using a method modified from the laboratory of Dr. Jeffrey M. Harmon (Uniformed Services University of the Health Sciences, Bethesda, MD; unpublished). Cells in log phase growth were counted and harvested by centrifugation at 800g for 10 minutes (4 °C). After two washes with RPMI 1640 medium (w/o FBS), cells were resuspended in cold RPMI 1640 (w/o FBS and containing 10 mM HEPES, pH 7.2) at 2.5 x 10⁷ cells/ml. 200 µl of cell suspension and 10 µg DNA were combined in a Bio-Rad 0.4 cm electroporation cuvette and the mixture was incubated on ice for 10 min. Electroporation was done with a Gene Pulser II (Bio-Rad) at 340 V/960 µF. Electroporated cells were then incubated on ice for 10 minutes before being transferred to 10 ml of culture medium (with FBS) at room temperature. The electroporated cells were allowed to grow in 5% CO₂ at 37 °C for 24 hours before DEX or ethanol treatment.

**Western Blotting** - Cells were lysed with 1x Laemmli sample buffer with a protease inhibitor cocktail (Sigma, St. Louis, MO). Proteins resolved on SDS-PAGE (8%) were transferred to Immobilon-Nitrocellulose (NC) membranes (Millipore, Bedford, MA). The membranes were blocked with 5% non-fat milk and developed using ECL (Cat# RPN2106; Amersham, Piscataway, NJ). Rabbit polyclonal actin, tubulin, hGR (H-300), c-Myb, Spi-B and PU.1 antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA).

**Chromatin Immunoprecipitation Assay (ChIP Assay)** - Formaldehyde cross-linking and chromatin immunoprecipitation assays of tissue culture cells were performed as described (36,37) with some modifications. IM-9 and CEM-C7 cells (1 x 10⁷) were treated for 24 hours with/or without 1 µM DEX. Cells were incubated in 1% formaldehyde (Sigma) for 10 min at room temperature. Cross-linking was stopped by adding glycine to a final concentration of 125 mM. The cells were spun down and washed three times with ice-cold PBS before resuspending in 200 µl of cell lysis buffer (5 mM Pipes-KOH, pH 8.0, 85 mM KCl, 0.5% NP-40) containing protease inhibitors (1 µg/ml leupeptin, 1 µg/ml aprotinin and 1 mM PMSF). After incubating for 10 min on ice, the nuclei were pelleted and resuspended in 200 µl nuclear lysis buffer (50 mM Tris-HCl, pH 8.1, 10 mM EDTA, 1% SDS with protease inhibitors), and incubated on ice for 10 minutes. The chromatin released from the nuclei was sonicated at 4 °C with a Branson Sonifier to obtain DNA lengths of 0.1-1.5 kbp. The sonicated cell lysate was clarified by centrifugation at 13,000g for 10 min at 4 °C. The supernatant containing the sheared chromatin was used for the immunoprecipitation assay.

40 µl of the supernatant (sonicated chromatin) was diluted 5-fold in ChIP dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl, pH 8.1, 167 mM NaCl plus protease inhibitors) and precleared with 6 µg Rabbit Normal IgG (Santa Cruz Biotechnology), followed by the addition of a salmon sperm DNA/protein A agarose slurry (Upstate, Charlottesville, VA). Six µg of antibodies (Normal Rabbit IgG, hGR H-300, or c-Myb) (Santa Cruz Biotechnology) were added to precleared chromatin and incubated overnight at 4°C. The immune complexes formed were collected using salmon sperm DNA/protein A agarose beads and washed as described by others (38). The beads were washed once in low salt wash buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8.1, 150 mM NaCl), once in high salt wash buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8.1, 500 mM NaCl), in LiCl wash buffer (0.25 M LiCl, 1% NP-40, 1% sodium deoxycholate, 1 mM EDTA, 10 mM Tris-HCl, pH 8.0), and twice in TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA). The chromatin DNA-protein-antibody complexes were eluted using elution buffer (1% SDS, 0.1 M NaHCO₃), and the DNA-protein formaldehyde crosslinks were reversed by incubating at 65°C with 0.3 M NaCl overnight.
For PCR amplification, the DNA was purified with a Qiaquick PCR Purification Kit (Qiagen) and eluted in 50 µl Tris-HCl elution buffer.

The PCR reaction mixtures included 1 µl purified DNA template, 0.04 µM of each primer, 2 mM MgCl₂, 0.25 mM dNTP (A,T,G,C), 1x PCR Buffer II (no Mg²⁺) and 1 unit of AmpliTaq Gold (Applied Biosystems, Foster City, CA) in a total volume of 25 µl. The primers used for PCR amplification were:

1) hGR 1A far upstream (control), spanning -3436/-3642 (3.7 kbp upstream of hGR 1A FP12 (9,31)), hGR1A -3436 AS (reverse, 5'-CCTCGTTGGCACTAATTC-3') and hGR 1A -3642/-3616 S (forward, 5'-CTTTGACATGCTTGGAGTGTGCCCTCT-3');
2) PGK gene (heterologous gene control), PGK AS (reverse, 5'-GGGTGACTTCGGGTGCTTTC-3') and PGK S (forward, 5'-GGGTGTGGGGCGGTAGTGT-3');
3) 1A promoter/exon +146/+316 (FP5-FP12) which contains binding sites for GR and c-Myb/Ets (31), hGR1A +294/+317 AS (reverse, 5'-CTCTTACCCTCTTTCTGTTTCTA-3') and hGR1A 146 (hGR 1A+55/+77, forward, 5'-CTTGCTCCCTCTCGCCCTCATTC-3').

