Quantification and Glucocorticoid Regulation of Glucocorticoid Receptor Transcripts in Two Human Leukemic Cell Lines†

Kim Brint Pedersen and Wayne V. Vedeckis*

Department of Biochemistry and Molecular Biology and Stanley S. Scott Cancer Center, Louisiana State University Health Sciences Center, 533 Bolivar Street, New Orleans, Louisiana 70112

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ABSTRACT: We have quantified the basal and glucocorticoid-regulated levels of different transcripts from the human glucocorticoid receptor (GR) gene in the T-cell acute lymphoblastic leukemia cell line, CEM-C7, and in the B lymphoblastoid cell line, IM-9. Highly specific quantitative, reverse transcription-polymerase chain reaction assays measured total GR transcripts, transcripts encoding the isoforms glucocorticoid receptor α (GRα) and glucocorticoid receptor *β* (GR*β*), and transcripts containing different forms of exon 1: 1A1, 1A2, 1A3, 1B, and 1C. GRα and GR*β* transcripts are coordinately upregulated in CEM-C7 cells and coordinately downregulated in IM-9 cells by dexamethasone. The concentration of GRα mRNA is more than a 1000-fold higher than that for GR*β* mRNA. Transcripts with different exon 1 forms are all upregulated in CEM-C7 cells and all downregulated in IM-9 cells by dexamethasone, but transcripts containing exons 1A1, 1A2, or 1A3 are regulated to a higher degree than transcripts containing exon 1B or exon 1C. However, exon 1B- and exon 1C-containing transcripts are substantially more abundant than exon 1A-containing transcripts, with exon 1A3-containing transcripts more abundant than exon 1A1- or exon 1A2-containing transcripts. Analysis using models for glucocorticoid receptor autoregulation kinetics suggests that the minor 1A3-containing transcript component could be important for GR protein upregulation, and hence apoptosis, in CEM-C7 cells. These studies suggest that GRα transcripts containing exons 1A3, 1B, and 1C contribute most to the intracellular level of GR mRNA and may be the most relevant for steroid-mediated apoptosis in T-lymphoblasts.

Glucocorticoids (GCs)[1] are commonly used in the treatment of lymphoid leukemias due to their ability to induce apoptosis of the malignant cells (*1*, *2*). They exert their effect by diffusing through the cell membrane and binding to the glucocorticoid receptor (GR). Upon ligand binding, the GR becomes activated as a transcription factor and modulates the transcription of glucocorticoid-responsive genes. The responsiveness of cells to GCs depends not only on the GC concentration, but also on the intracellular concentration of GR (*3*). The regulation of the GR gene is therefore of prime importance for glucocorticoid responsiveness. One of the factors regulating the GR gene is the hormone itself. In most cell types, GR is downregulated by GCs at both the RNA and protein level. However, some cell types, such as the T-cell acute lymphoblastic leukemia (ALL) cell line CEM-C7, display upregulation of GR at the RNA and protein level (*4*, *5*), and for cell lines derived from CEM-C7 this upregulation is essential for the apoptotic response to GCs (*6*).

Two proteins are generated from the human glucocorticoid receptor gene: glucocorticoid receptor α (GRα) and glucocorticoid receptor *β* (GR*β*). GRα consists of 777 amino acids and GR*β* of 742 amino acids, with the first 727 amino acid residues being identical in the two proteins (*7*). Transcripts for both proteins are generated from nine exons. Splicing of exon 8 to two alternative forms of exon 9 (exon 9α and exon 9*β*) gives rise to the mRNAs for GRα and GR*β* (*8*). GRα is able to bind to glucocorticoids, whereas GR*β* cannot (*7*). GRα is thus the protein mediating the transcriptional response of glucocorticoids. GR*β* is expressed in many human tissues and has been implicated as a dominant negative inhibitor of GRα activity (*9*−*11*). However, the physiological role of GR*β* remains controversial (reviewed in ref *12*).

In addition to the GRα- and GR*β*-encoding transcripts, at least three human GR promoters (promoter 1A, promoter 1B, and promoter 1C) exist, giving rise to at least five transcript variants in which an untranslated exon 1 (exon 1A1, exon 1A2, exon 1A3, exon 1B, and exon 1C) becomes spliced to a common splice acceptor site of exon 2 (*13*). All

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* To whom correspondence should be addressed. Phone: (504) 568−8175. Fax: (504) 568-6997. E-mail: wvedec@lsuhsc.edu

[1] Abbreviations: GC, glucocorticoid; GR, glucocorticoid receptor; QRT-PCR; quantitative reverse transcription-polymerase chain reaction; RT-PCR; reverse transcription-polymerase chain reaction; GRα, glucocorticoid receptor alpha; GR*β*, glucocorticoid receptor beta; ALL, acute lymphoblastic leukemia; FBS, fetal bovine serum; DEX, dexamethasone; EtOH, ethanol; PDAR, predeveloped Taqman assay reagent; CV, coefficient of variation; UTR, untranslated region; OD, optical density; PMSF, phenylmethylsulfonylfluoride; PBS, phosphate buffered saline; [RNA], RNA concentration; [P], protein concentration; S_{RNA}, RNA synthesis rate; S_P, protein synthesis rate; $T_{1/2RNA}$, RNA half-life; $T_{1/2P}$, protein half-life; Eq, equilibrium; max, maximum; k_{tr}, translational efficiency; IC_{50}, 50% inhibitory concentration; SC_{50}, 50% saturating concentration.

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of these are expected to be translated to the same proteins (GRα or GRβ), as the translation initiation codon in exon 2 is preceded by an in-frame stop codon in exon 2. Previously, promoter 1A was found to be highly responsive to GCs, while promoter 1B and 1C seemed to have low or no GC responsiveness (*13*).

To assess the role of different GR transcripts in overall GC regulation of the GR gene at the RNA level, we have studied the glucocorticoid regulation of individual transcripts and determined their absolute concentrations using highly specific real-time quantitative reverse transcription-polymerase chain reaction (QRT-PCR). We studied the transcripts in two leukemic cell lines: CEM-C7, derived from T-cell ALL (*14*, *15*), and IM-9, which is an Epstein−Barr virus-transformed B lymphoblastoid cell line (*16*).

We found that GRα and GRβ transcripts are coordinately upregulated in CEM-C7 cells and coordinately downregulated in IM-9 cells by the glucocorticoid dexamethasone (DEX). The concentration of GRα mRNA is more than 1000-fold higher than the concentration of GRβ mRNA, with less than 0.1 GRβ transcript per cell in either cell line. GR transcripts with different exon 1 forms are all upregulated in CEM-C7 cells and all downregulated in IM-9 cells, but transcripts containing exons 1A1, 1A2, or 1A3 are regulated to a higher degree than transcripts containing exon 1B or exon 1C. On the other hand, exon 1B- and exon 1C-containing transcripts are substantially more abundant than exon 1A-containing transcripts. Among the exon 1A-containing transcripts, those containing exon 1A3 are more abundant than those containing exons 1A1 or 1A2.

Models describing the kinetics of GR mRNA autoregulation are fitted to the observed regulation of GR transcripts and used to predict the kinetics of glucocorticoid regulation of GR protein. Comparisons of these predictions with the observed regulation of GR protein indicate that the minor transcript component of exon 1A3-containing transcripts might be an important contributor to the total pool of GR protein in CEM-C7 cells. It is further indicated that the GRβ-encoding transcripts and transcripts with exon 1A1 or exon 1A2 are present in such low relative and absolute amounts that they hardly can have a substantial effect at the protein level. The studies suggest that GRα transcripts containing exons 1A3, 1B, and 1C contribute most to the intracellular pool of GR mRNA in CEM-C7 and thus may be the most relevant for steroid-mediated apoptosis in T-lymphoblasts.

EXPERIMENTAL PROCEDURES

Cell Culture. CEM-C7 cells (a kind gift from Dr. E. Brad Thompson, University of Texas Medical Branch, Galveston, TX) were grown in RPMI 1640 supplemented with 10% dialyzed FBS. Human IM-9 cells were grown in RPMI 1640 supplemented with 10% FBS. For glucocorticoid treatment, cells were treated with dexamethasone (DEX) at a final concentration of 1 μM, which is sufficient for saturating the GR. Control cells were treated with vehicle alone (ethanol at 0.1 μL/mL). This ethanol concentration does not affect cell growth or protein content per cell.

Real-Time Quantitative RT-PCR. Primers and Taqman (Applied Biosystems, Foster City, CA) probes were designed using the Primer Express Software from Applied Biosystems such that the amplicons and the Taqman probes span the two exons involved in a splice site. Primers were from MWG Biotech, Inc. (Greensboro, NC). Assays for GRα- and GRβ-encoding transcripts measured transcripts with the exon 8/exon 9α and exon 8/exon 9β splice site, respectively. Assays for transcripts with different forms of exon 1 detected transcripts with splicing between the particular exon 1 form and the common splice acceptor site of exon 2. The assay for total GR transcripts quantified transcripts with the exon 5/exon 6 splice site. Taqman probes contained 6-carboxyfluorescein at the 5′ end and the quencher 6-carboxy-*N*,*N*,*N*′,*N*′-tetramethylrhodamine at the 3′ end. The sequences of primers and Taqman probes are listed in Table 1. Predeveloped Taqman assay reagents (PDAR) for measuring 18S rRNA were from Applied Biosystems (cat. no. 4310893E). The One-Step RT-PCR kit (Applied Biosystems) was used for the QRT-PCR assays. Each reaction for measuring GR transcripts contained 5 μL of RNA sample, 900 nM forward primer, 900 nM reverse primer, 100 nM Taqman probe, and 1× Master mix and Multiscribe enzyme mix from the One-Step RT-PCR kit in a total volume of 25 μL. Each reaction for measuring 18S rRNA contained 5 μL of RNA sample, 1× primers and probe mix from the PDAR kit, and 1× Master mix and Multiscribe enzyme mix from the One-Step RT-PCR kit in a total volume of 25 μL. The assays were conducted on an ABI Prism 7700 Sequence Detection system from Applied Biosystems at the following thermal cycling parameters: 30 min at 48 °C, 10 min at 95 °C, 40 to 45 cycles of 15 s at 95 °C, and 1 min at 60 °C. Amplification curves were visually inspected to set a suitable baseline range and threshold level. The fractional number of PCR cycles (Ct) required to reach the threshold fluorescence level was scored and used for generating standard curves and interpolating RNA concentration levels.

