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Association of the Glucocorticoid Receptor Alternatively-Spliced Transcript 1A With the Presence of the High Molecular Weight Membrane Glucocorticoid Receptor in Mouse Lymphoma Cells

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Abstract Using the combination of a cDNA library prepared from membrane glucocorticoid (mGR)-enriched S-49 cells and a mouse leukocyte genomic library, we have cloned a 7.3 kb full-length glucocorticoid receptor 1A cDNA. Primer extension, 5′RACE, and long distance PCR identified the transcription start site as being located at 1026 bp from the ATG codon. The first 1,013 nucleotides (nts) of the full length sequence constitute 5′ UTR sequence (exon 1), the next 2349 bp, the coding region, and the last 3,907 bp, the 3′UTR. The entire 5′UTR sequence is unique to transcript 1A. The 3′UTR sequence is ~88.5 % conserved with the rat 3′UTR. Western blot analysis compared the molecular weight of in vitro translation products from the cloned 1A cDNA with partially purified cellular mGR. Both preparations contained the novel 150 KD and the 94 KD classical GR peptides, suggesting that transcript 1A encodes both receptor forms. Transfection of mGR-less and glucocorticoid lysis-resistant AtT-20 and HL-60 cells with full-length GR 1A cDNA imparted both mGR expression and glucocorticoid lysis-sensitivity to these cells. J. Cell. Biochem. 74:430–446, 1999. © 1999 Wiley-Liss, Inc.

Key words: membrane glucocorticoid receptor; GR 1A; apoptosis; leukemia

Glucocorticoids (GCs) were one of the earliest recognized drug groups for the treatment of leukemias and lymphomas [Csoka et al., 1997; reviewed in Stevens and Stevens, 1985] and are still widely in use. In lymphoproliferative diseases, the mechanism by which the hormones impart their therapeutic effect is not well understood. However, the involvement of glucocorticoid receptors (GRs) in mediating the hormonal signaling is well accepted.

Currently, two forms of GRs are known: the intracellular (cytoplasmic/nuclear) GR (iGR), and plasma membrane-associated GR (mGR) [Gametchu, 1987; Gametchu et al., 1991a,b, 1993; Sackey et al., 1997]. The iGR was first described more than three decades ago [reviewed in Stevens and Stevens, 1985] and has been extensively characterized as a transcription factor belonging to the nuclear receptor superfamily. The mGR, on the other hand, has not been well studied, although first reported in the early 1970s, [reviewed in Szego and Pietras, 1981]. Our work has suggested that mGR is a modified form of iGR [Gametchu, 1987; Gametchu et al., 1991a, 1995], although this is still a major point of contention. The question of whether one or both of the two GR forms is involved in GC signaling in leukemia therapy is not yet settled. Early in vivo and in vitro studies correlated the level of iGR with positive hormonal response [Lippman et al., 1978]. However, as more data accumulated the imperfection of this correlation was noticed, suggesting that the iGR, important as it is to the mediation of hormone action, may not be the only factor which interprets GC signals in the apoptotic pathway. Work conducted in our laboratory with several murine and human lymphoid cell lines, produced evidence that mGR is more strongly

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correlated with GC-evoked lymphocytolysis than the iGR [Gametchu et al., 1994; Sackey et al., 1997]. That is, iGR was usually present, but mGR levels (very variable) more precisely predicted the lymphocytolytic behavior. To investigate this possibility further we have now turned to molecular biological approaches.

The molecular etiology of iGR has been extensively studied including the cloning of its gene and mapping of the different functional domains [Evans and Arriza, 1989; Gustafsson et al., 1987]. However, little is known about the manner in which its expression is controlled [Timothy et al., 1995]. Recent work from several laboratories show that GR expression is diversified by both promoter selection and alternative splicing of its primary transcript at the first exon, which contributes to the 5′ UTR of the message. In the murine system, the GR gene encodes at least five transcripts (named 1A, 1B, 1C, 1D, and 1E) with different 5’ ends that are alternatively spliced onto a single splice acceptor site 13 bp upstream of the ATG codon in exon 2 [Strähle et al., 1992; Chen et al., this issue, 74:418–429]. The effects of this molecular diversity are reflected in different tissue- or cell-specific patterns of expression for these transcripts. Divergence of the sequences in each exon 1 variant promoter region may enable specific patterns of interaction with other transactivation factors, leading to unique expression patterns for each transcript.

In the preceding accompanying paper [Chen et al., this issue, 74:418–429] we reported that the cellular presence of mGR is highly correlated with the expression of one of the alternative GR transcripts (1A). These data suggested that transcript 1A may be responsible for the expression of mGR. In this paper we test this hypothesis by constructing a plasmid expressing full-length GR transcript 1A, including the 5′ and 3′ UTRs. We then expressed this transcript and in an in vitro transcription/translation system to compare the size of purified iGR and mGR with the translation products. Finally, we transfected GR 1A cDNA into mGR-less, hormone-resistant cells and studied the expression and cellular localization of mGR and its role in GC-mediated cell death.

MATERIALS AND METHODS

Cell Selection and Culture

S-49 mouse lymphoma, COS-7 (a derivative of the simian monkey kidney cell line CV-1 transformed by a mutant of SV40 [Gluzman, 1981]), AtT-20 mouse pituitary tumor, and HL-60 human promyelocytic leukemia cell lines were purchased from the ATCC (Rockville, MD). Membrane GR-enriched (mGR^{++}) and mGR-deficient (mGR^{--}) S-49 cells were produced by multiple sequential cell-separation techniques (immunopanning, fluorescent cell sorting, and soft agar cloning) as we have been previously reported [Gametchu, 1987; Gametchu et al., 1993, and accompanying paper].

Isolation of Mouse GR Genomic Clones

A Balb/c mouse leukocyte genomic library (in EMBL3-Sp6/T7) purchased from Clontech (Palo Alto, CA) was screened with a 500 bp transcript 1A-specific probe complementary to nucleotides spanning the -542 to -34 region from the ATG translation initiation codon (accompanying manuscript). DNA fragments of these genomic clones were amplified using Clontech's EMBL3/λGEM1 LD insert screening primers, long distance amplification conditions, and the “hot start” TthStart antibody method (Clontech) [Barnes, 1994]. The PCR reaction products were digested with Bam HI, and then subcloned into the pSPL3 vector (Gibco BRL, Gaithersburg, MD). Direct PCR sequencing of both strands (USB, Cleveland, OH) was followed by analysis with the GCG (Madison, WI) sequence analysis software package.