PCR reaction mixtures were resolved on 5% PAGE and visualized by EtBr staining after 28-35 cycles of amplification.

RESULTS

Hormone responsiveness of the hGR 1A promoter depends upon footprints 11 and 12 - We previously identified a human glucocorticoid receptor (hGR) 1A promoter that can be significantly auto-upregulated in T-lymphoblasts (9,26), and it is much more sensitive than the other two major hGR promoters, 1B and 1C (9,26,31). Deletion analysis and in vitro DNase I footprinting, using nuclear extracts from DEX-treated CEM-C7 cells, identified the DNA sequences that mediate the hormone responsiveness in T-cells - footprint 11 (FP11), a non consensus GRE half site, and footprint 12 (FP12). Computer analysis of FP12 revealed perfect overlapping c-Myb and c-Ets binding sequences (Fig. 1A), and our previous analysis confirmed that these proteins do bind to this sequence (31). To more fully characterize the role of these DNA elements for both basal promoter activity and in hormone-responsiveness, we performed internal deletions of FP11 and FP12, singly or in combination. Deletion of either of these two footprints causes non-responsiveness of the 1A promoter to DEX, whether a full-length promoter (-964/+269; Fig. 1B) or a shorter promoter that retained about 60% of the basal promoter activity (+41/+269; Fig. 1C) is used. The cause of the apparent downregulation of promoter activity upon DEX treatment of the full-length promoter in which FP12 has been deleted (Fig. 1B) is unknown, but this may result from the influence of other upstream sequences that are lacking in the shorter promoter construct. These data clearly show that FP11 and FP12 are critical, and perhaps sole, sequences required for the hormone-induced upregulation of hGR 1A promoter activity in the Jurkat T-lymphoblast line.

Besides being critical for hormonal responsiveness, these two DNA elements also contribute much to the basal promoter activity, because their deletion causes a dramatic decrease in transcriptional activity (Fig. 1B and 1C). In fact, when a minimal hGR 1A promoter containing only FP11 and FP12 (+242/+269) was cloned into pXP1, it retained about 70% percent of the basal activity of the full length promoter and was completely hormone-responsive (data not shown). Thus, it appears that nearly all of the protein factors that control basal promoter activity and hormonal responsiveness can be recruited to this small DNA element (FP11/FP12) in the hGR 1A promoter.

Ets family members are selectively expressed in lymphoid cell lineages - Based upon sequence analysis of FP12, we previously showed that c-Myb and two c-Ets protein family members, c-Ets-1 and c-Ets-2, are able to bind to FP12 in vitro (31). Thus, we wished to determine which c-Ets protein family members are indeed expressed in the three lymphoid cell lines used in our studies. Although there are over 20 different Ets family members, various c-Ets proteins are selectively expressed in certain hematopoietic lineages (39). This allowed us to focus on the most likely candidates that might be present in the three cell lines. In particular, Spi-B and PU.1 are two Ets proteins that are expressed during, and important for, differentiation of lymphoblasts. Spi-B is selectively expressed during T-cell development and its level drops in mature cells,
and PU.1 is restricted to B-cells and macrophages (39). Using western blotting, we analyzed the relative levels of 4 Ets family members that were possibly expressed in our T-cell (CEM-C7 and Jurkat cells) and B-cell (IM-9 cells) model systems (Fig. 2). We found that: 1) c-Ets-1/2 are expressed at comparable levels in all three cell lines; 2) Spi-B is present in the three lines, but it is expressed at much higher levels in CEM-C7 and Jurkat T-cells than in the B-cells; and, 3) PU.1 is expressed at a high level in IM-9 B-cells while it is undetectable in T-cells. Electrophoretic mobility supershift assays using T- and B-cell lymphoblast nuclear extracts demonstrated that Spi-B and PU.1 can bind to FP12 in vitro (data not shown). Thus, it seemed feasible that Spi-B and PU.1 are the Ets-related proteins that may be involved in cell type-specific regulation of hGR 1A promoter expression in lymphoblasts. In particular, because PU.1 is only present at high levels in IM-9 Pre-B cells, it seemed likely that this transcription factor is responsible for DEX-mediated downregulation of the hGR 1A promoter in these cells. In addition, because the c-Ets and c-Myb binding sites in FP12 overlap (Fig. 1A), we postulated that c-Myb and certain Ets members (depending on the cell type) antagonize each other at this binding site to oppositely regulate the response of the hGR 1A promoter to hormone in different lymphoid cell types.

