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Three Mechanisms Are Involved in Glucocorticoid Receptor Autoregulation in a Human T-Lymphoblast Cell Line†

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ABSTRACT: Glucocorticoids up-regulate the glucocorticoid receptor (GR) in the human T-lymphoblast cell line CEM-C7. One mechanism for the up-regulation of the GR protein is the well-known up-regulation of GR transcripts. We have investigated the effect of other factors on the up-regulation. At least three promoters (1A, 1B, and 1C) exist, which give rise to GR transcripts with different exon 1 sequences. Transcripts with different exon 1 sequences have similar stabilities. Glucocorticoids have little, if any, effect on mRNA stability. In transfection experiments of the GR-deficient mouse fibroblast cell line E8.2, different exon 1 sequences furthermore caused no significant differences in the translational efficiencies of GR transcripts. However, the ratio between the concentrations of the glucocorticoid receptor B (GR-B) isoform and the glucocorticoid receptor A (GR-A) isoform was higher for transcripts containing the exon 1A3 sequence arising from promoter 1A than in transcripts containing exon 1 sequences from promoters 1B and 1C. Because the GR-B isoform is more active in transactivation then GR-A, this would tend to fine-tune glucocorticoid responsiveness of CEM-C7 cells, which express exon 1A3-containing transcripts. We also found that glucocorticoids do not decrease the stability of the GR protein in CEM-C7 cells. In contrast to other cell lines that downregulate GR expression in response to glucocorticoids, CEM-C7 lymphoblasts possess three mechanisms ensuring high glucocorticoid responsiveness: an up-regulation of GR mRNA by glucocorticoids, no destabilization of GR protein by glucocorticoids, and a high activity of promoter 1A with concomitant high expression of the GR-B isoform.

Glucocorticoids are used in the treatment of leukemias such as acute lymphoblastic leukemia because they can induce apoptosis of the leukemia cells (*1*−*3*). Glucocorticoids exert their effects by binding to the glucocorticoid receptor (GR),[1] which in turn becomes activated as a transcription factor and hence modulates the expression of glucocorticoid responsive genes. For cells of the CEM-C7 cell lineage, an

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[1] Abbreviations: GR, glucocorticoid receptor; GRα, glucocorticoid receptor α; GR*β*, glucocorticoid receptor *β*; GR-A, glucocorticoid receptor A; GR-B, glucocorticoid receptor B; Act D, actinomycin D; DEX, dexamethasone; EtOH, ethanol; QRT-PCR; quantitative reverse transcription polymerase chain reaction; bp, base pairs; PCR, polymerase chain reaction; CMV, human cytomegalovirus major intermediate early gene; RT-PCR; reverse transcription polymerase chain reaction; 5′-UTR, 5′-untranslated region; SEM, standard error of the means; st, standard; no tr, nontransfected.

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auto-up-regulation of GR protein is essential for an apoptotic response to glucocorticoids (*4*). Transcripts encoding full-length human GR protein consist of nine exons (*5*). Several GR isoforms are known. Splicing of exon 8 to two alternative forms of exon 9 (exon 9α and exon 9*β*) gives rise to the mRNAs for the isoforms glucocorticoid receptor α (GRα) and glucocorticoid receptor *β* (GR*β*) (*5*). GRα binds to glucocorticoids and thereby mediates the transcriptional response of glucocorticoids, whereas GR*β* cannot (*6*). Two translation initiation codons are located in exon 2 (*7*). Initiation from the 5′-most initiation codon results in a 777 amino acid GRα isoform termed glucocorticoid receptor A (GR-A), while initiation from the 3′-most initiation codon results in a 751 amino acid isoform termed glucocorticoid receptor B (GR-B) (*7*). GR-B is more active than GR-A in transactivation but not transrepression (*7*). At least three promoters (1A, 1B, and 1C) exist for the human GR gene (*8*). Each of these gives rise to an untranslated exon 1 form (exon 1A, exon 1B, and exon 1C) splicing to exon 2. Furthermore, three alternative splice donor forms of exon 1A were identified, all splicing the common exon 2 splice acceptor site. These were termed exons 1A1, 1A2, and 1A3 (*8*).

We have previously quantified and studied the glucocorticoid regulation of various GR transcripts in two leukemic cell lines, CEM-C7 and IM-9 (*9*). In both cell lines, exon 1B- and exon 1C-containing transcripts are more abundant than transcripts containing exon 1A. Among the exon 1A forms, exon 1A3 is by far the most abundant. The concentrations of GR*β*-encoding transcripts and of transcripts with exons 1A1 and 1A2 are so low that these transcripts only can affect glucocorticoid responsiveness in a very minor way. We previously also modeled the glucocorticoid regulation of GR protein based on the observed regulation of GR transcripts, an assumption that glucocorticoids decrease GR protein stability, an assumption that all GR transcripts are translated at the same rate, and an assumption that the translation rate is not affected by glucocorticoids. We observed that glucocorticoids increase the GR protein concentration in CEM-C7 cells more than predicted from this model (*9*). We decided to study factors that could explain this discrepancy in CEM-C7 cells.