Isolation of Full Length 1A cDNA by 5′- and 3′-RACE

Using a cDNA library constructed from $S\text{-}49^{++}$ cells, we have previously reported the isolation of the complete coding region and portions of the 5′ and 3′ UTRs of GR transcript 1A (see accompanying article). To obtain the full-length nucleic acid sequence of this transcript, we synthesized cDNAs from $poly(A)^+$ RNAs prepared from $S\text{-}49^{++}$ cells with QIAGEN's (Valencia, CA) Oligotex Direct mRNA kit. Then we used Clontech's Marathon cDNA amplification kit to prepare full-length cDNAs. Briefly, 2 μg of $poly(A)^+$ RNA and 10 pmol of the kit cDNA synthesis primer (a modified oligo dT primer with a 2 nt degenerate sequence at its 3′ end) directed reverse transcription. Then the second strand was synthesized [Gubler and Hoffman, 1983] and the cDNA blunt-ended with T4 DNA polymerase. The kit synthetic adaptor fragments (partially double-stranded adaptors with blocked 3′ends) were then ligated onto both ends of the double-stranded cDNA and the adap-

tor-modified cDNA was used as a template for PCR amplification, using kit-provided primers complementary to the adaptors and GR 1A-specific primers (primer positions are given relative to the translation initiation site ATG) and are shown diagrammatically in Fig. 4 [for 5′ RACE P27 (5′-CAAAGAACCAAATCTGGCTT-CGATGTGACGCAGGCTGC-3′, -295/-258); for 3′RACE P47 (5′-GGCACTCAGCTATCAGAA-GACCACAGAAATTGACT-3′, +3012/+3046)] and long-distance PCR conditions [Barnes, 1994]. A second round of PCR was performed using an anchor primer and internal primers [for 5′RACE P24 (5′-CAUCAUCAUCAUGCA-GATAACACACAGACATCC-3′ at -622/-602); for 3′RACE P76 (5′-CAUCAUCAUCAUCAUGT-GCCATAGAATCTAACACAAGTCTTGTGA-3′ at +3587/+3616)]. The 3′ and 5′RACE products were then cloned into pAMP1 vector per the manufacturer's procedures (Gibco BRL), screened by hybridization with a ^{32}P-labeled 500 bp 1A-specific cDNA probe, and sequenced. More than 20 positive clones were isolated for further PCR and size determination analyses and the clone with the longest insert was sequenced.

Transcription Start Site Determination by Primer Extension Analysis, PCR, and Genomic Clone Sequencing

Use of the "exon trapping" technique (Life Technologies, Gaithersburg, MD) [Gibson et al., 1994] did not isolate a complete exon 1A and this method is probably not appropriate for first exons. Primer extension analyses were performed as described [Jones et al., 1987]. A 26-base synthetic oligonucleotide (P79 primer: 5′-AGAGAGAGAGAGAGAGAGAGACATGG-3′) complementary to the transcribed strand of transcript 1A (located between position -919 and -944 from the ATG translation initiation site) was designed using Primer Premier software (Primer Biosoft International, Palo Alto, CA). The oligonucleotide was labeled at the 5′ end using ^{32}P-γATP (3,000 Ci/mmol, 10 mCi/ml; Amersham Corp., Arlington Heights, IL) and T4 polynucleotide kinase (Promega, Madison, WI), and 10^6 dpm of this preparation was mixed with 50 μg of total RNA from S-49^{++} cells. As a negative control, the same amount of yeast tRNA was used. The preparations were incubated for 1 h at 63°C to anneal the primer to the template; then reverse transcription was performed at 37°C for 1 h in 33 μl reaction mix containing 10 U of avian myeloblastosis virus reverse transcriptase (Gibco BRL), 16 mM Tris-HCl (pH 8.8), 75 mM KCl, 6.9 mM $MgCl_2$, 3.5 mM dithiothreitol, 230 μM each of all four deoxynucleoside triphosphates), and 2.3 μg of actinomycin D. The reaction was terminated by heating at 70°C for 15 min. 2.5 U of RNase H (Gibco BRL) was added to the mixture and incubated at 55°C for 10 min to degrade the RNA template. To serve as a control and size standard, genomic DNA was similarly extended with the same primer (see below). The extended products were electrophoresed on an 8% polyacrylamide sequencing gel in parallel with dideoxy sequencing reactions primed with the same oligonucleotide, dried in the gel, and exposed to X-ray film for analysis.

The correct initiation of the GR 1A mRNA was re-tested as follows. Two different oligonucleotides were synthesized and used in combination with four different downstream primers for PCR amplification (Fig. 3B). The oligo named 5′S (start) Primer corresponded to the nucleotide sequence −1 to +22 nt downstream from the GR 1A transcription start site; the oligo named 5′U (upstream) Primer contained the nucleotide sequence between −16 and +6 nt from the GR 1A transcription start site (Fig. 3B). The other 4 downstream primers prime from various positions near the 5′ end of exon 1A, downstream of the transcription start site at: +571 nt (P17), +425 nt (P24), +330 nt (P31), and +297 nt (P45). Anchor-adapted cDNAs from S-49^{++} cells were used as a template for PCR amplification as described above and products were analyzed on a 1.5% agarose gel. Finally, the start site designation was compared to sequences obtained from the genomic clones containing 1A cDNA sequences.

Southern Blot Analysis

Southern blot analysis was carried out essentially as previously described [Casey and Davidson, 1977]. Long distance PCR (LD-PCR) products were separated by electrophoresis on a 1.0% agarose gel, and transferred to a Genescreen nylon filter, hybridized with ^{32}P-labeled 500 bp 1A-specific cDNA probe overnight at 65°C, washed, and autoradiographed.