Regulation of the hormonal response of the hGR 1A promoter in T-lymphoblasts by c-Myb and Ets Proteins - To determine if c-Myb and members of the c-Ets family of transcription factors can, indeed, affect the response of the hGR 1A promoter to DEX, we performed transient cotransfection experiments with an hGR 1A promoter/luciferase reporter gene. The overexpression of c-Myb in Jurkat cells did not greatly affect the responsiveness of the hGR 1A promoter to DEX induction (Fig. 3A). Although this might suggest that the c-Myb site in FP12 is not functional in vivo, this is not the case. The C-terminal truncated c-Myb variant (c-Myb(Ct)), lacking the negative regulatory domain, can functionally stimulate the promoter activity by 3-4 fold (Fig. 3A), which means that the c-Myb binding site is specific and capable of recruiting c-Myb protein in vivo. Thus, the inability of transfected full-length c-Myb to further increase the hormonal response (Fig. 3A) may indicate that sufficient endogenous c-Myb is already present in Jurkat cells. This is supported by the fact that overexpressing a specific functional inhibitor of c-Myb, the c-Myb DNA Binding Domain (c-Myb DBD) dominant negative protein, in Jurkat cells effectively suppresses the hGR 1A promoter response to hormone (Fig. 3B). These data suggest that FP12 is a functional c-Myb binding site that mediates the hormonal response of the hGR 1A promoter activity in vivo.

Overexpression of c-Ets-1, c-Ets-2, or PU.1 in Jurkat cells substantially suppresses DEX induction of the hGR 1A promoter (Fig. 4A, 4B). However, this also causes large increases in basal promoter activity for c-Ets-1 (2-3 fold; data not shown) and c-Ets-2 (3-4 fold; data not shown), although overexpression of PU.1 only slightly affects the basal promoter activity (data not shown). These results suggest that although Ets family members may be involved in basal promoter activity, c-Ets-1, c-Ets-2, and PU.1 block DEX activation of the hGR 1A promoter in this T-lymphoblast cell line. Interestingly, overexpression of another c-Ets family member, Spi-B, does not block the response of the hGR 1A promoter to DEX (Fig. 4B, 5A), and this even results in a slight (though not statistically significant) increase in hormonal responsiveness of the promoter. PU.1 and Spi-B belong to the same Ets family subgroup, and, in some cases, they have overlapping activities and can be substituted for each other (40). However, Spi-B and PU.1 are able to interact with different cofactors, and they may have subtle differences in their preferred DNA binding sequence or different affinity for the same DNA binding site (41-44). We currently do not know if FP12 is a preferred DNA binding site for either PU.1 or Spi-B.

To determine if the slightly stimulatory effect (or at least the lack of inhibition) of Spi-B is due to its direct interaction with FP12, we transfected Jurkat cells with a mutant Spi-B (ΔSpi-B) that cannot bind to DNA because of a mutated DNA-binding domain. Surprisingly, we observed not only an increase in basal activity of the hGR 1A reporter gene (data not shown) but also a significant increase in the hormonal responsiveness of the promoter (Fig. 5A) compared to wild-type Spi-B. Thus, even though both Spi-B and PU.1 can efficiently bind to FP12 using an EMSA, a different mechanism,
independent of the binding of Spi-B to FP12, may be responsible for the Spi-B effect noted here. Nonetheless, DNA-binding of some transcription factor to FP12 is clearly required for the stimulatory response of the hGR 1A promoter to DEX, because (as was seen for the dominant negative c-Myb DBD; Fig. 3B), the overexpression of a c-Ets-2 dominant negative DBD mutant also blocks hormonal induction in the human Jurkat T-lymphoblast line (Fig. 5B).

These studies indicate that the various c-Ets family members can have complex effects upon both basal and hormone-regulated hGR 1A promoter activity, and these effects may involve both DNA-binding activity and DNA-binding independent effects. With regard to corticosteroid treatment of T- and B-lymphoblasts, the role of c-Ets family members does suggest a molecular mechanism for the opposite hormonal regulation seen in these cell types. Ets family members appear to be involved in maintaining basal hGR 1A promoter activity. The IM-9 pre-B-cell lymphoblast line, in which hGR 1A transcripts are downregulated upon hormone treatment (9,26), lacks Spi-B (which does not suppress promoter activity), but contains apparently high levels of PU.1 (Fig. 2), which strongly inhibits DEX-mediated hGR 1A promoter activity. T-cells (such as the Jurkat and CEM-C7 cell lines), in which DEX causes an auto-upregulation of hGR 1A promoter activity contain Spi-B, but do not contain detectable levels of PU.1 (Fig. 2), which strongly inhibits DEX-mediated hGR 1A promoter activity. T-cells (such as the Jurkat and CEM-C7 cell lines), in which DEX causes an auto-upregulation of hGR 1A promoter activity contain Spi-B, but do not contain detectable levels of PU.1. Thus, it seems likely that GR binding to FP11 and PU.1 binding to FP12 in the IM-9 B-cell line result in the formation of a complex on the hGR 1A promoter that inhibits its transcription.