Because the discrepancy could be explained by a ca. 10-fold higher translation rate from exon 1A-containing transcripts than from transcripts containing exons 1B and 1C, we investigated the effects of the different exon 1 sequences on the expression of the GR. Stabilities of GR transcripts with exons 1A3, 1B, or 1C were found to be very similar and hardly affected by the presence of glucocorticoids, indicating that for all three types of transcripts, the glucocorticoid regulation of GR transcripts occurs primarily at the level of transcription rate. A comparison of the translation rate of GRα-encoding transcripts containing exon 1A3, exon 1B, or exon 1C sequences was conducted by transfection studies of the GR-deficient mouse fibroblast cell line E8.2. The different exon 1 sequences caused no significant differences in translational efficiency.

The previously observed high glucocorticoid up-regulation of GR protein in CEM-C7 cells, thus, does not seem to be explained by a particularly high translation rate of exon 1A3-containing transcripts. Instead, we found that the GR protein stability in CEM-C7 cells is, surprisingly, not decreased by glucocorticoids, and this reconciles the observed and predicted up-regulation of GR protein by glucocorticoids in this cell line.

The exon 1A3 sequence causes expression of a higher amount of the GR-B isoform relative to the GR-A isoform than do the exon 1B or exon 1C sequences. Thus, transcription from promoter 1A may cause a somewhat higher glucocorticoid transactivation potential than transcription from promoters 1B and 1C. This will tend to fine-tune the glucocorticoid responsiveness of a cell type such as CEM-C7, which has a fairly high expression of exon 1A3-containing transcripts.

MATERIALS AND METHODS

Cell Culture. CEM-C7 cells and IM-9 cells were grown as previously described (*9*). Mouse E8.2 cells were a kind gift from Dr. Paul R. Housley, University of South Carolina, Columbia, SC. E8.2 cells were grown in Dulbecco's modified Eagle's medium (with 4.5 g/L glucose) supplemented with 5% Bovine calf serum. African green monkey CV-1 cells were grown in minimum essential medium Eagle supplemented with nonessential amino acids and 10% fetal bovine serum.

Quantitative Western Blots for Measuring GR Protein. Quantitation of GR protein by quantitative Western blots of whole cell lysates was performed as previously described (*9*). Band intensities were recorded on a Fluor-S MultiImager from Bio-Rad Laboratories (Hercules, CA). Bands at ca. 94 kDa and ca. 91 kDa represented full-length GR protein with the 94 kDa band corresponding to the GR-A form of GRα and the 91 kDa band corresponding to the GR-B form of GRα (*7*). Using the Quantity One software from Bio-Rad Laboratories, we quantified the combined intensity of these two bands and determined the GR concentration relative to the standards, which were varying amounts of whole cell lysate of CEM-C7 cells or IM-9 cells. Dependent on the Western blots in question, standard curves were generated by logarithmic or linear curve fitting to the data for the standards. Inverted images of the blots obtained from the imager are presented.

GR Transcript Stability. CEM-C7 and IM-9 cells were split into fresh medium 1 day before actinomycin D (Act D) treatment. The initial cell concentrations were ca. 5×10^5 cells/mL for CEM-C7 cells and ca. 2×10^5 cells/mL for IM-9 cells. Dexamethasone, to a final concentration of 1 μM, or ethanol vehicle, to a final concentration of 0.1 μL/mL, was added 4 h prior to Act D treatment. Act D was added to a concentration of 2 μg/mL, immediately followed by dispension of 5 mL aliquots into 25 cm^2 cell culture flasks. The flasks were harvested 0, 1, 2, 3, 4, 5, and 8 h after adding Act D, and total RNA was isolated from 4 mL cell culture by guanidinium thiocyanate−phenol−bromochloropropane extraction using TRI REAGENT from Molecular Research Center (Cincinnati, OH) according to the guidelines of the manufacturer. The RNA concentration of the 0 h samples was determined by an OD 260 nm measurement, and the samples were diluted to 40 ng/μL RNA. The remaining samples were diluted with the same dilution factor as the corresponding 0 h sample. GRα-encoding transcripts, GR*β*-encoding transcripts, and transcripts containing exon 1A3, exon 1B, or exon 1C were quantified by real-time QRT-

PCR assays as previously described (*9*). Greater than 90% of both CEM-C7 and IM-9 cells excluded trypan blue after 5 or 8 h of Act D treatment, indicating that there was not massive cell death occurring during the RNA stability studies (data not shown).