RNA Samples. Total RNA was isolated from cells by guanidinium thiocyanate-phenol-bromochloropropane extraction using TriReagent from Molecular Research Center, Cincinnati, OH, according to the guidelines of the manufacturer. The RNA was dissolved in diethyl pyrocarbonate-treated H_2O and stored at −80 °C.

RNA Standards. Absolute RNA standards were generated as follows. cDNA was made from total RNA isolated from IM-9 cells using the Advantage RT-for-PCR kit from CLONTECH Laboratories, Inc. (Palo Alto, CA). The reactions contained 1 or 5 μg of RNA, 1 μL of oligo(dT) primer, 1× reaction buffer, 0.5 mM each of dATP, dTTP, dCTP, and dGTP, 0.5 μL of recombinant RNAse inhibitor, and 1 μL of MMLV reverse transcriptase in a volume of 20 μL. The reactions were incubated at 42 °C for 1 h, followed by 94 °C for 5 min. A total of 80 μL of H_2O was added. From the cDNA, ca. 400 bp DNA fragments encompassing the amplicons used for real-time QRT-PCR were generated by PCR. The sequences of primers are listed in Table 1. The reactions contained 5 μL of cDNA, 1.5 mM $MgCl_2$, 0.2 mM each of dATP, dTTP, dCTP, and dGTP, 0.9 μM each of forward and reverse primer, and 1× PCR buffer II and 1.25 U AmpliTag Gold (both from Applied Biosystems) in a total volume of 50 μL. The thermal cycling parameters were 5 min at 95 °C; 30 to 42 cycles of 30 s at 94 °C, 30 s at 60 °C, and 30−60 s at 72 °C; followed by 10 min at 72 °C. The PCR products were separated by agarose gel electrophoresis followed by visualization by ethidium bromide staining. DNA fragments of the right size were cut out from

Table 1: Nucleotide Sequences of Primers and Probes Used for Real-Time QRT-PCR and for RNA Standards

	real-time QRT-PCR			RNA standard	
splice site	forward primer	reverse primer	Taqman probe	forward primer	reverse primer
exon 1A1−2	5′-TTTAAATGGCAG-AGAGAAG-GAGAAA-3′	5′-TCTACCAGGAGT-TAATGATTCTTT-GGA-3′	5′-TCCATCAGTGAA-TATCAACTTCTA-AGGTCCAGTGA-3′	5′-TGCTCCCTCTCG-CCCTCATTC-3-	5′-AAAAGTCTTCG-TGCTTGGA-3′
exon 1A2−2	5′-CGCATGTGTCCA-ACGGAA-3′	5′-TCCTGAGCAAGC-ACACTGCT-3′	5′-TTCTTTGGAGTC-CATCAGTGAATA-TCAACCTCT-3′	5′-TTCACTTCTGCT-GGGGAAAT-3′	5′-AAAAGTCTTCGC-TGCTTGGA-3′
exon 1A3−2	5′-GCCTGGCTCCTT-TCCTCAA-3′	5′-CAGGAGTTAATG-ATTCTTTGG-AGTCC-3′	5′-TCAGTGAATATC-AACTTCTTCTC-AGACACTTT-3′	5′-GAGGCAGGTGCA-GTTTGTGT-3′	5′-AAAAGTCTTCGC-TGCTTGGA-3′
exon 1B-2	5′-GCCCAGATGATG-CGGTG-3′	5′-TCTACCAGGAGT-TAATGATTCTTT-GGA-3′	5′-CCATCAGTGAAT-ATCAATTTGGGC-CCG-3′	5′-GCAGCTGAAGAC-CCGG-3′	5′-CCAGGTCATTTC-CCATCACT-3′
exon 1C-2	5′-GGGAACTGCGGA-CGGTG-3′	5′-GGAGTTAATGAT-TCTTTGGAG-TCCA-3′	5′-CGGCTCCTCTGC-CAGAGTTGAT-ATTCACT-3′	5′-ATTTCCCTCCTG-CTCCTTCT-3′	5′-CCAGGTCATTTC-CCATCACT-3′
exon 5−6	5′-AGTGATTGCAGC-AGTGAAATG	5′-TGCAGTAGGGTC-ATTTGGTCAT-3′	5′-CAAAGGCAATAC-CAGGTTTCAGGA-ACTTACACCT-3′	5′-CCTCTGAAAATC-CTGGTAACAAA-3′	5′-TGGTCGTACATG-CAGGGTAG-3′
exon 8−9α	5′-GGCAGCGGTTTT-ATCAACTGA-3′	5′-AATGTTTGGAAG-CAATAGTTAAGG-AGA-3′	5′-TTTCAACCACTT-CATGCATAGAAT-CCAAGAGTTT-3′	5′-TACCCTGCAT-GTACGACCAA-3′	5′-TTTTGGTATCTG-ATTGGTGATGA-3′
exon 8−9β	5′-AACTGGCAGCGG-TTTTATCAA-3′	5′-TGTGAGATGTGC-TTTCTGGTTTAAA-3′	5′-CATAACATTTTC-ATGCATAGAATC-CAAGAGTTTT-GTCA-3′	5′-TACCCTGCAT-GTACGACCAA-3′	5′-TTGTCGATGAGC-ATCAGTTG-3′

addtnl std	include splice sites:	forward primer	reverse primer
exon 5−9α std	exon 5−6 and exon 8−9α	5′-CCTCTGAAAATC-CTGGTAACAAA-3′	5′-TTTTGGTATCTG-ATTGGTGATGA-3′

the gels and extracted using the GENECLEAN SPIN kit from Qbiogene, Inc. (Carlsbad, CA) according to the guidelines of the manufacturer. DNA fragments were cloned into the pCRII-TOPO vector using a TOPO TA Cloning kit from Invitrogen (Carlsbad, CA) and were transformed into chemically competent TOP10 cells according to the guidelines of the manufacturer. Plasmid DNA was prepared from transformed colonies and checked for inserts and orientation of inserts with suitable restriction digestions. The orientation was chosen so that transcription from the T7 promoter in the pCRII-TOPO vector generates a segment of sense GR RNA. Plasmid DNA from selected clones was sequenced to ensure correctness of the inserted sequences. RNA was generated by in vitro transcription of Hind III-restricted plasmid DNA using the MEGAscript T7 kit from Ambion Inc. (Austin, Texas). The transcription reaction contained 1 μg of gel-purified DNA template, 7.5 mM each of ATP, CTP, GTP, and UTP, 1× reaction buffer, and 2 μL of T7 RNA polymerase enzyme mix in a total volume of 20 μL. The reactions were incubated at 37 °C for 4 to 24 h. 2 U of RNAse-free DNase 1 were added, followed by incubation at 37 °C for 15 min. The RNA was recovered by phenol/chloroform and chloroform extraction followed by 2-propanol precipitation. The RNA was redissolved in 50 μL of RNA storage buffer from Ambion Inc. The RNA was run on a 5% acrylamide/8 M urea gel together with RNA molecular weight markers generated by in vitro transcription of the RNA Century Marker Plus Template set from Ambion Inc. RNA fragments of the correct size were visualized by UV shadowing and cut from the gel. RNA was eluted from minced gel pieces by incubation for at least 2.5 h at 37 °C in 350 μL 0.5 M ammonium acetate, 10 mM EDTA, and 0.2% SDS. The RNA was recovered by EtOH precipitation and dissolving in RNA storage buffer or by gel filtration over a Micro Bio-Spin 30 Chromotography column (Bio-Rad Laboratories, Hercules, CA) according to the guidelines of the manufacturer. The concentrations of the RNA standards were determined by fluorometry using the RiboGreen RNA Quantitation Reagent and kit from Molecular Probes (Eugene, Oregon). Briefly, RNA was dissolved in TE buffer, and RiboGreen reagent was added to a final volume of 2.5 mL. Fluorescence was measured in an Aminco-Bowman Series 2 spectrometer from Spectronic Unicam (Rochester, New York). Excitation was at 485 nm with an 8-nm band-pass and emission was at 525 nm with an 8-nm band-pass. The concentrations were determined relative to the *Escherichia coli* RNA standard supplied with the kit.