Construction of a Full-Length 1A cDNA

The cloning of a large cDNA with complete 5′ and 3′ UTRs required a series of strategies. To begin cloning the full-length cDNA, 5′and

3′ RACE techniques were used to generate two overlapping cDNA sequences using the 5′ end cDNA primer 5′GSP (5′-TTCCTTCCATGAAGTGGGCAGAGTTGT-3′, +1/+27) with primer P1 (5′-CCCCATGGAAGCAGATATATACAGAGTAAG-3′, +4131/+4102) to generate one fragment, and the 3′ end cDNA primer 3′GSP (5′-GAAAAAGCAGATATTTTATTATGATGTTT-3′, +7285/+7257), with primer P47 (5′-GGCACTCAGCTATCAGAAGACCACAGAAATTGACT-3′, +4039/+4073) to generate the other fragment (refer to Fig. 5). To combine these two fragments an LD-PCR was performed using the above 5′ and 3′ RACE products as templates, and 5′GSP and 3′GSP primers. Agarose gel electrophoresis was used to estimate the size of amplified products, followed by DNA sequence analysis. These products were then cloned into the eukaryotic expression T/A cloning vector pCR 3.1-Uni vector essentially according to the manufacturer's specifications (Invitrogen). Two different clones were identified. One contained 4.1 kb of the 5′ end of the 1A cDNA (pCR1A-4). The other contained 2.7 kb of the 3′end of the 1A cDNA (pCR1A-16). These two clones were then combined as follows to construct a full-length cDNA: First, for the purpose of creating a restriction site, pCR1A-4 clone was further extended to 5.0 kb (to create clone pCR1A-5) by ligation with a 977 bp Bgl II(+4074)/EcoR V (+5050) fragment obtained by PCR from the cDNA library. A recombinant 1A cDNA clone was created by then using a 5.0 kb Afl II (−14 nt to +4771 nt) fragment from clone pCR1A-5, and clone pCR1A-16 cut with Afl II. This clone was later found to contain a 1.3 kb deletion from nt +1024 to nt +2350. In order to repair the deletion, a 3.5 kb cDNA fragment was generated from the cDNA library by 5′RACE, using the 5′GSP and an anti-sense primer (P77 5′-CTAGAGACCACATGTAGTGCGTATAGA-3′, from +3521 to +3495). The PCR product was then cloned into PCR 3.1-Uni vector. The final GR1AFC.5 expression vector containing the full-length 1A cDNA was then constructed by replacing the deleted part with the AccB7 I fragment (+13 to +3531). Each of these steps was checked by sequencing across the cloning junctions.

In Vitro Transcription and Translation

Both linearized and circular plasmids containing full-length GR 1A cDNA were transcribed (with similar results) with T7 RNA polymerase and translated in the TNT T7-coupled reticulocyte lysate system (Promega), labeling with [^{35}S]methionine, or [^{35}S] cysteine according to the manufacturer's instructions. Cytosol and plasma membrane fractions of S-49 cells were prepared by homogenization and differential centrifugation as previously described [Gametchu, 1987]. The translation products, detergent-extracted membrane proteins [Gametchu, 1987; Gametchu et al., 1991a], and cytosol samples were then immunoprecipitated with BUGR-2 antibody and protein A Sepharose 4B. Washed immune complexes were separated by SDS-PAGE (7.5%) and transferred onto a nitrocellulose membrane. Blocked membrane was incubated with BUGR-2 anti-mouse GR antibody followed by alkaline phosphatase-conjugated goat anti-mouse IgG antibody and then visualized by colored phosphatase product formation [Gametchu, 1987].

Transfection of Full-Length 1A cDNA Into COS-7, AtT-20, and HL-60 Cells

COS-7 and AtT-20 cells grown on 60 mm tissue culture dishes to 60–80% confluency were transfected with the full-length GR 1A cDNA expression vector using SuperFect transfection reagent according to the manufacturer's instructions (QIAGEN, Valencia, CA). Each 60 mm culture dish was exposed to 5 μg DNA in Opti-MEM I medium (Gibco BRL) for 6 h at 37°C. Following removal of the transfection reagent, fresh medium was added to the cells and the incubation was continued for 5 days. For stable transfections, cells were transferred after 4 days to medium containing G418 (800 μg/ml) and after 10 days surviving clones were screened for expression of GR message by RT-PCR. The transfection of HL-60 cells was performed by electroporation using the T820 electroporation system according to the procedures provided by the manufacturer (Genetronics, San Diego, CA). DNA (25–30 μg) was added to 5×10^6 cells/0.4 ml which were then subjected to a 0.6 kV electric pulse for 90 μS, allowed to recover, and replated.

Fluorescence-Activated Cell Sorting (FACS) Analysis of Transfected Cells for mGR

Live, stably-transfected Cos-7 or AtT-20 cells were incubated with 5.6 μg/ml of FITC-conjugated BuGR-2 antibody for 1 h at 4°C, followed by three washes with PBS as previously de-

scribed [Gametchu et al., 1991b, 1993], a method previously shown to label only mGR. Specific antibody binding to plasma membrane proteins was analyzed by flow cytometry using a FACS analyzer (Becton Dickinson) equipped with a FACS Lite laser. Negative control experiments involved cells transfected with an empty vector or stained with FITC-conjugated goat anti-rabbit IgG.

Glucocorticoid Sensitivity Experiments

Stably transfected mouse pituitary AtT-20 and transiently transfected HL-60 cells (3×10^5/60 mm dish) were seeded in the presence and absence of 10^{-6} M concentration of dexamethasone for 4 days. Using the trypan blue dye exclusion assay, cell viability was estimated every 24 h as previously described [Gametchu, 1987; Sackey et al., 1997]. Negative controls were cells transfected with empty vector in parallel experiments.

RESULTS

Cloning and Sequencing of the 5′-Flanking Region for Mouse GR Transcript 1A

When the mouse leukocyte genomic library in EMBL3 Sp6/T7 was screened with a 500 bp cDNA fragment specific to GR transcript 1A, three clones (1a1, 1a7, 1a23) were identified and purified by sequential screening. Each clone served as a template for LD-PCR using the vector insert-flanking sequences as primers. All contained an insert ~16 kb long (Fig. 1). The sequence of approximately 2.2 kb of 5′ flanking DNA and 1 kb of sequence downstream of the transcription start site (see below for analysis of start site) was determined, and is shown in Figure 2. DNA sequence analysis of one of these clones (1a1) revealed 2,140 bases of exon 1A upstream flanking sequence, the 1013 bases of GR exon 1A, and 112 bases of the following intron. Since there is approximately 32 kb of sequence in that intron (between exon 1A and exon 2), our clone contains about half of the