GR Protein Stability. CEM-C7 and IM-9 cells were split into fresh medium 1 day before cycloheximide treatment. The initial cell concentrations were ca. 5×10^5 cells/mL for CEM-C7 cells and ca. 2.5×10^5 cells/mL for IM-9 cells. Dexamethasone, to a final concentration of 1 μM, or ethanol vehicle, to a final concentration of 0.1 μL/mL, was added 4 h prior to cycloheximide treatment. Cycloheximide was added to a concentration of 50 μM, immediately followed by dispension of 5 mL aliquots into 25 cm^2 cell culture flasks. The flasks were harvested 0, 24, 48, and 72 h after adding cycloheximide, and whole cell lysate was prepared from 4.5 mL of the cell culture. The amount of GR in cell lysates (sum of bands at ca. 94 and ca. 91 kDa) was determined by quantitative Western blots.

Plasmid Construction. The plasmid pCMVhGRα (*10*) was kindly provided by Dr. John Cidlowski (National Institute of Environmental Health Sciences, Research Triangle Park, NC). The plasmids pCMV-1A3-hGRα, pCMV-1B(120bp)-hGRα, pCMV-1B(76bp)-hGRα, and pCMV-1C-hGRα contain 981 base pairs of exon 1A3, 120 base pairs of exon 1B, 76 base pairs of exon 1B, and 184 base pairs of exon 1C, respectively, upstream of the exon 2 sequence. Please note that the exon 1B sequences lack the cytosine at position −1056 of the 1B sequence published in ref *11*, because this cytosine is consistently absent from exon 1B sequences amplified from CEM-C7 or IM-9 cells. The plasmids were constructed in the following way. PCR products were amplified from cDNA from IM-9 cells. The forward primers introduced a *Bam*HI site just 5′ to the exon 1 sequences. The forward primers had sequences of 5′-GCCGGATC-CAGGTTATGTAAGGGTTTGCTTTCACC-3′ (1A3), 5′-AAAGGATCCGGTTCTGCTTTGCAACTTCTC-3′ (1B, 120bp), 5′-AAAGGATCCGGCAGCTGAAGACCCG-3′ (1B, 76bp), and 5′-AAAGGATCCGGAGACTTTCTTAAAT-AGGG-3′ (1C). The sequence of the reverse primer was 5′-CTTGTGAGACTCCTGTAGTG-3′. For construction of pCMV-1A3-hGRα, the PCR product was cloned into the pCRII-TOPO vector from Invitrogen (Carlsbad, CA), digested with *Bam*HI and *Cla*I, and ligated into pCMVhGRα digested with *Bgl*II and *Cla*I. For the remaining constructs, the PCR fragment was digested with *Bam*HI and *Cla*I and ligated into the vector backbone of pCMVhGRα digested with *Bgl*II and *Cla*I. Sequencing of the plasmids ensured that there were no mutations in the PCR-amplified sequence between the chimeric *Bgl*II/*Bam*HI site and the *Cla*I site.

Transfection. One day prior to transfection, cells were seeded in 6-well plates at a concentration of 2×10^5 cells per well. For E8.2 cells, the medium was changed at least 2 h before transfection to Dulbecco's modified Eagle's medium (with 4.5 g/L glucose) supplemented with 1% bovine calf serum. The transfection was done in 6-well plates using the SuperFect® transfection reagent from Qiagen GmbH (Hilden, Germany) essentially according to the guidelines of the manufacturer. Twenty-four hours after transfection, wells were harvested either for RNA isolation or for generating a whole cell lysate. The RNA samples were treated three times with the DNA-*free* kit from Ambion Inc. (Austin, Texas) according to the guidelines of the manufacturer to remove plasmid DNA. The concentration of GRα-encoding transcripts and 18S rRNA were determined by real-time QRT-PCR. The 18S rRNA values were determined relative to dilutions of total RNA prepared from untransfected E8.2 cells. The amount of GRα-encoding transcripts relative to the amount of 18S rRNA was calculated and expressed in arbitrary units. The GRα protein content in cell lysates was determined by quantitative Western blots relative to IM-9 cell lysate standards. The translational efficiency was calculated as the GRα protein amount/GRα transcript concentration.

Modeling. Modeling of glucocorticoid regulation of GR protein was conducted as described in ref *9* based on the observed glucocorticoid regulation of GRα-encoding transcripts likewise reported in ref *9*.

Statistical Analysis. Protein expression, RNA expression, and translation rates of transcripts with different exon 1 sequences were compared by a single-factor analysis of variance followed by pairwise comparisons by a least significant difference procedure. Differences were considered significant if the test probabilities were less than 5% ($p < 0.05$).