Quantitative Western Blots. Whole cell lysates were made by suspending harvested and PBS-washed cells in Laemmli Sample buffer containing 1 mM PMSF and 1/100 vol Protease Inhibitor cocktail (P-8340) from Sigma (St. Louis, MO), followed by sonication. Protein concentration was determined by the DC Protein Assay kit from Bio-Rad Laboratories with bovine serum albumin as a standard. As relative standards, we used different amounts of 24 h DEX-treated CEM-C7 cell lysates or glucocorticoid-untreated IM-9 cell lysates. Lysate aliquots containing 20 μg of protein and standards were separated by SDS−PAGE using 8% separating gels with 5% stacking gels. Proteins were transferred onto Immobilon-NC Transfer Membranes (Millipore Corporation, Bedford, MA) using semi-dry transfer. GR protein was detected using a 1:500 dilution of the rabbit polyclonal IgG antibody GR (H-300), cat. no. sc-8992 from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA) as primary antibody

and a 1:2500 dilution of peroxidase-labeled anti-rabbit secondary antibody (NIF 824) from Amersham Biosciences (Piscataway, NJ). The peroxidase activity was detected using ECL Western Blotting Detection reagents from Amersham Biosciences. Luminescence was recorded on a Fluor-S MultiImager from Bio-Rad. A major band at ca. 93 kDa and a much weaker band at ca. 89 kDa were interpreted as full-length GR protein, with the 93 kDa band representing the glucocorticoid receptor-A form of GRα and the 89 kDa band representing the sum of GRβ and the glucocorticoid receptor-B form of GRα (*17*, *18*). Using the Quantity One software, the combined intensity of these two bands was quantified, and the relative GR concentration was determined relative to the standard curve.

Time-Course Experiments. CEM-C7 and IM-9 cells were split into 30 mL of medium in a 150 cm^2 cell culture flasks 1 day before steroid treatment. The initial cell concentrations were 5×10^5 CEM-C7 cells/mL and 1.2×10^5 IM-9 cells/mL. DEX or ethanol vehicle was added to the flasks in 3 mL of additional cell culture medium. Immediately after addition and mixing, a 5 mL aliquot was taken as the 0 h sample, from which cells were immediately pelleted by centrifugation and dissolved in TriReagent. Additional 5 mL aliquots were taken at time points of 1, 2, 4, 24, and 30 h. Each experiment included a DEX- and an ethanol-treated cell culture flask, and the experiments were conducted four times for CEM-C7 cells and eight times for IM-9 cells. Transcript concentrations were determined by real-time QRT-PCR. RNA standards had concentrations ranging from 10^{-11} to 10^{-17} M specific RNA in a diluent consisting of 40 ng of yeast RNA/μL of H_2O. RNA samples for real-time QRT-PCR assays were, where possible, diluted to 40 ng of RNA/μL. For the least abundant GR transcripts, RNA samples were assayed undiluted. GR transcript concentrations were normalized to total RNA, either determined by the optical density at 260 nm or by determining the concentration of 18S rRNA by real-time QRT-PCR relative to known quantities of Taqman Control Total human RNA purchased from Applied Biosystems. GR transcript concentrations were also expressed as the number of transcripts per cell. The cellular RNA concentrations were determined in separate experiments from asynchronously growing CEM-C7 and IM-9 cells by counting the cells in a hemacytometer and determining the total amount of RNA extracted from a fixed number of cells.

Time-course experiments for detection of GR protein were conducted in a similar way, with 6 mL sample aliquots being taken at time points of 0, 4, 8, 24, 48, and 72 h for IM-9 cells and at time points of 0, 6, 12, 24, 36, and 48 h for CEM-C7 cells. To avoid entry into stationary phase, cell cultures were further split after the first 24 h of treatment. A DEX- and an ethanol-treated flask were included in each experiment, and the experiments were conducted four times each for both CEM-C7 and IM-9 cells. The GR protein content for each sample was determined using two separate Western blots. Corresponding dexamethasone- and ethanol-treated aliquots were analyzed on the same blots, and the GR concentration of the dexamethasone-treated samples were expressed in percentages of the GR concentration of the corresponding ethanol-treated samples.

Statistical Analysis. Differences in transcript concentrations between cells treated with DEX and ethanol vehicle were tested by the Student two-sample t-test. Differences in DEX-induced fold changes in transcript concentrations between two different GR transcripts were analyzed for the averages of the transcript concentrations at 4, 24, and 30 h time points by the paired-sample t-test. Differences were considered significant if the test probabilities were less than 5% ($P < 0.05$).

RESULTS

Characteristics of Real-Time QRT-PCR Assays. The splicing pattern of the various GR transcripts is shown in Figure 1A. To quantify GR transcripts with a specific splice site, we designed assays with a forward and reverse primer in each of the exons involved in the splice and a Taqman probe spanning the splice site, as shown in Figure 1B. RNA standards were generated as described in Experimental Procedures. The RNA standard generated for exon 1B-containing transcripts showed an absence of the cytosine residue at position −1025 published in ref *19*. The absent cytosine does not appear to be an amplification artifact, as it was consistently observed whether random hexamers or oligo(dT) primers were used in the RT step, whether amplification was done from CEM-C7 or IM-9 RNA, and when different DNA polymerases were used.

We validated each assay by running a 10-fold dilution series of standards starting from concentrations of 10^{-11} M. All assays worked well and with high efficiency in the PCR step. The sensitivity differed between assays. The most sensitive assays, illustrated by the GRα-specific assay in Figure 1C, allowed a reproducible amplification from as low as 10^{-16} M transcript, and concentrations as low as 10^{-17} M and occasionally 10^{-18} M (equivalent to three transcript molecules per reaction) could result in detectable amplification. Less sensitive assays such as the assay for total GR transcripts only allowed reproducible amplification signals from ca. 10^{-14} M transcripts with no detection at 10^{-16} M or less. The sensitivity of all assays turned out to be adequate for quantifying all the transcripts in IM-9 and CEM-C7 cells.

The reproducibility of the assay specific for GRα-encoding transcripts, i.e., transcripts containing the splice site between exon 8 and exon 9α, was assessed by measuring the concentration in a sample of IM-9 total RNA in different wells on different 96-well plates and by extrapolating the concentrations from different preparations of standards. The intra-assay (well-to-well) coefficient of variation (CV) was 13%, while the inter-assay (plate-to-plate) CV was estimated to be ca. 4%, and the inter-standard CV to be ca. 8%. The overall CV was 15%, so well-to-well variation seemed the major component of assay variability in this experiment (data not shown). It should be noted, however, that in the daily use of these assays, the combination of inter-assay and inter-standard variation can, in extreme situations, result in up to 2.6-fold differences in concentrations of GRα-encoding transcripts.

The variability of the real-time QRT-PCR measurements tends to increase with decreasing transcripts concentrations. That is, the lower the transcript concentrations were, the less precise the real-time QRT-PCR assays were. For example, at transcript concentrations above 2 pmol/g of RNA, the average CV was 18%, while it was 56% at transcript concentrations below 0.1 pmol/g of RNA (data not shown). The values of absolute transcript concentrations depend on