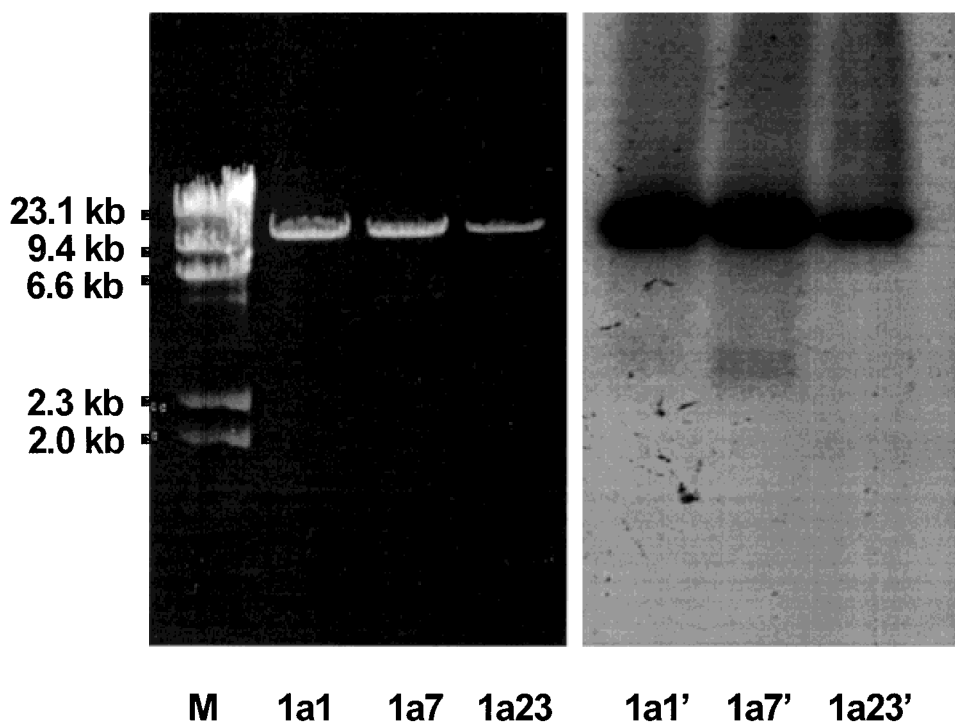

Fig. 1. LD-PCR amplification of three mouse genomic clones containing promoter 1A, exon 1, and flanking intronic sequence. **Lanes 1a1, 1a7,** and **1a23** represent DNA amplified from these three separate clones. The DNAs when blotted and probed with a 500 bp, ^{32}P-labeled, transcript 1A-specific probe demonstrated a major 16 kb amplified band (**lanes 1a1′, 1a7′,** and **1a23′**) containing transcript 1A sequences. Other minor PCR amplimer bands were not detected by hybridization, and thus are probably nonspecific amplification products. **M,** Marker HindIII fragments of λ phage DNA: 23.1 kb, 9.4 kb, 6.6 kb, 2.3 kb, 2.0 kb.

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-2140  TTACTAAGAC TATGGAGCAC TCACAAAAAG GGATCATCTT CATCCTGTCT
-2090  TATATTTAAA TGAACTACCA ATGCTCATTA CAAATTTCTC TACCTTCACC
-2040  ACCCCAGGTC AGCTGTATTT ACAGGTCAAC TTGTCAATCT AAGTTCTCTG
-1990  CAGCTGGGAC TCATGGAGTC CGTTCTGCCT TCTTGACAGG AGTGTGGGCA
-1940  TCCCAGACTG AGTTTAGGGC TGCAGTCACA GGTAGATTTC TGTTGCAATT
-1890  ATTTGATCAT CCTTTACAAT GGTTCCCAGG AGTTCACATA GCTTCCAACA
-1840  GCGCATATGA AAGCCTGTTG TTACCTTTAA GCCTGTAGAG AACCGTAGAT
-1790  GAGGAGAAGG AATGATCAGA CTGACAGATG AAATATGAGA AAGATAAATG
-1740  TGGGCAGAGT CTAGAGCTGG GGAGGGGGCA ATCAAGCGAG AGGGAGAATG
-1690  AGAAAAGATA TGAAATGGCC AAGCACTAAA TAATTATTAT GGGTTAGATA
-1640  GTGAAAAATA AATGCAAGGA CACAGTCACT GGACAGGGTA GAAGAACGTG
-1590  ATGAATCTGA GGAGATTAAT CAGGCCTCCT TGAACTGTCA AGGAAAGATA
-1540  TTCATTGAGA CGGAGGCTGT GGTGTTCATT CCCCTGACGA TGATTAATAG
-1490  AAGCTAAGGT CAGAGTTAGC ATTCATTCTT TTTGAGAGAA AGAATGTCAA
-1440  CAAGAAAACA CATAACGTAA AAAGCCTCCG AAGAAAACTC AAGTTCAATG
-1390  GACACAGATT CAGGGAATAG TTAAGCTACT TTGGAGCATG GAGAGGGAGG
-1340  ACAGCTGTCT AAATGGATTG TTCTGCAGAG TGAGGAGAGG CAAGTGCAGG
-1290  ACGATGCCTT TAGCTACTTA TCGCTTTAAG ACTTATGTAA CTTGTATTTC
-1240  TTCTCCGTCT GCTGCAGCTC TGAGGAACAC ATCTAACAGA AAGTGGAAAG
-1190  TCAGGCAATT GAAAAGAACC AAAAGCAAAG AGGAAATAAT GGCAAGAAAA
-1140  TAAAGCCCGA GGCAGAATTA ATATACACCT TCCATATACT GCCCTGATAG
-1090  GGGGAAAAGA CAAACAAACA AACAAGGTCA AATAGACAGT CTGTAGTCAC
-1040  TGGAGAGAAT CTGTTAACTG GCCTCTCATC TTTCTATCCA GCAAGCACAG
 -990  AAAAACAAAA ATGACTACTG CTAAAAGTCT ACTTTCTAGA AACCAACACG
 -940  GAACATTTTA GGATGTTAAC ATTAAAGTCT AAAAAAGTTC TGTTCAGCAG
 -890  AGCCTCATGA TATGGGCAAG GACTTTGCCA ATAATCTGTC AGCAAACATA
 -840  GCATCTGCTT TCTTAAAGTC ACACTCTTCT GTTCTTCCTA ACTTGCAAGT
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Fig. 2. Nucleotide sequence of the mouse GR gene exon 1A with 5′ and 3′ flanking sequences. The nucleotide sequence of exon 1A is shown in bold. The solid and open arrowheads demarcate the 5′and 3′ends of this exon, respectively. Position +1 represents the transcription start site for transcript 1A as determined by primer extension analysis and RT-PCR confirmation. All priming sites used for primer extension, PCR and 5′RACE are double underlined. The positions of a TATA box, CAAT site, and an Sp-1 recognition sequence in the proximal promoter are indicated by a single underline and label above the sequence. Lowercase letters indicate intron sequence.