RESULTS

Stability of GR Transcripts. The stability of GR transcripts with exon 1B, exon 1C, and the most abundant exon 1A form (exon 1A3) was determined in the absence and presence of glucocorticoids after treatment with Act D. An Act D concentration of 2 μg/mL was chosen because this concentration blocks RNA accumulation in the cells and because treatment with 1 and 5 μg/mL Act D gave similar degradation curves in a preliminary experiment (data not shown). The transcripts were quantified by real-time QRT-PCR as the transcripts with a splice between the exon 1 form in question and exon 2. The degradation curves for CEM-C7 cells are shown in Figure 1. The 4 h pretreatment with 1 μM DEX was sufficient to increase the concentrations of exon 1C-, exon 1B-, and exon 1A3-containing transcripts 3.4-, 2.7-, and 6.8-fold, respectively (Figure 1, "0 h"). The degradation curves in the absence and presence of DEX are close to parallel, indicating no major effect of glucocorticoids on the stability of the different GR transcripts. Estimates of the half-lives of the different transcript forms are calculated from the slopes of regression lines for the 0−5 h time points. There is no large difference in the stability of the transcripts with different exon 1 sequences. Glucocorticoids also have no effect on the stability of GRα-encoding transcripts and GRβ-encoding transcripts in CEM-C7 cells (data not shown). In IM-9 cells, glucocorticoids likewise do not affect stability of any of the GR transcript forms, and transcripts containing exon 1C, exon 1B, and exon 1A3 have similar stabilities (data not shown).

Translation Rates of Exon 1B-, Exon 1C-, and Exon 1A3-Containing Transcripts. A comparison of the translation rates of exon 1B-, exon 1C-, and exon 1A3-containing transcripts was done by transient transfections of mouse E8.2 cells with GRα expression vectors, in which transcription is under the control of the human cytomegalovirus major intermediate early gene (CMV) promoter and in which the GR exon 2 is

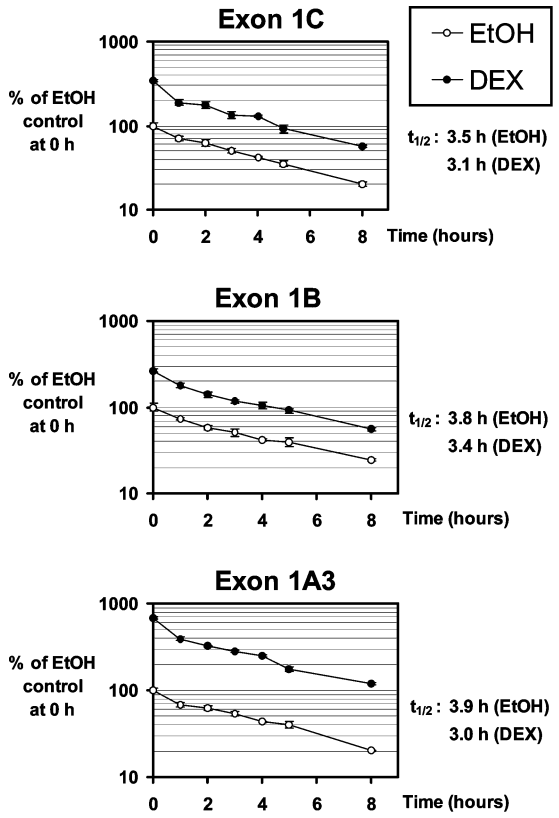

FIGURE 1: GR transcript stabilities. Stabilities of transcripts containing exon 1C, exon 1B, or exon 1A3 in CEM-C7 cells in the presence of 2 μg/mL actinomycin D added at the 0 h time point are shown. Transcript concentrations were measured by real-time QRT-PCR. The stabilities were assayed in cells that were pretreated for 4 h with 1 μM DEX or the ethanol (EtOH) vehicle. The data represent the means $\pm$ standard error of the means (SEM), $n = 3$. The half-lives ($t_{1/2}$) are calculated from the slope of the regression lines to data for time points from 0 to 5 h.

preceded by each of the exon 1 sequences. The mouse E8.2 cell line was chosen because this cell line was reported to express essentially no GR RNA or protein (*12*), which we have confirmed (data not shown). Thus, the analysis would not be confounded by endogenous GR mRNA or protein. The E8.2 line is a spontaneous glucocorticoid-resistant variant of the mouse L929 fibroblast cell line. Also, this cell line is easy to transfect, whereas the human CEM-C7 cells are very resistant to transfection. Human GR promoters may have multiple transcription start sites. The cloned exon 1A3 and exon 1C sequences were chosen according to major start sites (*5, 8*). For exon 1B, a short (76 nucleotides) form corresponds to exon 1B as listed in ref *8*, while the longer (120 nucleotides) form corresponds to the form with the 5′-most start site (*11*).

We initially observed that the isolated fraction of total RNA from transfected cells was contaminated with plasmid DNA. We found that three consecutive treatments with the DNA-*free* kit were necessary for reducing the plasmid DNA in RNA preparations to an inconsequential level (data not shown).