c-Myb is critical in the auto-upregulation of the hGR 1A promoter by hormone - Because FP12 has a consensus c-Myb binding site, we determined if c-Myb might be responsible for the hormone-induced activation of the hGR 1A promoter in T-lymphoblasts. Western blotting revealed that high levels of c-Myb protein were present in the CEM-C7 and Jurkat T-cell ALL lines, but that it was undetectable in the IM-9 B-cell line (Fig. 6A). DEX treatment had no effect on the c-Myb levels in the T-lymphoblasts. This observation suggests a role for c-Myb in hGR 1A promoter upregulation in T-lymphoblasts. If this were true, then supplying c-Myb to c-Myb-deficient IM-9 cells should result in an upregulation of the hGR 1A promoter after DEX-treatment in these cells. IM-9 cells were cotransfected with a c-Myb expression construct and the hGR 1A promoter luciferase reporter gene. Indeed, an increase in hormone-stimulated hGR 1A reporter upregulation was obtained with increasing levels of transfected c-Myb cDNA (Fig. 6B). These data suggest that the recruitment of the GR to FP11 along with c-Myb recruitment to FP12 is critical for the auto-upregulation of hGR 1A promoter in T-lymphoblasts, whereas hormone-mediated downregulation of the 1A reporter is due to the recruitment of PU.1 to FP12 in IM-9 B-lymphoblasts.

c-Myb and PU.1 are involved in regulating hormonal responsiveness of the hGR 1A promoter in lymphoblast cells in vivo - Using chromatin immunoprecipitation analysis, we investigated the involvement of specific transcription factors in hormone-treated CEM-C7 and IM-9 cells in vivo. After 24 hours of DEX treatment, both the GR and c-Myb were recruited to the hGR 1A promoter region containing FP11 and FP12 in CEM-C7 cells, compared to the ETOH-treated sample (Fig. 7A). No recruitment of GR or c-Myb was observed in the negative controls (hGR 1A promoter upstream region and the phosphoglycerate kinase (PGK) exon sequence), indicating that the recruitment of the GR and c-Myb was specific for the hGR 1A +146/+316 sequence (which contains FP 11 and FP12). Together with the results obtained using overexpression and reporter gene analysis, it appears that the hGR and c-Myb, acting in concert, may assist in forming an active transcription complex on the hGR 1A promoter during DEX induction in CEM-C7 cells.

As expected, no c-Myb was recruited to the hGR 1A promoter in IM-9 cells, which lack this transcription factor, while the GR was recruited in response to hormone treatment (Fig. 7B). However, an increased recruitment of PU.1 to this hGR 1A promoter region was observed in DEX-treated IM-9 cells (Fig. 7B). Because the overexpression of PU.1 can repress hGR 1A +41/+269 promoter activity in T-lymphoblasts (Fig. 4B), the DEX-induced increase of PU.1 recruitment to the hGR 1A promoter in IM-9 B-lymphoblasts may represent the in vivo mechanism for hormone-mediated downregulation of the hGR 1A promoter in this cell line. Similar to the co-
recruitment of GR and c-Myb in CEM-C7 cells, the simultaneous recruitment of the GR to FP11 and PU.1 to FP12 may assist in forming a complex capable of repressing transcription of the hGR 1A promoter in IM-9 B-cells. Thus, we propose that the expression pattern of specific transcription factors, such as PU.1 and c-Myb, in different lymphoblast cell lineages may be largely responsible for determining the direction of the autoregulatory response of the hGR 1A promoter to hormone.

**c-Myb may also regulate hormonal responsiveness of the hGR 1B and 1C promoters in lymphoblasts**

At least three promoters (1A, 1B, and 1C) control the expression of the human GR gene (9,10,29). In contrast to the hGR 1A promoter, the 1B and 1C promoters are GC rich and resemble promoters found in “housekeeping” genes (10,29,30,45,46). Although DEX induces significant *in vivo* upregulation of all three promoters in CEM-C7 cells, no consensus GREs have been identified in any of them (9,26,27,31). The hGR 1A promoter is the most hormone-sensitive in T-lymphoblasts. Because all three promoters are coordinately up-regulated (albeit to different levels), we wished to determine if the same molecular mechanism involving c-Myb may be operative for all three promoters. Figure 8 shows that luciferase reporter genes containing hGR promoter 1A, 1B, or 1C are all up-regulated by hormone in Jurkat T-ALL cells. The coexpression of the c-Myb DBD dominant negative mutant protein was effective in blocking the upregulation of all three promoters. Thus, it appears that a similar molecular mechanism as that proposed for the hGR 1A promoter (see below) may control expression of the 1B and 1C promoters as well. Future experiments will determine if composite response elements, as was found for the hGR 1A promoter (Fig. 1A), may exist for the hGR 1B and 1C promoters as well.

**DISCUSSION**

Corticosteroids are effective agents in treating certain types of leukemia, as they trigger apoptosis in sensitive cells. Whereas the absolute concentration of GR in cells prior to hormone treatment correlates somewhat to the sensitivity of the cell (13,16,21,24,25,28), the relationship between the steroid sensitive phenotype and the GR level after auto-upregulation by hormone treatment is much stronger (9,22,23,26,31,47). Indeed, the overexpression of functional GR can convert hormone resistant T-ALL cells to a hormone-sensitive phenotype (18,21,23). Thus, it is important to understand the molecular mechanism for auto-upregulation of hGR promoters in lymphoblasts (1,15,22,23,26,27,31,48).