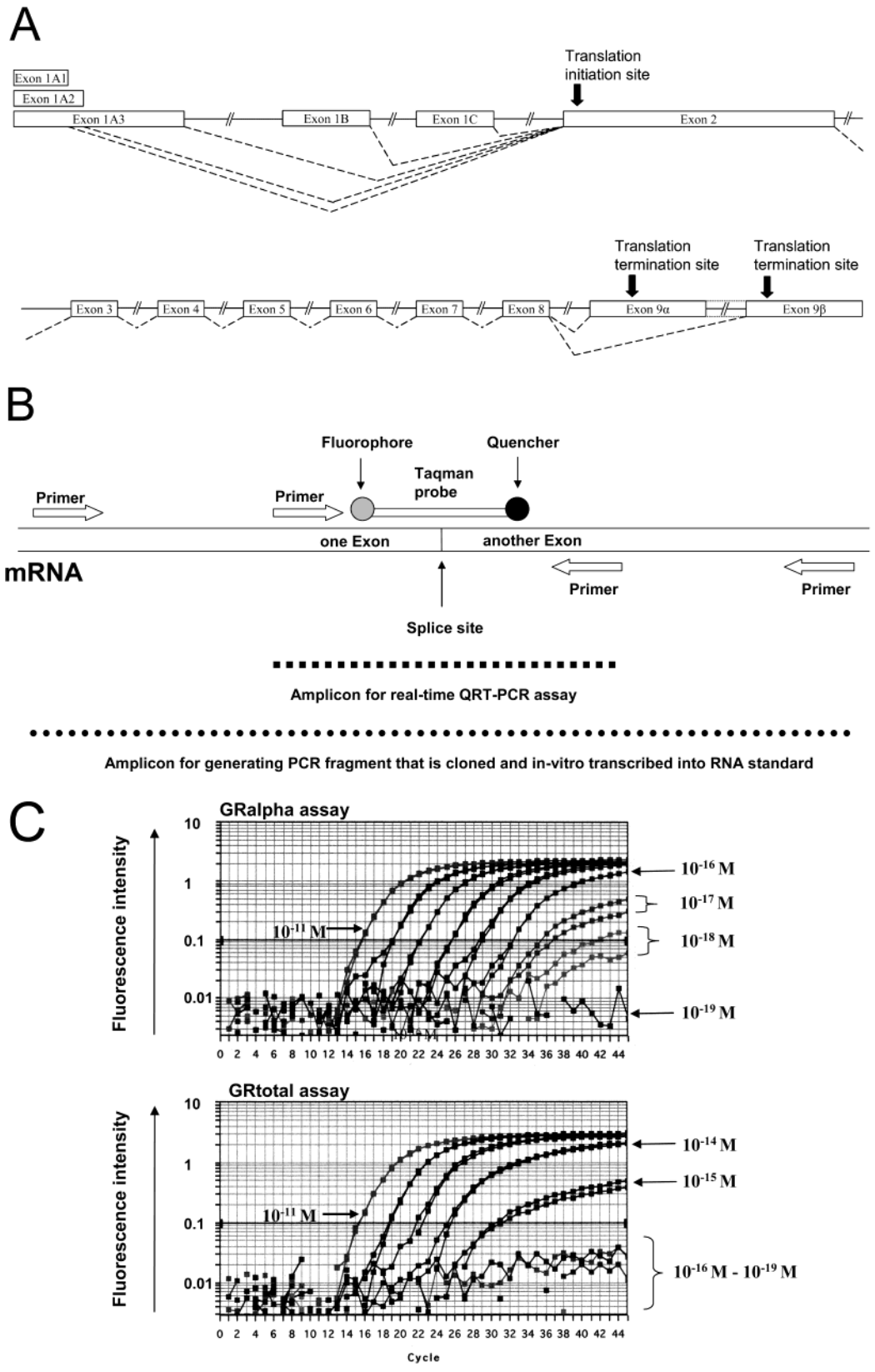

FIGURE 1: Design of sensitive real-time QRT-PCR assays specific for a particular splice site. (A) Splicings involved in generating an mRNA encoding full-length GRα or GRβ protein. We designed assays specific for each of the exon 1 forms splicing to exon 2 and for exon 8 splicing to each of the exon 9 forms. Note that exon 9α may include exon 9β (*11*). For measuring total GR mRNA, we designed an assay specific for the common exon 5- exon 6 splice site. (The different exons are not drawn to scale.) (B) Specificity of real-time QRT-PCR assays was achieved by having primer sites in the two exons involved in a splice in combination with a Taqman probe spanning the splice site. A larger amplicon encompassing the real-time QRT-PCR amplicon was used for generation of an RNA standard. (C) The range of sensitivities for real-time QRT-PCR assays is illustrated with the assays for GRα-encoding transcripts and for total GR transcripts in which standard RNA samples at concentrations from 10^{-11} to 10^{-19} M are measured. The panels show the intensity of the generated fluorescence during the assays as a function of the PCR cycle number.

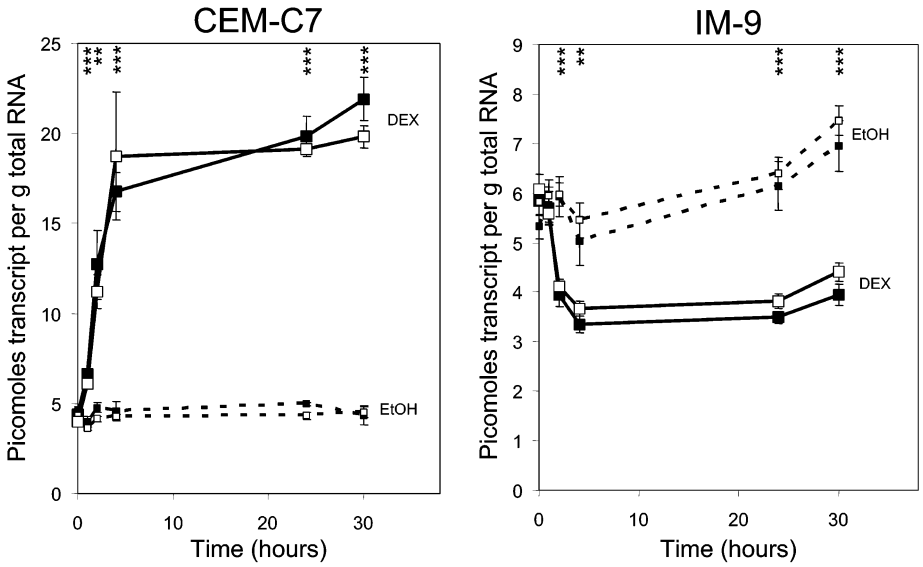

FIGURE 2: Dexamethasone upregulates total GR transcripts in CEM-C7 cells and down-regulates them in IM-9 cells. Time course experiments of total GR transcript concentrations in cells treated with 1 μM DEX or the ethanol vehicle. The transcript concentrations were normalized to g of total RNA determined either by absorbance at 260 nm (open symbols) or by real-time QRT-PCR assay of 18S rRNA relative to total human calibrator RNA (closed symbols). Data represent the mean and standard error of the mean (SEM) of four experiments for CEM-C7 cells and eight experiments for IM-9 cells. For the data normalized relative to 18S rRNA, the significant differences in the transcript concentration between cells treated with DEX and cells treated with the ethanol vehicle are indicated by ** ($p < 0.01$) or *** ($p < 0.001$).

the measured concentration of the RNA standards. We measured the concentration of each standard in three separate Ribogreen assays with triplicates in each assay. The overall precision expressed as 95% confidence intervals was ±11% of the means.

Real-time QRT-PCR assays for measuring GRβ transcripts might potentially amplify a 3244 bp amplicon from GRα transcripts with a 3′ UTR that includes exon 9β (Figure 1A). The assays for exon 1A1 might amplify segments of exon 1A2- and exon 1A3-containing transcripts, as they contain both up- and downstream primer sites. Likewise, the assay for exon 1A2 might amplify a segment of exon 1A3-containing transcripts. Furthermore, since all exon 1-containing transcripts share downstream primer binding sites in exon 2, nonspecific binding of an upstream primer to a "wrong" exon 1 sequence might generate an amplification product. The same possibility exists for the GRα- and GRβ-encoding transcripts. We tested the specificity of the real-time QRT-PCR assays by running the assays with 10^{-11} M of potentially interfering transcripts. We found no, or exceedingly low, interference (data not shown).

We further analyzed amplification products of samples from CEM-C7 cells, IM-9 cells, and appropriate standards generated in the real-time QRT-PCR reactions by agarose gel electrophoresis. For all assays, CEM-C7 and IM-9 RNA generated amplification products of the same size as those generated from standards (data not shown). In the assay for exon 1A2-containing transcripts, an additional amplification product was consistently generated, but cloning and sequencing revealed that it was a nonspecific amplification product. In the assay for exon 1A3-containing transcripts, we occasionally observed an additional faint amplification product. Additional studies indicated that these products did not markedly interfere with the quantification of the specific transcripts being measured (data not shown).

Total GR Transcripts Are Upregulated in CEM-C7 Cells and Downregulated in IM-9 Cells. The absolute concentrations of total GR transcripts, represented by transcripts with the exon 5−exon 6 splice site, after treatment with DEX and the ethanol vehicle are shown in Figure 2. The transcript concentrations were normalized to grams of total RNA determined either by an OD 260 nm measurement or by real-time QRT-PCR assay of 18S rRNA relative to control total human RNA. Figure 2 shows that the two types of normalization give similar results. In the remainder of the results section, only normalizations based on 18S rRNA are presented. It is further apparent that DEX-treatment increases the transcript concentration in CEM-C7 cells, and decreases the transcript concentration in IM-9 cells. The upregulation in CEM-C7 cells becomes statistically significant after 1 h of DEX treatment, while the downregulation in IM-9 cells becomes statistically significant after 2 h of DEX treatment. The normalizers, OD 260 nm and 18S rRNA, are practically unaffected by DEX within the first 4 h of treatment, in which most of the regulation of the GR transcripts takes place. Even after 24 h of DEX treatment, the values of the normalizers are changed by less than 30% of those of the ethanol vehicle controls (data not shown).

Autoregulation of GRα- and GRβ-Encoding Transcripts. The autoregulation of GRα, GRβ, and total GR transcripts is compared in Figure 3A. Like total GR transcripts, both GRα- and GRβ-encoding transcripts are significantly upregulated in CEM-C7 cells and significantly downregulated in IM-9 cells after at least 2 h of DEX treatment. The GRα- and GRβ-encoding transcripts have remarkably similar time course profiles. There is no significant difference in the fold upregulation between total GR transcripts, GRα-encoding, and GRβ-encoding transcripts. We conclude that there is a coordinate glucocorticoid regulation of GRα and GRβ at the transcript level.

The absolute concentrations of GRα- and GRβ-encoding transcripts are shown in Figure 3B. For both cell lines, whether treated with DEX or the ethanol vehicle, the concentrations of GRα-encoding transcripts are more than