We performed initial transfection experiments with varying amounts of the exon 1C-containing expression plasmid. For transfections with up to 2 μg of plasmid per transfection in the well of a six-well plate, there was proportionality between the amount of GRα-encoding transcripts as determined by real-time QRT-PCR and the amount of GR protein as determined by quantitative Western blots (data not shown). The ratio of protein concentration to RNA concentration is thus a good measure of the translational efficiency, independent of the transfection efficiency. For the transfection experiments comparing the translation efficiencies of different exon 1 sequences, we subsequently chose transfections with 1.5 μg of plasmid per well. An example of the quantitative Western blots is shown in Figure 2A. Two closely spaced bands represented full length GRα, the upper band corresponding to GR-A and the lower band to GR-B (*7*). The total intensity of these bands was scored. Because the expression plasmids do not cause expression of GRβ, this receptor species is not present in the Western blots.

Transfection with equal amounts of plasmid (1.5 μg) resulted in lower expression of GRα protein for the exon 1A3-containing plasmid than for those containing exon 1B or exon 1C (Figure 2B). However, the expression of GRα RNA for the exon 1A3-containing plasmid was also lower (Figure 2C). Normalization of the GR protein level to the GR RNA level, which is an indication of the relative translational efficiency, is illustrated in Figure 2D. Exon 1C had a slightly higher value for translational efficiency than exon 1A3 and exon 1B, but there was no statistically significant difference in the translational efficiencies among the plasmids with different exon 1 sequences.

Exon 1A3 Causes an Increase in the Ratio of GR-B Protein to GR-A Protein. For E8.2 cells transfected with 1.5 μg of GRα expression plasmid, there was a difference between the different plasmids regarding the relative intensity of the two bands representing the GR-A and GR-B proteins. While the GR-A band is by far the more intense for the exon 1B- and exon 1C-containing plasmids, the two bands are of roughly the same intensity for the exon 1A3-containing plasmid (Figure 2A). This was consistently observed in all the Western blots, and it is not an artifact caused by unequal loading of GR protein. That is shown in the Western blot in Figure 3A, in which approximate equal amounts of GR protein were loaded per lane and for which the exon 1A3-containing plasmid still shows higher expression of GR-B relative to GR-A than the exon 1B- or exon 1C-containing plasmids. This phenomenon is furthermore not restricted to E8.2 cells. We transfected the cell line CV-1, which is an African green monkey kidney fibroblast line, with the GRα expression plasmids. The analysis in CV-1 cells is complicated by the fact that the cells express a protein at ca. 90 kDa that can be detected in Western blots for human GR protein. Still, the expression is so low that transfection with GRα-encoding expression plasmids consistently gives bands of higher intensity in Western blots than untransfected cells. Exon 1B- and exon 1C-containing expression plasmids result in detectable GR-A and GR-B protein with more GR-A than GR-B protein. By contrast, transfection with the exon 1A3-containing plasmid results in a clearly detectable GR-B band, and a much weaker, barely detectable GR-A band (Figure 3B).

Dexamethasone Does Not Decrease GR Protein Stability in CEM-C7 Cells. Exon 1A3-containing transcripts obviously do not cause an inherently ca. 10-fold higher translational rate than those of exon 1B- and exon 1C-containing transcripts, which could have explained the previously observed higher than expected up-regulation of GR protein