Steroid-treatment of the hormone-sensitive, CEM-C7, T-cell ALL line causes auto-upregulation of GR expression and apoptosis. Conversely, the IM-9, pre-B-cell, lymphoblastoid line auto-downregulates GR expression and is resistant to hormone-mediated cell death (9,19,26,27,31). We had identified a composite hGR 1A promoter element (FP11/FP12) containing a non-consensus GRE located adjacent to a DNA site containing overlapping core sequences for c-Myb and c-Ets protein family members. This element appears to be necessary and sufficient in conferring hormone-responsiveness in T-lymphoblasts (9,15,26,31). We proposed that the direction of GR gene regulation by the binding of the GR to FP11 was determined by which transcription factor (c-Myb, c-Ets family member) simultaneously bound to FP12 (31). The results reported here strongly support this hypothesis.

c-Myb and c-Ets family members play pivotal roles in development and gene expression regulation in hematopoietic cells (39,42,44). Based upon the selective expression of c-Ets members in certain lymphoid cells, we focused on Ets-1, Ets-2, PU.1, and Spi-B in our studies. Of these, PU.1 appears to be the best candidate for controlling the auto-downregulation of GR gene expression, as it is present in a cell line that exhibits downregulation (IM-9) and absent in T-cell lines (CEM-C7, Jurkat) that auto-upregulate the hGR 1A promoter. PU.1 can interact with numerous other transcription factors and cofactors including TATA binding protein (TBP) (51), pRb (52), NF-EM5/Pip (53) and NF-IL6β (C/EBPδ) (54), CREB binding protein (CBP) (55), c-Myb, C/EBP α (56), c-Fos (57), c-Jun (58), AML1 (59) and GR (60). PU.1 also causes transcriptional repression via its binding to the corepressor mSin3A and the formation of a complex including HDAC1, and via a direct interaction of PU.1 with MeCP2 in a mSin3A-HDAC repression complex.
Thus, PU.1 is a bi-functional transcription factor that can activate or repress gene expression, depending upon the specific cofactors that it recruits to a promoter in a certain cell type. Because PU.1 is recruited to the hGR 1A promoter in DEX-treated IM-9 cells that auto-downregulate promoter activity and suppresses hGR 1A promoter upregulation in Jurkat T-cells, it is likely that PU.1 acts as a transcriptional repressor by recruiting corepressors that inhibit transcription of the hGR 1A promoter (Fig. 9). Future studies will reveal the other protein cofactors that are recruited to the hGR 1A promoter in IM-9 cells, and which contribute to hGR 1A promoter auto-downregulation.

In contrast to PU.1, c-Myb is expressed in Jurkat and CEM-C7 T-cell lymphoblasts (which exhibit DEX-mediated auto-upregulation hGR1A promoter activity), while it is absent in IM-9 B lymphoblasts. ChIP analysis in Jurkat cells shows the recruitment of c-Myb to the hGR 1A promoter after hormone-treatment, and the expression of c-Myb in c-Myb-naïve IM-9 B lymphoblasts via transient transfection results in the hGR 1A promoter becoming auto-upregulated by steroid treatment. Thus, we propose that upregulation of the hGR 1A promoter in T-lymphoblasts involves the simultaneous binding of the GR to FP11 and c-Myb to FP12, followed by the recruitment of coactivators to the hGR 1A promoter (Fig. 9).

Because of the overlapping c-Myb and c-Ets consensus binding sites, the configuration of FP12 lends itself to act as a “molecular switch” (Fig. 9). Thus, either c-Myb or a c-Ets family member, but not both, could bind to FP12 in a mutually exclusive manner. Because these transcription factors would compete for binding to the same DNA sequence, the relative levels, and cell type-specificity, of c-Myb and c-Ets family members could largely control the direction of auto-regulation of GR gene expression when the GR binds to FP11. We have clearly shown that c-Myb is a transactivator of hGR 1A promoter expression, while PU.1 acts as a repressor. While PU.1 (because of its restricted expression in the cell types tested) remains a prime candidate for the c-Ets family member involved in auto-downregulation of the hGR 1A promoter, other c-Ets family members found in hematopoietic cells, such as Erg and Fli-1 (44)), must also be tested. Further, the role (if any) of the unusual, DNA-binding independent activity of the c-Ets family member, Spi-B, in T-lymphoblasts must be resolved. It will also be important to identify the coactivators and corepressors that are recruited to the hGR 1A promoter by the GR/c-Myb and GR/PU.1 complexes, respectively. These studies also need to be extended to additional leukemia cell lines that respond to steroid treatment by undergoing apoptosis (as well as those that are steroid-resistant), and to fresh human patient samples, to determine if the molecular switch mechanism that we have proposed is a fundamental, common molecular mechanism for hGR auto-regulation. Our preliminary results indicate that a similar mechanism may be involved in hGR promoter 1B and 1C auto-regulation as well.