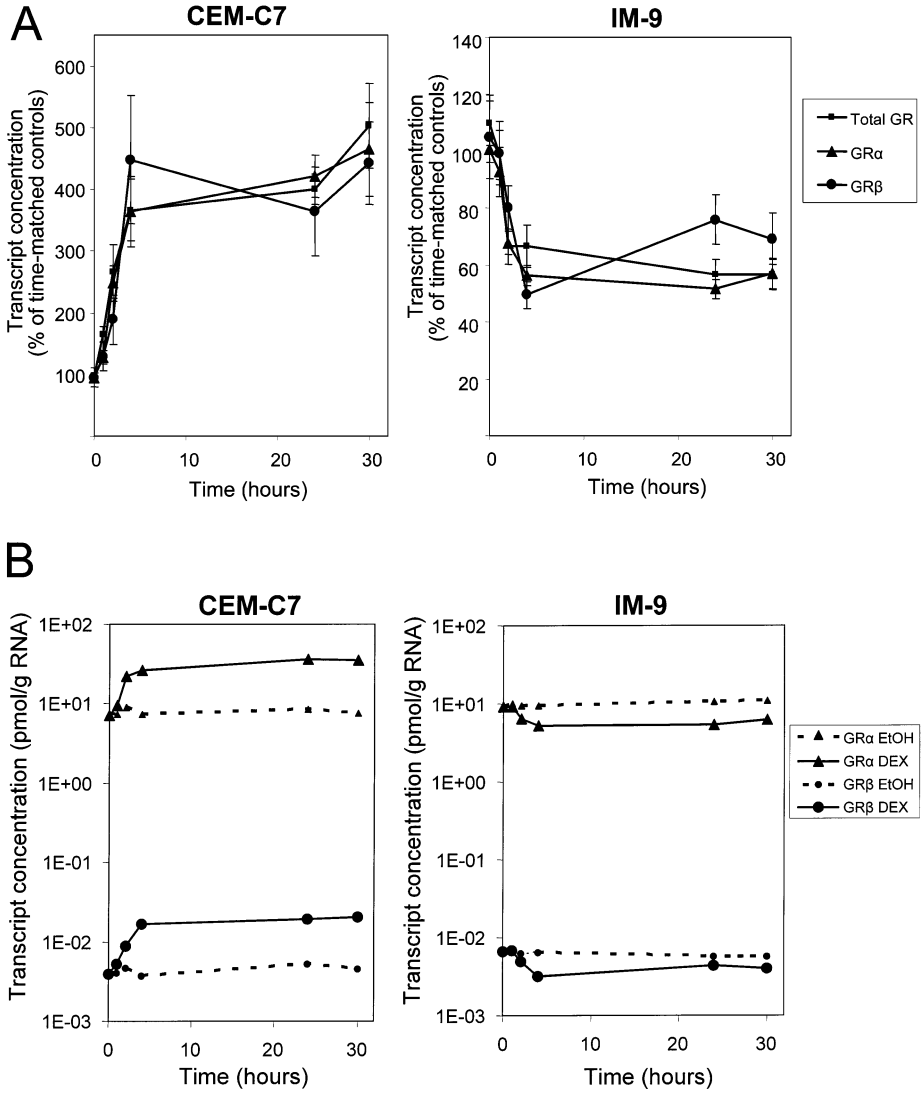

FIGURE 3: GRα- and GRβ-encoding transcripts are coordinately regulated by dexamethasone. (A) Dexamethasone regulation of total GR transcripts, GRα-, and GRβ-encoding transcripts in CEM-C7 and IM-9 cells. The transcript concentrations of cells treated with dexamethasone are expressed in % of transcript concentrations of cells treated with the ethanol vehicle. The error bars represent the standard errors of the transcript concentration ratios. The DEX-induced up-regulation of transcripts in CEM-C7 cells becomes significant after 1 h for GRα-encoding transcripts and after 2 h for GRβ-encoding transcripts. The DEX-induced downregulation of transcripts in IM-9 cells becomes significant after 2 h for both GRα- and GRβ-encoding transcripts. (B) The absolute concentrations of GRα- and GRβ-encoding transcripts in CEM-C7 and IM-9 cells treated with DEX or the ethanol vehicle.

1000-fold higher than the concentrations of GRβ-encoding transcripts. A similar large difference in concentrations between the two transcripts has been observed for glucocorticoid-untreated HeLa cells and Jurkat T-lymphoblast cells (data not shown). To assess how accurately the GRβ transcript can be quantified in the presence of a high concentration of GRα transcripts, we performed real-time QRT-PCR of dilution series of the GRβ RNA standard diluted either in the normal diluent of 40 ng/μL yeast RNA or in the same diluent spiked with 10^{-11} M GRα RNA standard. The presence of 10^{-11} M GRα RNA standard did not markedly affect amplification curves of GRβ RNA standards at concentrations from 10^{-12} to 10^{-16} M (data not shown). Thus, not even a 100 000-fold excess of GRα-encoding transcripts interferes with the measurement of GRβ-encoding transcripts.

Auto-Regulation of GR Transcripts Containing Different Forms of Exon 1. Transcripts containing exons 1A3, 1B, or 1C could be measured for samples diluted to 40 ng of RNA/μL. The transcripts containing exons 1A1 or 1A2 could not be detected at this RNA concentration, so undiluted RNA samples with RNA concentrations ranging from 0.17 to 1.9 μg/μL were used for quantification. Even so, the concentrations of these transcripts were close to the assay sensitivity and at a range (<0.1 pmol/g of RNA) where the assays become less precise. The 1A1- and 1A2-containing transcripts are therefore measured with less precision than the other transcripts.

Glucocorticoid regulation of the transcripts with different forms of exon 1 is compared in Figure 4A. All transcripts are significantly upregulated by DEX relative to ethanol-treated controls in CEM-C7 cells and significantly downregulated in IM-9 cells. Transcripts with exons 1A1, 1A2, and 1A3 are significantly more upregulated in CEM-C7 cells than are transcripts containing exon 1B (test probabilities $p = 0.008$, $p = 0.032$, and $p = 0.006$, respectively) or exon 1C ($p = 0.007$, $p = 0.03$, and $p = 0.005$, respectively). Transcripts with exons 1A1, 1A2, and 1A3 are significantly

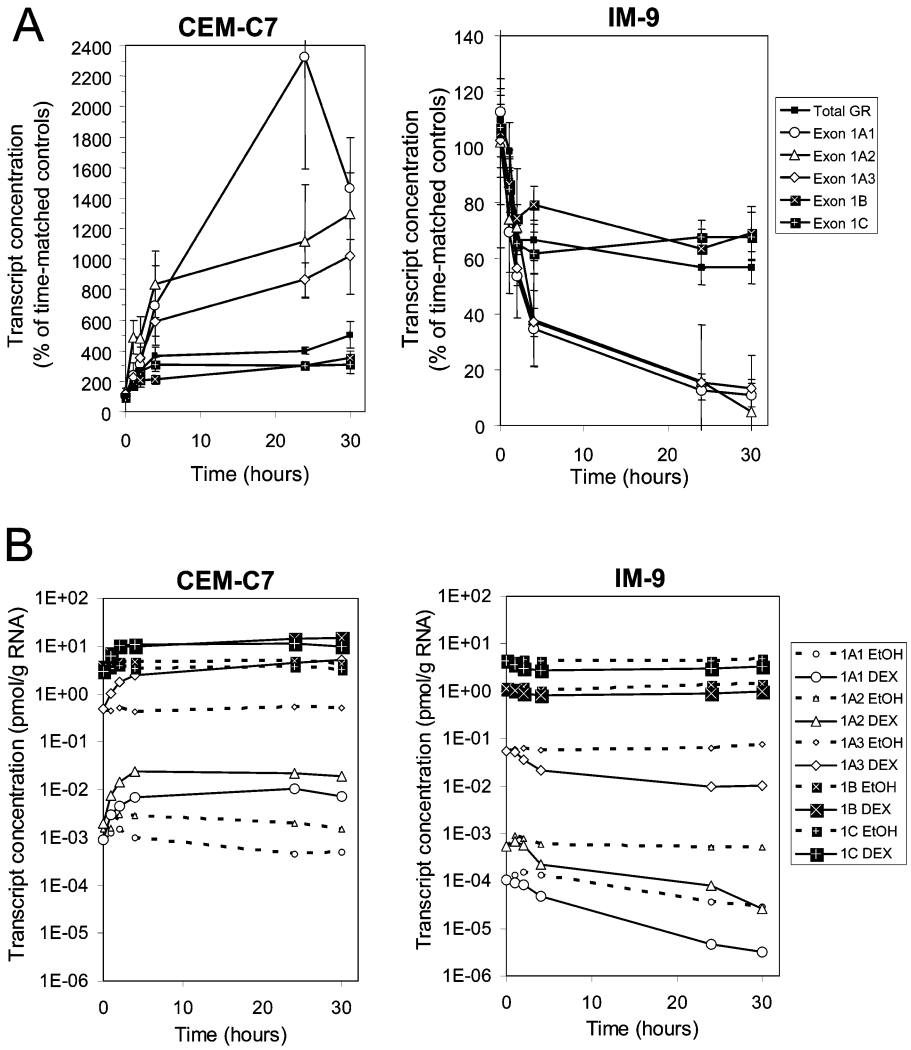

FIGURE 4: Transcripts with exon 1A1, 1A2, or 1A3 are more highly regulated by dexamethasone, but less abundant, than transcripts with exon 1B or exon 1C. (A) Dexamethasone regulation of GR transcripts with different forms of exon 1 spliced to exon 2 in CEM-C7 and IM-9 cells. The transcript concentrations of cells treated with dexamethasone are expressed in % of transcript concentrations of cells treated with the ethanol vehicle. The error bars represent the standard errors of the transcript concentration ratios. The DEX-induced up-regulation of transcripts in CEM-C7 cells becomes significant after 1 h for exons 1A2, 1A3, 1B, and 1C and after 2 h for exon 1A1. The DEX-induced downregulation of transcripts in IM-9 cells becomes significant after 2 h for exons 1A3, 1B, and 1C, after 4 h for exon 1A2, and after 24 h for exon 1A1. (B) The absolute concentrations of GR transcripts with different forms of exon 1 spliced to exon 2 in CEM-C7 and IM-9 cells treated with DEX or the ethanol vehicle.

more downregulated in IM-9 cells than are transcripts containing exon 1B ($p = 0.008$, $p < 0.001$, and $p < 0.001$, respectively) or exon 1C ($p = 0.013$, $p < 0.001$, and $p < 0.001$, respectively). The GRα-encoding and total GR transcripts are upregulated slightly more (ca. 4.5-fold; Figure 3A) at 24 and 30 h in CEM-C7 than the exon 1B- and exon 1C-containing transcripts (3−3.3-fold; Figure 4A).

The absolute concentrations of transcripts with different forms of exon 1 are shown in Figure 4B. Exon 1B- and exon 1C-containing transcripts are more abundant than exon 1A-containing transcripts. The highest relative abundance of an exon 1A-containing transcript occurs for the exon 1A3-containing transcript in CEM-C7 cells after DEX treatment for 24−30 h, where the concentration reaches 30−50% of the levels of exon 1B- and exon 1C-containing transcripts.