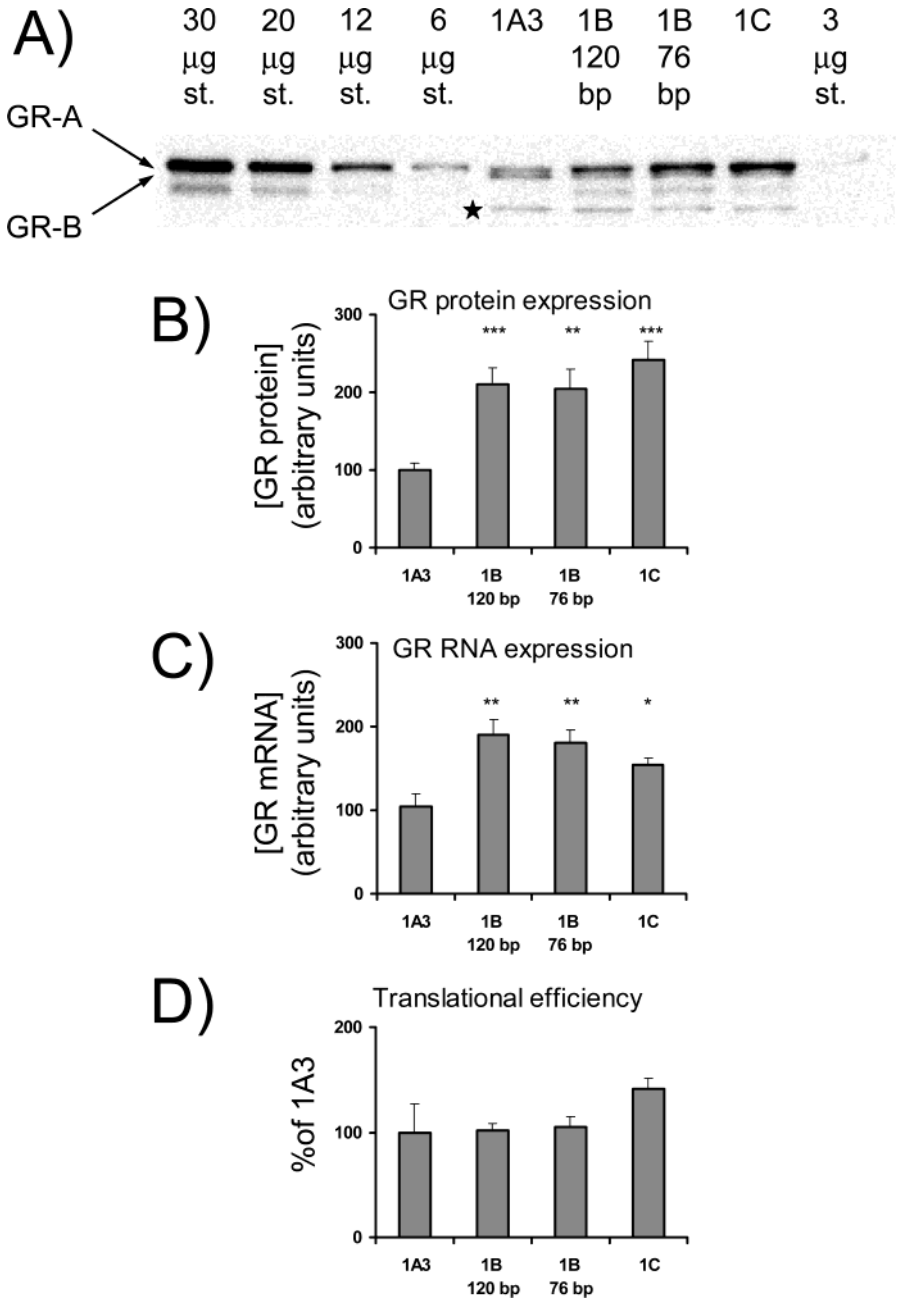

FIGURE 2: The translational efficiencies of transcripts containing exon 1A3, exon 1B, or exon 1C in E8.2 cells are of the same magnitude. Panel A presents an example of a quantitative Western blot for measuring the concentration of GR protein in E8.2 cells transfected with hGRα expression plasmids containing different forms of the exon 1 sequence (exon 1A3, a 76 bp and a 120 bp form of exon 1B, and exon 1C). Twenty-five micrograms of protein of whole cell lysate of the transfected E8.2 cells was added per lane. The star indicates a nonspecific band that also appears in untransfected E8.2 cells and confirms equal loading of protein in each lane. For this Western blot, the standards (st) were 3−30 μg of protein of an IM-9 whole cell lysate. Panel B shows the expression level of GRα protein in lysates of E8.2 cells transfected with 1.5 μg of expression plasmid per well. The expression level was set to 100 for exon 1A3. The error bars represent SEM, $n = 9$. Panel C shows the expression level of GRα-encoding transcripts of E8.2 cells transfected with 1.5 μg of expression plasmid per well. The expression level was set to 100 for exon 1A3. The error bars represent SEM, $n = 9$. Panel D shows the relative translational efficiencies in E8.2 cells of transcripts containing exon 1A3, exon 1B, or exon 1C. For each transfection experiment, the relative translational efficiency for an exon 1 form is calculated as the average GRα protein level of three transfected wells divided by the average GRα transcript level of three transfected wells. The expression level was set to 100 for exon 1A3. The error bars represent SEM for three separate transfection experiments. The *p*-value for no difference in translational efficiency among the exon 1 forms is 0.28. For panels B and C, significant differences compared to exon 1A3 are indicated by ∗ ($p < 0.05$), ∗∗ ($p < 0.01$), and ∗∗∗ ($p < 0.001$). No other pairwise comparisons were significant.

in dexamethasone-treated CEM-C7 cells (*9*). The higher than expected up-regulation of GR protein in these cells was based on an assumption of a destabilizing effect of glucocorticoids on GR protein as had previously been seen in many other hormone-treated cell lines (*13*−*16*). We tested the validity of this assumption by measuring the stability of GR protein of CEM-C7 lymphoblast cells treated with 50 μM cycloheximide. Surprisingly, dexamethasone did not decrease the GR protein stability in these cells (Figure 4A) but may even have stabilized the GR protein. By contrast, dexamethasone did seem to decrease the GR protein stability in IM-9 cells (data not shown).