Lastly, by identifying other signaling pathways that can increase hGR promoter activity and the cellular level of GR protein, it may be possible to improve the response of certain leukemias to therapy. If the level of GR can be elevated in a hormone-independent way by stimulating the activity or levels of transcription factors that activate the various hGR promoters, then it would be possible to achieve a more complete steroid-induced apoptotic response in the blast cells at initial presentation, and perhaps even convert hormone-resistant disease to the hormone-sensitive phenotype by elevating the GR above the threshold level needed to trigger steroid-mediated apoptosis.

REFERENCES


FOOTNOTES
We thank Dr. John A. Cidlowski (NIEHS, Research Triangle Park, NC) for the PCYGR construct, Dr. Giuseppe Raschellà (Ente Nuove Tecnologie Energia Ambiente (ENEA), Rome, Italy) for the pcDNA3-c-MybHA and c-Myb DNA Binding Domain (DBD) expression constructs, and Dr. E. Premkumar Reddy and Dr. Ramana V. Tantravahi (Temple Univ., Philadelphia, PA) for the pC75 and pCt expression plasmid constructs. Also we thank Dr. Craig A. Hauser (The Burnham Institute, La Jolla, CA) for kindly providing the plasmids, pFN Ets-1, pFN Ets-2 and pFN Ets-2 DBD and Dr. Françoise Moreau-Gachelin for PU.1 and Spi-B expression constructs.

The abbreviations used are: GR, glucocorticoid receptor; hGR, human glucocorticoid receptor; GC, glucocorticoid; DEX, dexamethasone; FP, footprint; GRE, glucocorticoid response element; T-ALL, T cell acute lymphoblastic leukemia; β-gal, beta-galactosidase.

**FIGURE LEGENDS**

**Fig. 1.** FP11 and FP12 are critical for hormonal responsiveness of the hGR 1A promoter in lymphoblasts. A) The hGR 1A promoter/exon sequence, containing FP11 and FP12, is shown. The c-Ets and c-Myb consensus binding sequences that match with the overlapping sequences in FP12 are indicated. B) Activity of the full-length hGR 1A promoter. 1.5 µg of pXP1-hGR 1A c/p -964/+269 or deletion constructs were cotransfected into Jurkat cells together with 1 µg of pCYGR. ΔFP11, ΔFP12, and ΔFP11/12 are the respective deletion mutants of these sequences from promoter hGR 1A c/p -964/+269. C) Activity of the +41/+269 hGR 1A promoter. 1.5 µg of pXP1-hGR 1A c/p +41/+269 or FP11, FP12, or FP11/12 deletion constructs of this plasmid were cotransfected into Jurkat cells with 1 µg of pCYGR. 3 x 10^6 cells were used for each transient transfection and 1 µg of a pCMV-β-galactosidase (pCMV-β-gal) construct was included to normalize the transfection efficiency. The luciferase activity of each sample was measured with a luminometer and normalized to the β-galactosidase activity. Three or four individual experiments were combined to obtain the mean and calculate the standard error of the mean (SEM). The steroid responsiveness is plotted as the percentage of the ETOH control. The basal promoter activity is given as relative luminescence units normalized to β-galactosidase activity. *: P<0.05, **: P<0.01 and ***: P<0.005 for steroid responsiveness versus the respective ethanol control.

**Fig. 2.** Expression of c-Ets family members in lymphoblast cell lines. Depicted are western blots showing the expression pattern of c-Ets-1/2, Spi-B, and PU.1 in Jurkat (T-cell acute lymphoblastic leukemia), CEM-C7 (T-cell acute lymphoblastic leukemia), and IM-9 (B-cell lymphoma) cells. 2 x 10^6 cells were lysed in Laemmli sample buffer, and the lysates were loaded and separated via 8% PAGE. Proteins were transferred to nitrocellulose membranes and blotted with c-Ets-1/2, Spi-B, or PU.1 antibodies. The blots were developed using an ECL kit. The three lanes per cell line are samples from three separate experiments, indicating the reproducible levels of these transcription factors in the various cell lines.