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-790  AATCTCCATT TCTCAAGCAA CAATCCCCAC TTGATCCTGA TAACCTTTTA
-740  TTTCATAGTA AACTCTTGAA AACAGTTGCA CATGTACCAG AAAATGATGC
-690  TTGACACCAT GGAAATGATT TGACACAGCA GTTACTTTGT CTCCACCAGT
-640  TACACCTTTC TATAGAGTTC CCATAGAGGC TTTATGTAAA GCACTGGGGT
-590  GTTTTTGTTT GTTTGTTTGT TTTAACAGCT CTACCATTTA ATCAGCCTAG
-540  AGAATGATCT AGAAACCAGT CTATCTGAAG GATTCTACTG AGTTTAGAAT
-490  TTAGTATAAC AGGAGAGAGT GGGTGAGACC CTTCTGATGG GCCCTGAAGC
-440  CAAGAGCATT GGCTCAGCTC TGAGTGCCCA AGCCAAGAGC ATTGGCTCAG
-390  CTCTGAGTGC CCAAGCCATT GCACACTGCT GTGTTGGCAT GGCGTTTCTG
-340  CAGGCCATTG GTACTCTTAC TGTTTTGGCC ATGTAATTCA TCGCTCACTA
-290  TTCAACTGTG ACAGGTGTGC TTAAAACGAC ATACCTGTTC ACAGCCTATA
-240  TGGTGACCAG GACCCTGAAC TAACTTGGAC CTTATGTCAG AAGCAACAAA
                  CAAT
-190  AGACATACCA ATATTTTCTT GATTTTCAAA TTGGTAAGTT AAATTGTCTA
-140  CCCTTGCGTA GATTCTCTTC AGGCAAATGA GGAAGTGCCA GTTAAAGGTA
 -90  GTGTGTAAAA TCAAAACAAA AATTAAACTG GCACCTGCGT GATGAACAAA

         TATA                           5'U Primer   +1▼  5'GSP
 -40  AATTATAATC AATGGTACAA CTGTCTGAAG TCATTTTCAT TTCCTTCCAT
                                             5'S Primer
  11  GAAGTGGGCA GAGTTGTGGG GCTAACTCTC TTCTCTCCTC CCTTTCCCTC
                                P79
  61  TCGGTCCTCC CCCCCCCAAC CCCCATGTCT CTCTCTCTCT CTCTCTCTCT

 111  CTCTCTCTCT CTCTCTCTCT CTCTCTGTTT TGGTTCCTTC TTGCTCTTTT

 161  TAGGGCCTGC ACAGACCCAC GGGTAGCCAC TGATCACTCT CACTTCTGCT

 211  GAGGAAATGA CAGCATGCTT CTCTAAATGG TGGAACGAAA GAGAAAACAG
                         P45                              P31
 261  ATGTTCTGAT ACCAAATGGT CAGACTTTGG AGGGTTAGCA GTATTCTCAG

 311  GACCAGTAGG ACCTAGAATA TCTGAGTCTG AAGAGCACTG GGAGTCACT
                                                 Sp-1   P24
 361  GGGGGGTGGG TCAGAAGACT AAGACAAGAA AAAGGGAGGT GGGCGGATGT

 411  CTGTGTGTTA TCTGCTCTGC TGATCCCTCT CGTGCATGAT GCAACACCTG
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Figure 2. (Continued.)

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461   ACTTTAACCC TCTTGCTATG GTTTCTATTT GGGTCTGACT TGGGGACTAT
                                                     P17
511   CTGCTGAATC AGTATCTCTG AGCAGAACCA AGAAATTCAC CCCCAAAGAG

561   GAGTCACTGT ATTAGTCAGG GTCTCTGAAC AAAGTTAGAC CCAAGAAACA

611   GAATCCTCTG GTCTGAAATG GTCTCTTGTG TGAAATTCTC TGCTTTGTAC

661   GCAAAGGAAA GAACATGCCG GTAGGAGCCT GCTCGTCAAA CGAGGTGTGA
                                 P27
711   ATCTAGCTTC TTCTAGAAAA AGCAGCCTGC GTCACATCGA AGCCAGATTT

761   GGTTCTTTGC TCTGAGAGCG GTTAGGCTAG TGGAGGGCAG GCTTCCGTGA

811   CAACTGGTAC AGGGACAGGT GCAGTGTGGG TCCCACAGAT ATGAACTCTG

861   ATAAATCGTG CATGAGCTAC TCTGCGTAAG AATGGAGAAG AGAGCAGCCC

911   AGCTCCCACC CTCCTGGGGT TCCCATCGCA GCCTGATCAT CTGCAGCCTT

961   CTCAGCCAGG AAGATGTTTC AGATCCTGCT TCGTTAGAGT GTCTGGGAGG
         ▽
1011  AAGgtaagtg gtactttgat tcccattctg cagctgggta atgtggattc
 Exon 1A/intron
1061  caacaaaatt agatgagggt cacacagagg tcagagcatc tgaaaaaaat

1111  tatcacaaga ttttt
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Figure 2. (Continued.)