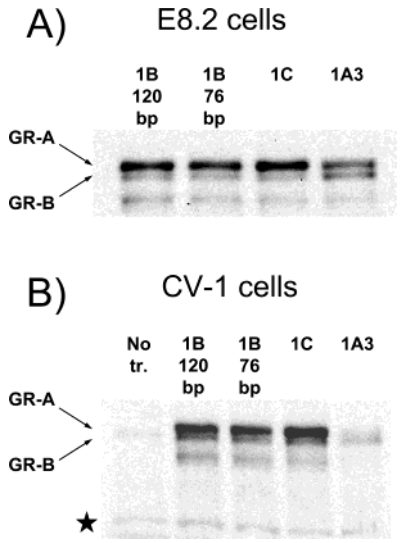

FIGURE 3: Exon 1A3 increases GR-B/GR-A ratio. Panel A shows a Western blot of whole cell lysates of E8.2 cells transfected with GRα expression plasmids containing different exon 1 sequences. Based on previous Western blots, loading was adjusted to allow approximately equal amounts of total GR protein to be loaded per lane. Panel B shows a Western blot of whole cell lysates of CV-1 cells transfected with 1.5 μg per well of GRα expression plasmids containing different exon 1 sequences. A lysate of nontransfected CV-1 cells (no tr) is also shown. Fifty micrograms of lysate protein was loaded per lane. The star indicates a nonspecific band that also appears in untransfected CV-1 cells and confirms equal loading of protein in each lane.

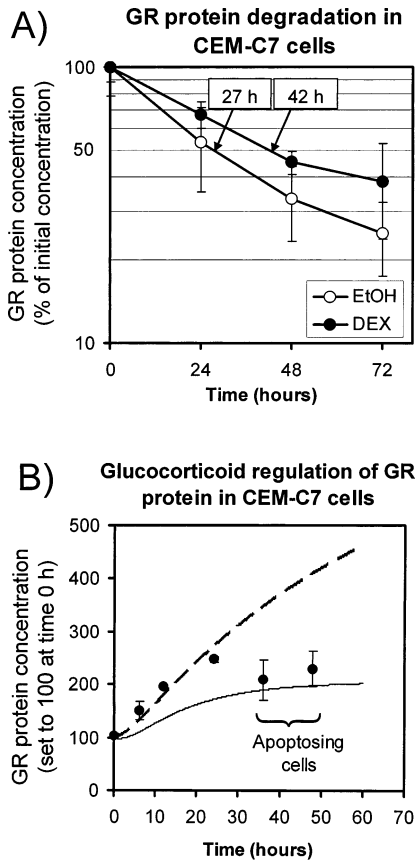

FIGURE 4: GR protein stability in CEM-C7 cells is not decreased by DEX. In panel A, CEM-C7 cells were pretreated for 4 h with either 1 μM DEX or the ethanol (EtOH) vehicle followed by addition of 50 μM cycloheximide at the 0 h time point. For both DEX- and EtOH-treated samples, the GR protein concentration was set to 100% at the 0 h time point. The error bars represent the SEM, $n = 3$. Panel B demonstrates glucocorticoid regulation of GR protein. The filled circles with their corresponding error bars indicate the mean and SEM of the observed glucocorticoid regulation of GR protein reported in ref *9*. The curves are models of the glucocorticoid regulation of GRα protein based on the observed glucocorticoid regulation of GRα transcripts reported in ref *9*. The thin solid curve represents a model based on GR protein half-life estimates of 24 and 11 h in the absence and presence of DEX, respectively. The thick dashed curve represents a model based on GR protein half-life estimates of 27 and 42 h in the absence and presence of DEX, respectively.

Modeling of glucocorticoid regulation of GR protein using the apparent half-lives of the GR protein seen in Figure 4A, under the assumptions of all GRα-encoding transcripts translated at the same rate and of glucocorticoids not affecting translational efficiency, is shown in Figure 4B. Under these conditions, the modeled up-regulation of GRα protein fits the previously observed up-regulation of GRα protein well for the first 24 h of glucocorticoid treatment.

DISCUSSION

It has previously been reported that total GR mRNA stability is unaffected by glucocorticoids in IM-9 and CEM-C7 derived cells (*17*, *18*). We confirm that the stabilities of both GRα-encoding and GRβ-encoding transcript are unaffected by dexamethasone in these two cells lines. Furthermore, the stabilities of GR transcripts with exons 1A3, 1B, or 1C are very similar and hardly affected by the presence of glucocorticoids. This indicates that for all types of GR transcripts, the glucocorticoid regulation occurs primarily at the level of transcription and that the relative concentration of the transcripts reflects the relative transcription rate from the corresponding promoters.

We have previously reported that under the assumptions of glucocorticoids decreasing GR protein stability, all glucocorticoid transcripts being translated at the same rate, and the translation rate not being affected by glucocorticoids, the GR protein levels during the first 24 h of dexamethasone treatment in CEM-C7 cells increase more than expected from the increase in GR transcripts alone (*9*). We have tested whether this higher up-regulation of GR protein could be explained by the exon 1A3 sequence causing a higher translation rate than the exon 1B and exon 1C sequences. This was done by comparing the influence of exon 1A3, a long and a short form of exon 1B, and exon 1C on the translational efficiency of GRα mRNA expressed in mouse E8.2 cells after transfection with GRα expression plasmids. The translational efficiencies are of similar magnitude, although exon 1C-containing transcripts may be translated at a slightly higher efficiency. The long and short forms of exon 1B have the same translational efficiency, indicating no effect of the 44 nucleotide sequence at the 5′-end of the long exon 1B sequence on translational efficiency.