**Fig. 3.** c-Myb modulates hGR 1A promoter expression. A) Effect of c-Myb overexpression on hGR 1A promoter activity. Jurkat cells were cotransfected with pXP1-1A +41/+269-Luc plus pcDNA3 (empty vector), pcDNA3-c-MybHA, or carboxyl-terminal truncated c-Myb, pCt. Luciferase assays were performed as described in Materials and Methods. B) Over expression of a c-Myb dominant negative inhibitor blocks hGR 1A promoter stimulation by DEX. Jurkat cells were cotransfected with 1.5 µg pXP1-1A +41/+269-Luc, 1 µg of pCYGR, 1 µg of pCMV-β-gal (for normalizing transfection efficiency), and either 1.5 µg pcDNA3 or 1.5 µg pcDNA3-c-Myb DBD. The luciferase activity was normalized to β-galactosidase activity, and the steroid responsiveness is plotted as the percentage of the ETOH control. Data from four separate experiments was used to obtain the mean and calculate the standard error of the mean (SEM). **: P< 0.01 and ***: P<0.005 for steroid responsiveness versus the respective ethanol control.
Fig. 4. The hormonal responsiveness of the hGR 1A promoter is modulated by c-Ets protein family members. A, Overexpression of c-Ets-1 and c-Ets-2 repress steroid-responsiveness of the hGR 1A promoter. Jurkat cells were cotransfected 1.5 µg of pXP1-1A +41/+269-Luc and 1.5 µg of pcDNA3 (empty vector), pFN-c-Ets-1, or pFN-c-Ets-2. B) Effects of the c-Ets subfamily members, PU.1 and Spi-B, on hormonal responsiveness of the hGR 1A promoter. Jurkat cells were cotransfected with 1.5 µg of pXP1-1A +41/+269-Luc and 1.5 µg pEVX (empty vector), pEVX-PU.1, ∆EB (empty vector) or ∆EB-Spi-B. For all of the samples, 1 µg pCMV-β-gal (normalization control) and 1 µg pCYGR (to provide functional GR protein) were also cotransfected. Three or four individual experiments were combined to obtain the mean and calculate the standard error of the mean (SEM). The steroid responsiveness is plotted as the percentage of the ETOH control. **: P< 0.01 and ***: P<0.005 for steroid responsiveness versus the respective ethanol control.

Fig. 5. Spi-B and ΔSpi-B affect DEX induction of the hGR 1A promoter in T-lymphoblasts. A) Jurkat cells were cotransfected with 1.5 µg of pXP1-1A +41/+269-Luc and 1.5 µg of ΔEB (empty vector), ΔEB-Spi-B, or ΔEB-ΔSpiB (alternative spliced Spi-B variant without the Ets domain that is required for DNA binding). All cultures were also transfected with 1 µg pCYGR (to provide functional GR) and 1 µg pCMV-β-gal (normalization control). B) Overexpression of the dominant negative Ets protein inhibitor, Ets-2 DNA Binding Domain (DBD) blocks the DEX-mediated upregulation of the hGR 1A promoter. Jurkat cells were cotransfected with 1.5 µg of pXP1-1A +41/+269-Luc and 1.5 µg pcDNA3 (vector control) or pFN-Ets-2 DBD. All cultures were also transfected with 1 µg pCYGR (to provide functional GR) and 1 µg pCMV-β-gal (normalization control). Three or four individual experiments were combined to obtain the mean and calculate the standard error of the mean (SEM). The steroid responsiveness is plotted as the percentage of the ETOH control. **: P< 0.01 and ***: P<0.005 for steroid responsiveness versus the respective ethanol control.

Fig. 6. c-Myb is required for steroid-mediated upregulation of the hGR 1A promoter in lymphoblasts. A) Western blot analysis of c-Myb protein levels in CEM-C7, Jurkat, and IM-9 cells, which were treated with ETOH vehicle or 1 µM DEX for 24 hours. 40 µg of total protein were subjected to 8% PAGE. After transfer to nitrocellulose membrane, the blots were probed with anti-c-Myb or anti-actin antibodies (loading control) and developed using ECL. B) IM-9 cells were electroporated with 3 µg of pXP1-1A +41/+269-Luc, 2 µg of pCMV-β-gal (for normalization) and increasing amounts of the c-Myb expression construct, pcDNA3-c-MybHA (0, 0.5, 1.0, 2.0 or 5.0 µg). For each transfection, the pcDNA3 empty vector was added to maintain the total amount of transfected DNA at 10 µg/reaction. Three individual experiments were combined to obtain the mean and calculate the standard error of the mean (SEM). The steroid responsiveness is plotted as the percentage of the ETOH control. *, P<0.05 for steroid responsiveness versus the respective ethanol control.

Fig. 7. Chromatin immunoprecipitation (ChIP) analysis of transcription factors recruited by the hGR 1A promoter in lymphoblasts during hormone treatment. The human CEM-C7, T-ALL, and IM-9, pre-B cell, lines were treated with ETOH or 1 µM DEX for 24 hours. Cells were collected for ChIP analysis using c-Myb, GR, and PU.1 specific antibodies. Normal purified rabbit IgG was used as a background control. To analyze the specificity of the ChIP assay, two additional controls for each sample were included: an hGR 1A upstream (-3416/-3616) amplicon is located 3.6 kbp upstream of hGR the 1A (+146/+316) sequence, and it contains no known GRE, c-Myb, or c-Ets binding site; and 2) a PGK (phosphoglycerate kinase 1) coding exon (+396/+798) sequence, which serves as a negative control. The PCR was performed as described in Materials and Methods. For the hGR 1A (+146/+316) and hGR 1A upstream sequences, 28 cycles (IM-9 cells) or 31 cycles (CEM-C7 cells) were used. For the PGK gene coding exon, 24 cycles were used. After gel electrophoresis, the EtBr stained PAGE gels were photographed. A) ChIP assays of ETOH- or DEX-treated CEM-C7 cells using the GR and c-Myb antibodies. B) ChIP
analysis of ETOH- or DEX-treated IM-9 cells using a c-Myb (top panel) or PU.1 and GR antibodies (bottom panels). Each ChIP experiment has been repeated at least 4 times to confirm the constant and reproducible patterns shown here.