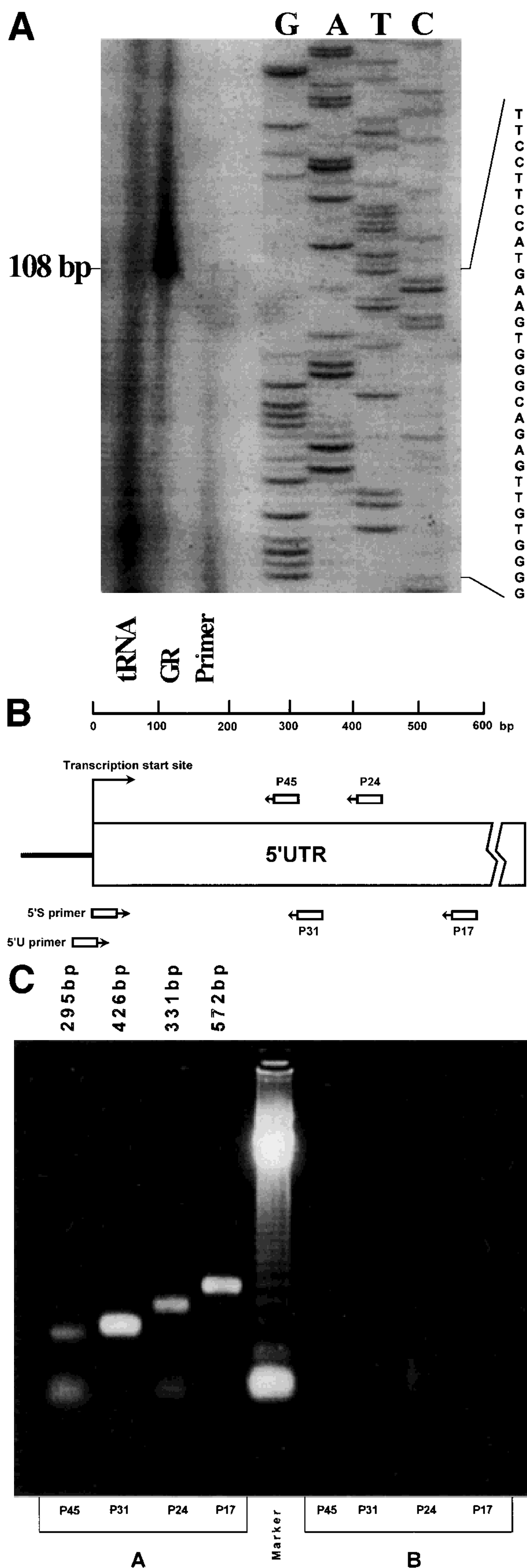
intron. Other known exon 1 variant sequences (B through E) [Strähle et al., 1992, and accompanying paper] and their promoters are contained in this region.

Determination and Confirmation of the Transcription Initiation Site

To determine the start of transcription for GR transcript 1A, we used the 5' RACE technique. Sequencing independent clones extended the known sequence at the 5′-end to 1010 nucleotides upstream from the translation initiation ATG (Fig. 2). To confirm the mapping of the 5′end of the GR transcript 1A, we performed primer extension analysis. The major abundant extension product has a length of 108 bp (Fig. 3A). No extended products were visible in the yeast tRNA control analysis. Therefore, the 5′ RACE result identified nucleotides encoding the 5′ end 16 nt upstream of the longest cDNA sequence. This new site, therefore, probably corresponded to the real 5′ end of the GR transcript 1A cDNA. PCR amplifications were then performed to confirm the location of the transcription initiation site determined by the primer extension. Two different 5′ primers were used in combination with four different downstream primers for PCR amplification. When amplifications included the start site primer partner called 5′S Primer in Figure 3B, downstream primers produced amplified DNA products with sizes of 572 bp, 426 bp, 331 bp, and 298 bp, respectively (Fig. 3C). In contrast, the further upstream primer (named 5′U Primer in Fig. 3B) did not produce any amplified DNA when used in partnership with the four downstream primers. These results confirmed that the primer-extension reaction correctly identified the transcription initiation site as represented by the 5′S Primer in Figure 3B.

Analysis of the 3′UTR

The 3′UTR was examined by DNA sequence and Figure 4 shows the results of these studies.



Conserved with greater than 88.5% homology between the rat [Miesfeld et al., 1986] and mouse GR genes, the sequence at the 3′ end of the mouse 1A GR cDNA is 3,907 bp long. There are three canonical AATAAA polyadenylation signals located starting at positions 5570 nt, 7039 nt, and 7266 nt from the transcription start site in this region.

Cloning of Full-Length GR 1A cDNA

Several cloning and deletion repair strategies were necessary to finally obtain the full-length 1A cDNA (Fig. 5). Agarose gel analysis showed that a single PCR product of ~7.3 kb was eventually obtained (not shown). Subsequent cloning of this cDNA into the eukaryotic expression T/A cloning vector pCR 3.1-Uni vector, and digestion of the resulting clone GR1AFC.5 with Hind III and EcoR I restriction enzymes, revealed the expected size and fragment pattern (not shown). Final verification was established by DNA sequence analysis, showing that the full-length GR 1A cDNA contained 7,285 bp, including 1,026 bp of the 5′-UTR, 2,349 bp in the coding region, and 3,907 bp in the 3′UTR.

In Vitro Transcription/Translation Analyses of Cloned GR 1A cDNA

Western blot analysis of in vitro transcription and translation products using the cloned full-

Fig. 3. Analysis of the transcription start site for transcript 1A. **A:** Primer extension analysis. Labeled primer products extended from oligo P79 were isolated and resolved on a 8% urea-acrylamide DNA sequencing gel. DNA sequencing reactions were performed by using the genomic clone as a template (GR) with the same oligonucleotide as a primer. The letters above the lanes indicate which dideoxy nucleotide was used in that sequencing reaction. The dash indicate the sizes, in nucleotides, of the extension products. Extension products were not visible with the yeast RNA (tRNA) and primer alone (Primer) controls. **B:** Diagram illustrating the strategy used to identify the transcription start by PCR analysis

5′S PRIMER	5′-TTTCCTTCCATGAAGTGGGCAGA-3′
5′U PRIMER	5′-CTGAAGTCATTTTCATTTCCTT-3′
P17 PRIMER	5′-TACAGTGACTCCTCTTTGG-3′
P24 PRIMER	5′-GCAGATAACACACAGACATCC-3′
P31 PRIMER	5′-TATTCTAGGTCCTACTGGTCC-3′
P45 PRIMER	5′-TAACCCTCCAAAGTCTGACC-3′

C: PCR analysis of the mouse GR 1A transcription initiation site. Anchor-ligated cDNAs were annealed with either the GR 5′S Primer (A) or 5′U Primer (B) in combination with four downstream primers (P17, P24, P31, or P45) for PCR amplification. Amplimer sizes, where they exist, are given at the top of the lane. The size marker used was the 123 bp ladder (Gibco BRL).