Estimation of the Number of GR Transcripts per Cell. The RNA content of asynchronously growing cells of CEM-C7 and IM-9 cells was found to be 7.0×10^{-12} and 2.5×10^{-11} g of RNA/cell, respectively. These values were used to estimate the number of transcript molecules per cell. The values for the averages of the 24 and 30 h samples are shown in Table 2.

Table 2: Estimation of the Number of GR Transcript Molecules Per Cell

	CEM-C7		IM-9	
splice site	EtOH	DEX	EtOH	DEX
exon 1A1−exon 2	0.002	0.038	0.00051	0.00006
exon 1A2−exon 2	0.007	0.087	0.0079	0.0008
exon 1A3−exon 2	2.2	20.5	1.1	0.2
exon 1B−exon 2	19	62	21	14
exon 1C−exon 2	15	45	71	48
sum of exon 1s−exon 2	36	128	93	62
exon 5−exon 6	20	88	100	57
exon 8−exon 9α	34	150	163	89
exon 8−exon 9β	0.021	0.083	0.088	0.064

The concentrations of GRβ transcripts and transcripts with exon 1A1 and 1A2 are not only low compared to the other GR transcript forms, but there are also less than 0.1 of each of these transcripts per cell. The total concentration of GR transcripts can be estimated by the concentration of tran-

scripts with the exon 5−6 splice site, by the sum of the concentrations of GRα- and GRβ-encoding transcripts, or by the sum of the concentrations of transcripts with the different forms of exon 1. These three estimates differ by up to a factor of 1.8. The differences may reflect assay variability, systematic error associated with the determination of absolute transcript concentrations, and/or the presence of additional transcripts not quantified in the current studies. Such additional transcripts include splicing variants that we have observed in our laboratory, in which exon 1A1 and exon 1A2 become spliced to alternative splice acceptor sites downstream in exon 2, or in which exon 1A3 becomes spliced directly to exon 3 (C.-d. Geng, B. S. Nunez, and W. V. Vedeckis, unpublished). However, preliminary real-time QRT-PCR measurements of these splicing variants indicate that they are present at a very low concentration in IM-9 and CEM-C7 cells (data not shown). To eliminate variation associated with having different RNA standards for quantifying different isoforms, we made an exon 5−9α RNA standard stretching from exon 5 to exon 9α and containing both regions amplified in the assays specific for the exon 5 − exon 6 splice site and the exon 8−exon 9α splice site. When transcripts containing the exon 5−6 and transcripts containing the exon 8−9α splice sites were quantified in CEM-C7 and IM-9 cells using this inclusive, long, double standard, we did not consistently measure higher concentrations of GRα transcripts compared to transcripts with the exon 5−6 splice site (data not shown). This illustrates that errors associated with having different standards for each assay are at least partially responsible for the 1.8-fold difference in total GR transcript estimates.

Modeling of Glucocorticoid Auto-Regulation of GR. The autoregulation of GR is modeled in the following way:

Let [RNA](t) and [P](t) describe the concentrations of GR RNA and protein at time t, let $S_{RNA}(t)$ and $S_P(t)$ describe the synthesis rates of GR RNA and protein at time t, and let $T_{1/2RNA}$ and $T_{1/2P}$ describe the half-lives of GR RNA and protein. Suffixes Eq, DEX, EtOH, and max denote the entities at equilibrium, in the presence of DEX, in the presence of EtOH, and at maximum, respectively.

The change in concentration for GR RNA and protein over time can then be described by a set of differential equations:

$$\text{For GR RNA, d [RNA]/dt} = \text{synthesis rate} - \text{degradation rate} = S_{RNA}(t) - [\text{RNA}](t)\ln(2)/T_{1/2RNA} \quad (1)$$

$$\text{For GR protein, d [P]/dt} = \text{synthesis rate} - \text{degradation rate} = S_{P}(t) - [\text{P}](t)\ln(2)/T_{1/2P} \quad (2)$$

The synthesis rate of GR protein is likely to be proportional to the GR mRNA concentration:

$$S_P(t) = k_{tr}[\text{RNA}](t) \quad (3)$$

with k_{tr} representing the translational efficiency of the RNA into protein.

At equilibrium conditions, defined by d [RNA]/dt = 0 and d [P]/dt = 0,

$$S_{RNA}^{Eq} = [\text{RNA}]^{Eq}\ln(2)/T_{1/2RNA} \quad (4)$$

$$S_P^{Eq} = [\text{P}]^{Eq}\ln(2)/T_{1/2P} \quad (5)$$

If the concentrations of both GR protein and RNA are at equilibrium, then from eq 3:

$$S_P^{Eq} = k_{tr}[\text{RNA}]^{Eq} \quad (6)$$

Comparing cells at equilibrium with either a saturating concentration of DEX or with the ethanol vehicle, the following expression (isolating $[P]^{Eq}$ from eq 5) and inserting S_P^{Eq} from eq 6) can be obtained:

$$[\text{P}]^{DEX,Eq}/[\text{P}]^{EtOH,Eq} = ([\text{RNA}]^{DEX,Eq}/[\text{RNA}]^{EtOH,Eq})\text{x}(T_{1/2P}^{DEX}/T_{1/2P}^{EtOH})\text{x}(k_{tr}^{DEX}/k_{tr}^{EtOH}) \quad (7)$$

which describes how the dexamethasone-induced regulation of GR at the protein level depends on the regulation at the RNA level, the ratio of GR protein half-lives in the presence of DEX and EtOH vehicle, and the ratio of translational efficiencies in the presence of DEX and EtOH vehicle.

There have, to our knowledge, not been any reports of glucocorticoids affecting the translation of GR transcripts, so we can assume that $k_{tr}^{DEX} = k_{tr}^{EtOH}$ at least for each transcript form. It is possible that the translational efficiencies vary among different transcripts. A glucocorticoid-induced change in the composition of the GR transcript population could then affect the overall translational efficiency. There is a coordinate glucocorticoid regulation of GRα- and GRβ-encoding transcripts, and transcripts with exon 1B and exon 1C also display similar glucocorticoid regulation. Thus, the only change in the transcript population that seems to be able to affect overall translational efficiency is the change in exon 1A-containing transcripts relative to exon 1B- and exon 1C-containing transcripts.

Experimental evidence for glucocorticoid-induced changes in the synthesis rate of GR mRNA comes from nuclear run-on experiments, whereas the GR mRNA stability is unaffected by glucocorticoids in IM-9 and CEM cells (*20*−*22*). For the models, we have thus presumed that transcript stabilities are unaffected by glucocorticoids. However, it is not known how S_{RNA}^{DEX} depends on the concentration of GR protein. It is possible that a sufficient level of GR protein to saturate the transcriptional response to glucocorticoids exists throughout the glucocorticoid treatment. In this case, S_{RNA}^{DEX} is a constant. A model for GR RNA downregulation in a situation where S_{RNA}^{DEX} is not constant, adapted from ref *23* is as follows:

$$S_{RNA}^{DEX} = S_{RNA}^{EtOH,Eq}(1 - [\text{P}]/([\text{P}] + IC_{50})) \quad (8)$$

where IC_{50} is the protein concentration at which $S_{RNA}^{DEX} = S_{RNA}^{EtOH,Eq}/2$.

A similar model for upregulation is

$$S_{RNA}^{DEX} = S_{RNA}^{EtOH,Eq} + (S_{RNA}^{DEX,max} - S_{RNA}^{EtOH,Eq})[\text{P}]/([\text{P}] + SC_{50}) \quad (9)$$

where SC_{50} is the protein concentration at which $S_{RNA} = (S_{RNA}^{EtOH,Eq} + S_{RNA}^{DEX,max})/2$ and $S_{RNA}^{DEX,max}$ is the transcription rate when the transcriptional response is saturated with GR.

If S_{RNA}^{DEX} is constant in the presence of glucocorticoid, an exact solution for the transcript concentration over time

is (cf. ref *24*)

$$[\mathrm{RNA}]^{\mathrm{DEX}}(t) = [\mathrm{RNA}]^{\mathrm{DEX,Eq}} - ([\mathrm{RNA}]^{\mathrm{DEX,Eq}} - [\mathrm{RNA}](0))\exp(-(\ln(2)/T_{1/2\mathrm{RNA}})t) \quad (10)$$

If further $k_{\mathrm{tr}}^{\mathrm{DEX}}$ is constant in the presence of glucocorticoid, an exact solution for the protein concentration over time is (cf. ref *24*)

$$[\mathrm{P}]^{\mathrm{DEX}}(t) = [\mathrm{P}](0)\exp(-(\ln(2)/T_{1/2\mathrm{P}}{}^{\mathrm{DEX}})t) + k_{\mathrm{tr}}[\mathrm{RNA}]^{\mathrm{DEX,Eq}}T_{1/2\mathrm{P}}{}^{\mathrm{DEX}}(1 - \exp(-(\ln(2)/T_{1/2\mathrm{P}}{}^{\mathrm{DEX}})t))/\ln(2) + k_{\mathrm{tr}}([\mathrm{RNA}](0) - [\mathrm{RNA}]^{\mathrm{DEX,Eq}})(\exp(-(\ln(2)/T_{1/2\mathrm{RNA}})t) - \exp(-(\ln(2)/T_{1/2\mathrm{P}}{}^{\mathrm{DEX}})t))/(\ln(2)/T_{1/2\mathrm{P}}{}^{\mathrm{DEX}} - \ln(2)/T_{1/2\mathrm{RNA}}) \quad (11)$$

Assuming $k_{\mathrm{tr}}^{\mathrm{DEX}} = k_{\mathrm{tr}}^{\mathrm{EtOH}}$, $k_{\mathrm{tr}}^{\mathrm{DEX}}$ can be calculated from eqs 5 and 6 as

$$k_{\mathrm{tr}}{}^{\mathrm{DEX}} = \ln(2)[\mathrm{P}]^{\mathrm{EtOH,Eq}}/(T_{1/2\mathrm{P}}{}^{\mathrm{EtOH}}[\mathrm{RNA}]^{\mathrm{EtOH,Eq}}) \quad (12)$$

Modeling the regulation of GR RNA and protein over time for other situations is done numerically by iterative calculation of $[\mathrm{RNA}](t + \Delta t)$ and $[\mathrm{P}](t + \Delta t)$ for small Δt, e.g., $\Delta t = 0.05$ h.