**Fig. 8.** Suppression of auto-upregulation of the hGR 1A, 1B, and 1C promoters in T-lymphoblasts by a dominant negative c-Myb DNA Binding Domain. Jurkat cells transfected with 1.5 µg of pXP1-1A +41/+269-Luc, pXP1-1B or pXP1-1C were also cotransfected with either 1.5 µg pcDNA3 (vector control) or pcDNA3-c-Myb DBD. All transfection reactions included 1 µg pCYGR (to provide functional GR) and 1 µg pCMV-β-gal (for normalization). Three or four individual experiments were combined to obtain the mean and calculate the standard error of the mean (SEM). The steroid responsiveness is plotted as the percentage of the ETOH control. *: P< 0.05 and **: P<0.01 for steroid responsiveness versus the respective ethanol control.

**Fig. 9.** Molecular switch model for the opposite steroid regulation of GR 1A promoter activity in lymphoblast cells from different lineages. In the basal state, the hGR 1A promoter expresses a basal activity that drives transcription of the GR gene. This may or may not involve the binding of a c-Ets family member to FP12 in the hGR 1A promoter. Upon binding the glucocorticoid ligand, the GR is activated and binds to FP11, probably as a monomer. In T-lymphoblasts, such as CEM-C7 cells, this causes the recruitment and stabilization of c-Myb binding to FP12 of the hGR 1A promoter. The GR/c-Myb complex then recruits coactivators to the hGR 1A promoter, resulting in the observed auto-upregulation of hGR 1A promoter activity by hormone. In B-lymphoblasts, such as IM-9 cells, the binding of the liganded GR protein to FP11 recruits and stabilizes the binding of the c-Ets family member, PU.1. The GR/PU.1 complex then recruits corepressors to the hGR 1A promoter, resulting in the observed auto-downregulation of hGR 1A promoter activity by hormone seen in this cell type. Thus, the relative expression and level of c-Myb or c-Ets family members are largely responsible for the direction and magnitude of GR promoter auto-regulation seen in different cell types.
A)  
FP11: +241  
FP12: +251

CAGTAAAATGCGCATGTGTCACACGGAAGCATGCG........

---CGGAAG---  c-Ets Consensus Sequence

---CAACGG---  c-Myb Consensus Sequence

B)  

<table>
<thead>
<tr>
<th>Percentage</th>
<th>ETOH</th>
<th>DEX</th>
</tr>
</thead>
<tbody>
<tr>
<td>300</td>
<td>**</td>
<td></td>
</tr>
<tr>
<td>200</td>
<td></td>
<td></td>
</tr>
<tr>
<td>100</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

C)  

<table>
<thead>
<tr>
<th>RLU (x10^-4)</th>
<th>ETOH</th>
<th>DEX</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basal</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Full Length</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ΔFP11</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ΔFP12</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ΔFP11/12</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Figure 1
<table>
<thead>
<tr>
<th>Jurkat</th>
<th>CEM-C7</th>
<th>IM-9</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image1.png" alt="Ets-1/2" /></td>
<td><img src="image2.png" alt="Ets-1/2" /></td>
<td>Ets-1/2</td>
</tr>
<tr>
<td><img src="image3.png" alt="Spi-B" /></td>
<td><img src="image4.png" alt="Spi-B" /></td>
<td>Spi-B</td>
</tr>
<tr>
<td><img src="image5.png" alt="PU.1" /></td>
<td><img src="image6.png" alt="PU.1" /></td>
<td>PU.1</td>
</tr>
</tbody>
</table>

**Figure 2**
Figure 3

A) RLU x 10^-4% of ETOH Control

- Vector
- c-Myb
- c-Myb(Ct)

B) % of ETOH Control

- Vector
- c-Myb DBD

***
**
Figure 4
Figure 5

A) 

Vector         Spi-B  \[\Delta\text{Spi-B}\]

B) 

Vector         c-Ets-2 DBD

ETOH  DEX  ETOH  DEX  ETOH  DEX
Figure 6

A) Western blot analysis of c-Myb and Actin expression in IM-9 cells treated with ET, DEX, ET, DEX, ET, and DEX in CEM-C7, IM-9, and Jurkat cells.

B) Bar graph showing the percentage of ETOH control for c-Myb expression in IM-9 cells treated with ETOH and DEX.
Figure 7
Figure 8

% of ETOH Control

**

* * *

100

ETOH DEX

c-Myb DBD

hGR Promoter

1A 1B 1C

Figure 8
Figure 9

CEM-C7 Cells

IM-9 Cells

Figure 9
C-MYB and members of the C-ETS family of transcription factors act as a molecular switch to mediate opposite steroid regulation of human glucocorticoid receptor 1A promoter

Chuan-dong Geng and Wayne V. Vedeckis

J. Biol. Chem. published online November 1, 2005

Access the most updated version of this article at doi: 10.1074/jbc.M508245200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at
http://www.jbc.org/content/early/2005/11/01/jbc.M508245200.citation.full.html#ref-list-1