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3379 CTGCCTTACT AAGAAAGGCT GCCTTAAAGA AAGTTGAATT TATAGCTTTT
3429 ACTGTACAAA CTTATCAACT TGTCTTGTAG ATGTTTTGTC GTTCTTTTTG
                                 P77
3479 TTTGTCTTGT TTGTTTTCTA TACGCACTAC ATGTGGTCTC TAGAGGGCCA
3529 AGACTTGGCA ACAGAAGCAG ATGAGCCATC ACTTTTCAGT GACAGGAAAG
3579 CAGACAGTGA TGTGCATTGG CTGGTGTATC ACAGAAACTA GAACAGTTAG
3629 TGGAGACATG TCCACTATCA GAGAAGGACC GCACCTGAAC CACCAGTGCC
3679 CAAAGTCCAT GTGATCAACT TTCTGCTCAA CTTTCAGTTG GCTGGATAAC
3729 ACTTTCTAGA CTTTTCTGTT GGTGTATTTT TCCCATGTAT AGTTAGGATA
3779 GCATTTTGAT TTATGCATGG AAACCTGAAA AAAGTTTACA CGTGTATATC
3829 AGAAAAGGGA AGTTGGTGCC TTTTATAGCT ATTACTGTCT GGTTTTAACA
3879 ATTTCCTTTA TATTCAGTGA ACTATGCTTG CTCGTTTTTC TTAAATAATT
3929 TTTGTATTCC AGTTATTGTA TAGCTGTTTA AGATGGGCAG CTGCCTCACA
3979 GCTCTCCTAG ACGCTAACAT TAATTTCCGT GTGAAAATGG GTCGGTGCTC
                P47
4029 CTACCCTGAT GGCACTCAGC TATCAGAAGA CCACAGAAAT TGACTCAGAT
                                   P1
4079 CTCCAGTATT CTTGTCAAAA GCTCTTACTC TGTATATATC TGCTTCCATG
4129 GGGAATTATA TAGGTTGTGC AGATTAACCG TCCTAACTGG TATAGAGCAC
4179 CTAGTCCAGT GACCTGCTGG GTAAACTGTG GATGATGGTT ACAAAAGACT
4229 AATTGTAAAA CAGTGCCCAC CAACAGGCCC CGTTTGCACC CAATGCACCA
4279 TCTCTTCAGT GGTGCGATAG CAACAAAGTT TGTAACTCAG CTCTTTCAGG
4329 ACTTCGGGAG TAGTTTGTGT AACATTTTAA AATGTATTAT TCCAGATAAC
4379 CAGCTGTGAT AAAGCCGAGA GATTGTTTTA ATCAGACCAA GTAACTTCTC
4429 TCATTAAACG TTACCCTCAA CTAAGTCTCT AATATGGCAA GAATGGCTAG
4479 ACACCCATTT TCACATCCCA CCTGTCACCA ATTGGTCTAG CTTTCCTGGT
4529 GGTACAGGAA AATCAGCTAC TGATTTTTTG TTATTTAGAA CTGAATGTCA
                                                P76
4579 GGCATCCATG TTTGTCCAAC TATACATCCC TACATGTGCC ATAGAATCTA
4629 ACACAAGTCT TGTGAACCAT TCACACTGAG AGTTATCATT TAAACAAAAC
4679 AGAAGCTGTA GTAGCCCTTT CTGTGTGCAC CTTACCAACT TTCTGTGACT
4729 CAAAGCTTAA CACACTTACT AAGCCACAAG AAATCTGATT TCTACTTAAG
4779 GTGGCCAAAT TATTTGTGTA ATAGAAAACT GAAAATCTAA TATTAAAAAT
4829 ATGAAACTTC TAATATATTT TTATATTTAG TTCTAGTTTC AGATATATAT
4879 CATATTGGTA TTCACTAATC TGGGAAGGGA AGGGCTACTG CAGCTGTACA
4929 TGCAATTTAT TAACATGATT GTAAAATAGC TGTATAGTGT AAAATAAGAA
4979 TGATTTTTAG ATGAGATTGT TTTATCATGA CATGTTATAT ATTTTTTGTA
5029 GGGGTCAAAG AAATGTTGAT GGATATCCTA TAAGATTTAT AGTATATAAG
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Fig. 4. The cDNA sequence of the 3′-UTR of the mouse GR. Putative polyadenylation signals (AATAAA) in the 3′-untranslated sequence are underlined. Primers used in the 3′RACE, and PCR are double underlined.