Transcript concentrations measured by real-time QRT-PCR provide values for $[\mathrm{RNA}](t)$. In asynchronously growing cells, in the absence of glucocorticoids (with the EtOH vehicle), we assume that the system is at equilibrium with regard to GR RNA and protein. The relatively flat time-course profiles of transcript concentrations for cells treated with EtOH (Figures 3B and 4B) attest to the validity of this assumption. The slight peak seen at 1−2 h is likely due the disturbance to the cell culture system associated with the introduction of a treatment. A slight increase (or decrease) in transcript levels over time might be associated with effects related to increased cell density or depletion of media components. These slight changes are factored out when considering fold increase or decrease in transcript concentrations for DEX-treated cells relative to EtOH-treated cells.

We fitted the models to observed transcript concentrations by optimizing the unknown parameters by the least squares deviation method. For the model assuming S_{RNA} is constant, the unknown parameters are $T_{1/2\mathrm{RNA}}$ and $[\mathrm{RNA}]^{\mathrm{DEX,Eq}}$. For the models allowing S_{RNA} to vary with the [P], the unknown parameters are $T_{1/2\mathrm{RNA}}$ and IC_{50} for downregulation, and they are $T_{1/2\mathrm{RNA}}$, SC_{50}, and $S_{\mathrm{RNA}}{}^{\mathrm{max}}$ for upregulation. Figure 5A shows that both models for either S_{RNA} being constant or for S_{RNA} varying with the [P] can be fitted to the observed data for GRα-encoding transcripts very well. Interestingly, for CEM-C7 cells the two models are identical, as the least squares deviation estimate of SC_{50} was 0, indicating that the RNA synthesis rate is maximal throughout the glucocorticoid regulation. The $T_{1/2\mathrm{RNA}}$ estimate was 2.5 h and the $[\mathrm{RNA}]^{\mathrm{DEX,Eq}}$ estimate was 4.5-fold higher than the initial concentration. For total GR transcripts in CEM-C7 cells, the two models were similar but not identical (models not shown). Assuming that $S_{\mathrm{RNA}}{}^{\mathrm{DEX}}$ is constant, the $T_{1/2\mathrm{RNA}}$ estimate was 2.2 h and the $[\mathrm{RNA}]^{\mathrm{DEX,Eq}}$ estimate was 4.5-fold higher than the initial concentration. Assuming that $S_{\mathrm{RNA}}{}^{\mathrm{DEX}}$ is dependent on [P], the estimate of SC_{50} was 28% of the initial RNA concentration, the $T_{1/2\mathrm{RNA}}$ estimate was 2.0 h, and the $[\mathrm{RNA}]^{\mathrm{DEX,Eq}}$ estimate was found to be 4.7-fold higher than the initial concentration. The models indicate that the transcripts are close to their equilibrium concentration (4.5−4.7-fold higher than the initial concentration) after 24 and 30 h of DEX treatment. For the downregulation model

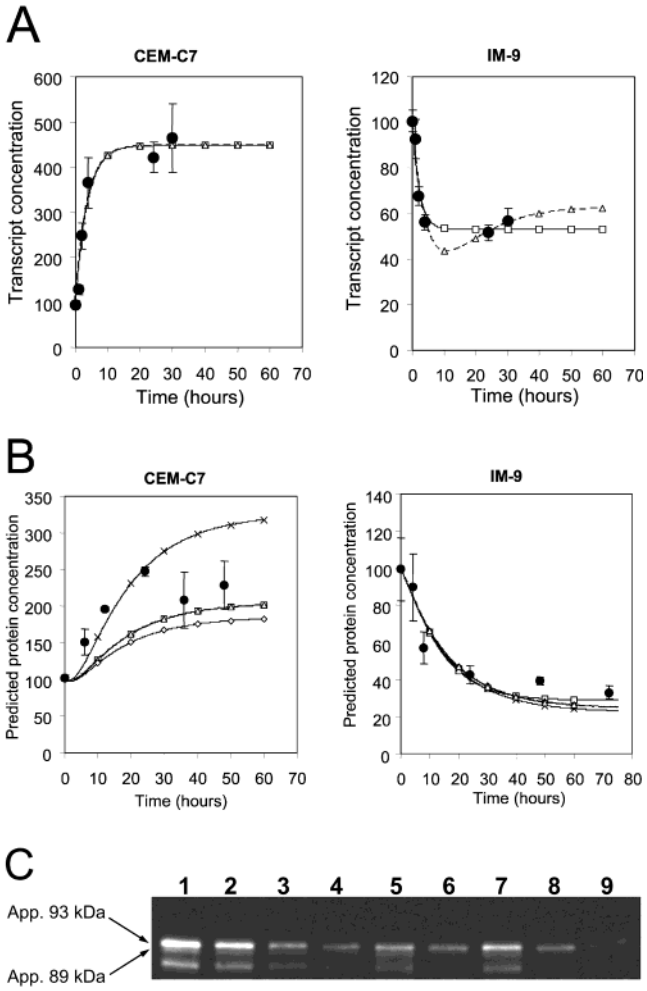

FIGURE 5: Glucocorticoid autoregulation of GRα mRNA and GRα protein. (A) Models of dexamethasone-induced regulation of GRα mRNA fitted to the observed regulation of GRα-encoding transcripts (filled circles) for CEM-C7 and IM-9 cells. Error bars represent standard errors. The initial transcript concentration is set to 100. Open squares indicate the model in which $S_{\mathrm{RNA}}{}^{\mathrm{DEX}}$ is constant and open triangles indicate the models in which $S_{\mathrm{RNA}}{}^{\mathrm{DEX}}$ depends on the protein concentration. (B) Models predicting the dexamethasone-induced regulation of GRα protein and the observed regulation of GR protein (filled circles) for CEM-C7 and IM-9 cells. Error bars represent SEM. The initial protein concentration is set to 100. Open triangles indicate the model in which $S_{\mathrm{RNA}}{}^{\mathrm{DEX}}$ is constant and open squares indicate the models in which $S_{\mathrm{RNA}}{}^{\mathrm{DEX}}$ depends on the protein concentration. Open diamonds and crosses indicate the models in which the translational efficiency of exon 1A-containing transcripts is 10-fold lower and 10-fold higher, respectively, than the combined efficiency for exon 1B- and exon 1C-containing transcripts. (C) Example of quantitative Western for GR protein. For this gel, lanes 1−4 and lane 9 contained 30, 20, 12, 6, and 3 μg protein aliquots of the relative standard, respectively, which in this case was a CEM-C7 sample treated for 24 h with DEX. Remaining lanes contained 20 μg protein aliquots of CEM-C7 cells treated for 48 h with DEX (lane 5), 48 h with EtOH vehicle (lane 6), 36 h with DEX (lane 7), and 36 h with EtOH vehicle (lane 8).

with S_{RNA} being constant, the GRα-encoding transcripts in IM-9 cells are likewise close to their equilibrium concentration (53% of initial concentration) after 24 and 30 h of DEX treatment. A slightly higher equilibrium concentration (63% of initial concentration), which has not been reached yet at the 30 h time point, is predicted from the model with S_{RNA} dependent on [P]. The half-life estimate was 1.5 h for the model assuming that $S_{RNA}{}^{DEX}$ is constant, and it was 2.6 h for the model assuming that $S_{RNA}{}^{DEX}$ is dependent on [P]. Similar values for total GR transcripts were 1.8 and 3.1 h (data not shown).

The dexamethasone-induced regulation of GRα protein is modeled based on reported protein half-lives of ca. 24 h in the absence of glucocorticoids and 11 h in the presence of glucocorticoids (*17*, *20*). Figure 5B shows the observed regulation of GR protein as determined by quantitative Western blots and the predicted regulation of GRα protein according to the models. An example of one of our quantitative Western blots is shown in Figure 5C. The combined intensity of the ca. 89 kDa band and the ca. 93 kDa band was measured as full-length GR. For IM-9 cells, the protein concentration is predicted to decrease to equilibrium concentrations of 24 and 29% of initial concentrations when $S_{RNA}{}^{DEX}$ is constant and $S_{RNA}{}^{DEX}$ is dependent on [P], respectively, and all transcripts have the same translational efficiency. The effect of the combined k_{tr} of the exon 1A-containing transcripts being 10-fold higher or lower than the combined k_{tr} of exon 1B and 1C, under the assumption of $S_{RNA}{}^{DEX}$ being constant, is also illustrated in Figure 5B. For these curves, individual models for each of the transcripts with different forms of exon 1 were fitted to the observed data, and the modeled GRα-encoding transcripts were partitioned according to the relative concentrations of transcripts with exon 1A1, 1A2, 1A3, 1B, and 1C. The effect in IM-9 is negligible due to the relatively low concentration of exon 1A-containing transcripts. It can be noted that the models describe the observed downregulation fairly well with GR protein being downregulated to a higher degree than the GRα-encoding transcripts.