We certainly did not observe a markedly higher translation rate of exon 1A3-containing transcripts than of exon-1B or exon-1C that could explain the higher than expected up-regulation of GR protein in CEM-C7 cells. A higher translation rate of exon 1A3-containing transcripts than of the other exon 1-containing transcripts in human T-lymphoblasts (as compared to the nonlymphoblast cells used here) cannot be ruled out, but clearly the exon 1A3 sequence does not in general confer a higher translation rate on GRα-encoding transcripts.

Open reading frames located in the 5′-untranslated region (5′-UTR) of transcripts of the nuclear receptor superfamily, of which GR is a member, have previously been reported to affect translation of the receptor. The 1026 bp mouse GR exon 1A sequence, which is homologous to the human exon 1A3, contains five upstream open reading frames. One of these starts at position 217 and has the potential to encode a 93 amino acid peptide. This open reading frame appears to be essential for GR expression from the murine exon 1A-containing GR transcript by in vitro transcription/translation in reticulocyte extracts (*19*). Human exon 1A3 has a similarly located open reading frame starting at position +154 with the potential to encode a 70 amino acid peptide. But open reading frames can also inhibit translation. This was observed in a study of the effects of different 5′-UTRs of the human and murine estrogen receptor α on the translation of a luciferase reporter gene (*20*). Open reading frames in the 5′-UTR could decrease expression, especially when the termination codons were close to the translation initiation codon of the luciferase gene. We have found that the human exon 1A3 sequence causes a higher GR-B/GR-A ratio than the other exons, at least in the two cell lines, E8.2 and CV-1. An intriguing possibility for explaining this result is an open reading frame starting at position +846 in exon 1A3. This has the potential for encoding a 49 amino acid peptide with a termination codon that overlaps the start codon for GR-A. Translation of this novel exon 1A open reading frame could possibly inhibit translation initiation of GR-A, which in turn might enhance initiation from the GR-B initiation codon.

GR-B has been reported to be nearly twice as effective as the GR-A species in gene transactivation but not in transrepression (*7*). CEM-C7 cells up-regulate exon 1A3-containing GR transcripts to a higher degree than transcripts containing exons 1B or 1C in CEM-C7 cells (Figure 1 and ref *9*), which would further tend to increase the GR-B/GR-A ratio and hence the transactivation potential of glucocorticoids. Among 12 human cell types, CEM-C7 cells were previously found to possess the highest expression of exon 1A3-containing transcripts (*8*). After 24 h dexamethasone treatment of CEM-C7 cells, the exon 1A3-containing transcripts comprise 16% of the population of exon 1-containing GR transcripts (*9*). CEM-C7 cells clearly express a GR isoform corresponding in size to GR-B (see Figure 5C in ref *9*), but it is less abundant than GR-A. The physiological importance in these cells of an increased GR-B/GR-A ratio caused by the exon 1A3-containing transcript thus seems to be mainly a fine-tuning of the transactivation potential of glucocorticoids.

Previously we based the modeling of glucocorticoid-regulated GR protein on estimates of the half-life of GR protein of 24 h in the absence and 11 h in the presence of glucocorticoids corresponding to values based on earlier studies (*13*, *21*). We tested the validity of the assumption that glucocorticoids decrease the stability of GR protein. Surprisingly, dexamethasone did not destabilize GR protein in CEM-C7 cells. Figure 4B illustrates that the apparent half-lives of the GR protein derived from Figure 4A (27 h in the absence of DEX and 42 h in the presence of DEX) can explain the previously observed high up-regulation of GR protein within the first 24 h of dexamethasone treatment. At times past 24 h of hormone treatment, apoptosis of the CEM-C7 cells is prominent, perhaps explaining why GR protein levels do not continue to increase in cells past this time. To our knowledge, this is only the second report of glucocorticoids not causing a decreased half-life of the GR protein. Interestingly, the other example is for mouse S49 T-lymphoma cells, which are also killed by corticosteroid hormones (*22*), suggesting that an inability of corticosteroids to decrease GR stability may be a general characteristic for immature T-lymphocytes and T-lymphoblasts.

CEM-C7 T-lymphoblasts undergo apoptosis in response to glucocorticoids, and an up-regulation of the GR protein is essential for this apoptosis to occur (*4*). These cells appear to possess three mechanisms contributing to this biologically relevant glucocorticoid response: a glucocorticoid-induced up-regulation of GR mRNA, increased expression of a more transcriptionally potent glucocorticoid receptor (GR-B) via a particularly robust induction of exon 1A3-containing transcripts, and no decrease in the stability of the liganded GR protein. All three of these mechanisms can render the T-lymphoblasts more sensitive to the corticosteroid hormone and thus result in a strong apoptotic response.

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