The GRα protein concentration in CEM-C7 cells is predicted to increase 2.1-fold when the RNA concentration increases 4.5-fold and $k_{tr}{}^{DEX} = k_{tr}{}^{EtOH}$ is constant. There is a marked effect of k_{tr} of exon 1A-containing transcripts being 10-fold higher or lower than the combined k_{tr} of exon 1B and 1C, with the protein concentration predicted to increase 3.3- and 1.8-fold, respectively, rather than 2.1-fold. Within the first 24 h of dexamethasone treatment, the observed GR protein concentration (solid circles) increases more than predicted from the model assuming $k_{tr}{}^{DEX} = k_{tr}{}^{EtOH}$ is constant. As indicated in the figure, a possible explanation is that the exon 1A-containing transcripts could be more efficiently translated than transcripts containing exon 1B or exon 1C.

The observed GR protein concentration does not seem to increase after 24 h of dexamethasone treatment. This may be due to glucocorticoid-induced apoptosis, which starts occurring after 24 h and is pronounced after 48 h, at which time more than 40% of the cells typically are dead or dying (data not shown). The apoptosis, thus, probably hinders the GR protein concentration in the cell population from reaching a new equilibrium in the presence of glucocorticoid.

DISCUSSION

The purpose of these studies was to quantify and study the autoregulation of several GR transcript isoforms to determine the most important transcripts. Total GR transcripts, represented by transcripts with the exon 5−exon 6 splice site, are significantly upregulated in CEM-C7 cells and significantly downregulated in IM-9 cells. Glucocorticoid-induced downregulation of GR mRNA in IM-9 cells has previously been shown by Northern blot analysis (*22*, *25*). Likewise, Northern blotting and, more recently, oligonucleotide array analysis have been used to show glucocorticoid-induced upregulation of GR mRNA in CEM-C7 cells or their variants (*4*, *25*−*27*).

Transcripts encoding GRα and GRβ (transcripts with exon 8−exon 9α and exon 8−exon 9β splices, respectively) are coordinately upregulated by DEX in CEM-C7 cells and coordinately downregulated by DEX in IM-9 cells. Coordinate regulation of transcripts encoding GRα and GRβ has previously been suggested using a human bronchial epithelial cell line and rat gastrocnemius muscle (*28*, *29*). In these studies, a 5-kb transcript detected by Northern hybridization was interpreted as GRβ transcript, but this was not confirmed at the nucleotide sequence level. In human respiratory epithelial cells, GRα and GRβ transcripts, quantified by RT-PCR, were both downregulated by DEX-treatment, at least after 6 h of treatment (*30*). Our real-time QRT-PCR assays that are specific for each transcript form unequivocally show the coordinate regulation of GRα- and GRβ-encoding transcripts. This implies that the splicing process joining exon 8 to either exon 9α or exon 9β is not differentially affected by glucocorticoid treatment.

All transcripts with different forms of exon 1 are upregulated by DEX in CEM-C7 cells and downregulated in IM-9 cells. This contrasts with earlier findings (*13*), where transcripts with exon 1B and exon 1C were not significantly downregulated in IM-9, and transcripts with exon 1C were not significantly upregulated in CEM-C7. This discrepancy is likely due to the less accurate and less sensitive competimer RT-PCR assay used in the previous study. On the other hand, the earlier finding of exon 1A-containing transcripts being more highly regulated by DEX than exon 1B- and exon 1C-containing transcripts has been substantiated by our new real-time QRT-PCR data.

The concentrations of GRα-encoding transcripts are more than 1000-fold higher than the concentrations of GRβ-encoding transcripts. The rarity of GRβ transcripts in human cells and tissues relative to GRα transcripts has also been noticed by other groups (*11*, *31*−*33*).

Of transcripts containing different forms of exon 1, we found exon 1B- and exon 1C-containing transcripts to be the most abundant. The exon 1A1- and exon 1A2-containing transcripts were even less abundant than GRβ-encoding transcripts. The greatest abundance of an exon 1A form was for exon 1A3, which in the T-cell line CEM-C7 reached 30−50% of the concentrations of the exon 1B and exon 1C forms. Interestingly, of the 11 different exon 1 forms of rat GR transcripts, the 1A3 homologue (rat exon 1_1) reaches its highest level in thymus, with 25−27% of the total transcript concentration (*34*).

We fitted GR autoregulation models to the observed regulation of GRα-encoding transcripts and total GR tran-

scripts. Dependent on the model and the cell type, the half-life estimates were between 1.5 and 3.1 h. These estimates are in the range of half-lives of 1−6 h previously reported for endogenous GR mRNA (*17*, *20*, *22*, *25*, *35*). The models further predict a regulation of GRα protein that for IM-9 cells is in fair accordance with the observed downregulation of GR protein. In CEM-C7 cells, the GR protein increased more within the first 24 h of dexamethasone treatment than predicted under the assumption that the translational efficiency is the same in the presence and absence of glucocorticoids. This higher observed upregulation cannot be explained by the presence of GR*β* protein that also is predicted to increase less than 2-fold in 24 h (model not shown). In contrast, the observed upregulation can be explained by more efficient translation of exon 1A-containing transcripts than exon 1B- and exon 1C-containing transcripts. Another possibility is a general higher translational efficiency of all GR transcripts in the presence than in the absence of glucocorticoids. The observed upregulation could also be explained if GR protein stability (ca. 24 h) is unaffected by glucocorticoids, but this scenario is in discord with previous stability studies (*17, 20*).

The biological role of GR*β* is controversial. GR*β* protein has been described as a dominant negative inhibitor of GRα present when present at higher concentration than GRα (*9*−*11*), while others have failed to observe the dominant negative effect of GR*β* (*36*−*38*). A high GR*β* RNA or protein level has been clinically associated with glucocorticoid insensitivity (*39*−*45*), but these studies have not generally included precise simultaneous measurement of GRα and GR*β* protein concentrations (*12*). For CEM-C7 and IM-9 cells, where GR*β* transcripts are coordinately regulated with, and very rare compared to, GRα transcripts, it is difficult to envision how the GR*β* protein concentration could become in excess and interfere with glucocorticoid responsiveness. In mammalian cells, the translational elongation rate is ca. 5 amino acids per second per ribosome (*46*−*51*). Since a ribosome physically covers at least 30 amino acids (10 codons) of an mRNA (*52*, *53*), the protein synthesis rate cannot exceed 5/10 = 1 protein molecule per 2 s per mRNA. Assuming that GR*β*-encoding transcripts are translated at this maximal rate, then the equilibrium concentrations of GR*β* protein in the absence of glucocorticoids would be at most 2580 molecules per CEM-C7 cell and 10 920 molecules per IM-9 cell. The content of wild-type GRα protein in CEM-C7 cells, based on ligand binding assays, is ca. 17 000 (range 14 000−20 000) receptors per cell (*54*−*57*). For IM-9 cells, the GRα protein content is ca. 57 000 (range 30 000−100 000) receptors per cell (*58*−*60*). The ratio of GR*β* protein/GRα protein is, thus, at most 0.18 and 0.36 for CEM-C7 and IM-9 cells, respectively, in the absence of glucocorticoids. GRα protein stability decreases, while GR*β* protein stability is unaffected when cells are challenged with glucocorticoids. Still, using eq 7, the ratio of GR*β* protein/GRα protein should only increase, at most, 2.2-fold when the transcripts are coordinately regulated and assuming that the ratio between the translational efficiencies in the presence and absence of DEX is the same for GRα and GR*β*. Thus, the estimated ratios of GR*β*/GRα protein in CEM-C7 and IM-9 cells in the presence of glucocorticoids are, at most, 0.41 and 0.80, respectively, which is insufficient for a dominant negative effect of GR*β*. It should also be noted that, in the quantitative Western blots, we never saw the ca. 89 kDa band become more intense than the band at ca. 93 kDa. We, thus, conclude that GR*β* has no role in glucocorticoid responsiveness of CEM-C7 and IM-9 cells during autoregulation. Along the same lines of reasoning, we can calculate that exon 1A1- and exon 1A2-containing transcripts, even if translated at 1 protein molecule per 2 s per mRNA, must each contribute to less than 9% and less than 2% of the total pool of GRα protein in CEM-C7 and IM-9 cells, respectively.

For derivatives of the CEM-C7 cell line in which GRα expression was regulated by tetracycline, it has previously been shown that upregulation of GR protein by glucocorticoids is necessary for inducing an apoptotic response, with 48 000 receptors per cell being sufficient (*6*), corresponding to a fold upregulation of between 2.4 and 3.4. We observed that GRα and total GR transcripts increased ca. 4.5-fold by DEX-treatment, while exon 1B- and exon 1C-containing transcripts increased 3−3.3-fold. This modest difference may be of great importance. The observed upregulation of exon 1B- and exon 1C-containing transcripts would increase their associated protein levels by only 38−50%, if they are translated with the same rate in the presence and absence of DEX. We observed a 150% GR protein increase within 24 h. The contribution of highly induced transcripts such as the exon 1A3-containing transcript to the total population of GR transcripts could thus be crucial by allowing the GR protein level to exceed the threshold required for a glucocorticoid-induced apoptotic response. All in all, our studies strongly suggest that GRα-encoding transcripts containing exons 1A3, 1B, and 1C are the most relevant transcripts for glucocorticoid mediated apoptosis in T-lymphoblasts.

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