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5079 AGCATCCATA CAGGCCTCAG TGGTCTTGGA AATTAAAACA GGTTTGCTCT
5129 AAGCTAGGGA GAGGGAGCTG GGACTGGCCC TGTGTGCAGT GCAGGTCCTG
5179 AGGGTTTGAC CCGATCAGAT CACAGGGGAA CTAATTCCCT CCCATCTAAC
5229 CATCCTCATC CGACCATGGC CCTGTCAGTG CAGGCTGGCT TTATTAAATC
5279 CAGGACAGAA AGGTGGCGCT TATGTACTTA GAGGCACCGT CCAGTAACAG
5329 GGTTGTTCCC ACATGCAGCC TCCGCACGGG GTAACAGAAA CGGAGGCTTT
5379 AGAAGTTTGG CAATAATGTG CATAGAGGTT CCAGCAATAT GTAAATACTA
5429 AAGAATCGCA TAGGAAGCCA ATAATACACT AATCCTCTCC ATCCTACAAG
5479 AGTCCATTTC CAAGTAAGAT GAGGACATGT TTATGTTATC TTTGAATGCT
5529 TTTTGAATGT TGTTATTTTC AGTATTTTGC AGAAATTATT TAATAAAAAA
5579 AAGTATAATC ATTTGCTTTT TGAATTCTCC TCTAAAAGGG AATGTTCAGT
5629 TTGTAATGGT TTAAATTGGT CTCAAAGTAC TTTAAATATG TACCCAGCTG
5679 GATGTGAAAT TTAGTGCCTA AGAAATACCA CTTGAAGATT ATCAATGACA
5729 GTGTTAAGTT TCAAAATGAG CTTCTCAAAA ATAGATTATT GTACATTTAT
5779 GGAATGTTAT ATGGTTAAAC CCAAAAAGCA CATCACACAT AAATCTGCTT
5829 TCAGTTCCAA CCAGCTTGGC TTTCAAAAAT AGAGCTCCA AAAAAAAAAAA
5879 AGGAAAAAAA AGATATATAT GCTTTGTTAT TAACAGAAGG CAGCAGACAT
5929 TCATAAAACT ACTATCGGAA GTTTTCCATT AGATGTATAA AGAGCTATCC
5979 TTTGGTATGT GGGAAAGAAG AAAGCTGTCA TAATTCTGAT TGAGTATAAG
6029 TGAGAGAGAT ACGGTACTGT TTGAGAGCAG CTCCTTTTCT GCGTGTGGCT
6079 TCATACCGTT CCAAACTATG TAGATTTTAT AATAGCTTCA GTGAGAATTG
6129 GTAACATGCC TGTATGACTC ACAACAGATC TTGAAAACTA TCTTTAATTA
6179 CTGGTAGGAC AAAAAGGGAC ATTCTGGTTA TTTTAGGCAC TGGCTTGGAA
6229 CACTGTATAT GCAGAAGAAA GAAGACAGGC AATCTGGGGA AAGAAGGGAC
6279 CTTGGAGCAC TGCCATCTTT AAGGAAAGAC ACACCAATAG ATGAGATCAT
6329 CCCAAAGCAC AGGACCACAG AGTGTGAGTC CTAGTGACGA GTCAGTGAGC
6379 TCTGGTGAGC TTGGAGAAGC CAGCCCCACC AGCAGAGCAG GCACGGCAGG
6429 GATGGGACAA GCAGGGACGA CAATTCCAGC TGGACACTGG TCCCAGTATT
6479 TTGCTCCCTC TTATATACCG TGAGGCAGTA TCACCGTGGG ATGAACCATG
6529 GTAGCACGTT TTGATCTGTC AGCACTCAAG GATCATGGTA GCCTTCGGGA
6579 GCTTTAGGTT TTGGTTGGTC ACCCCAACGA TCAGCTGTAG TTGAATGTGT
6629 TTCTTATGTG CCTGGTTTCA GTGTTAGAAG GTGAAATAGA GTGTGCAAAG
6679 GACACTGCAA ACCACTTCGG ATGGAAGTTT TCTCATTTTC CAGACTATTT
6729 TCGGTCAGCC TGGTCTATCA AGATCGGTAA CCAGGTCTTC AGGAAAGGGT
6779 TGGCTTCTAT CTAGGACATG CCTGAAAGGA TTTTATTTTC TGATAAATGG
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Figure 4. (Continued.)

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6829 CTGTATGAAA ATACCCTCCT AAATACCCTG CTTAACTACA TATAGATTTC
6879 AGTGTGTCAA TATTCTATTT TGTATATTAA ACAAATGCTA TATAATGGGG
6929 ACAAATCTAT ATTATACTGT GTATGGCATT ATTAAGAAGC TTTTTCATTA
6979 TTTTTTATCA CAGTAATTTT TAAATGTGTA AAAATTAAAA ACCAGTGACT
7029 CCTGTTTAAA AATAAAAGTT GTAGTTTTTT ATTCATGCTG AATAACCTGT
7079 AGTTTAAAAA CCTGTCTTTC TACTACACAG TGAGATGTCA GACTGTAAAG
7129 TTTTGTGTGG AAATGTTTAA CTTTTATTTT TCATTTCAAT TTGCTGTTCT
7179 GGTATTACCA AACCACACAT TTGTAATGAA TTGGCAGTAA ATGTTAGTCA
                                               3'GSP
7229 GCCATTTACA GCAATGCCAA ATATGGATAA ACATCATAAT AAAATATCTG
7279 CTTTTTC
```

Figure 4. (Continued.)

length 1A GR cDNA sequence compared to plasma membrane extracts of S-49 cells [Gametchu, 1987] are shown in Figure 6 (lanes 2 and 1, respectively). GR peptides of similar molecular sizes (150 KD, 97 KD, 94 KD, and 70 KD) were present in both preparations. Samples from the cytosol of S-49 cells contained the classical 94 KD GR only (lane 3). The presence of similar M_r GR immunoreactive bands in preparations from membrane extracts and the translation products, including the novel high M_r 150 KD band, suggest that transcript 1A encodes both mGR and iGR. The 24 KD (which runs with the tracking dye on a 7.5% agarose gel) and 54 KD immunoreactive bands seen in all lanes are the light and heavy IgG chains. These bands vary in intensity directly related to antibody concentration required for immunoprecipitation, which differs for these very different preparations.

FACS Analysis of Cos-7 and AtT-20 Stably-Transfected Cells

The 1A cDNA-associated plasma membrane localization of GR was further investigated by FACS analysis of live Cos-7 or AtT-20 cells that had been transfected with either full-length 1A cDNA-containing plasmid or empty vector (Fig. 7). High levels of mGR expression were observed in both Cos-7 and AtT-20 GR 1A cDNA-transfected cells (Fig. 7B and D, respectively). Cells transfected with empty vector (Fig. 7A and C), however, showed no significant specific fluorescence, suggesting that transcript 1A contains information for both expression and plasma membrane localization of the GR.

Glucocorticoid Sensitivity Assay in Stably or Transiently GR1A-Transfected Cells

To test the hypothesis that mGR, encoded by transcript 1A mediates the lymphocytolytic effect of GCs, we conducted hormone sensitivity studies on mGR-less cells usually resistant to lysis by GCs, which were transfected with 1A cDNA or empty vector. One mGR-less cell line chosen for these assays was the stably-transfected AtT-20, because their response could be correlated to the FACS analysis of expression (above). We originally also tried to do these analyses in Cos-7 cells, but discovered that they had an inherently high level of apoptosis due to their growth characteristics and were therefore unsuitable for analysis of lysis. Therefore the transiently-transfected HL-60 cells were chosen as an alternative. The results shown in Figure 8 indicated that cells transfected with 1A cDNA were partially lysed (by ~40%), while cells receiving empty vector were not lysed at all. The 4-day survival value was significantly lower for 1A cDNA-containing cells ($P = 0.001$) when assessed by a Student's *t*-test. These results suggest that expression of transcript 1A permits GC signaling leading to cell death.

DISCUSSION

Since the early 1970s, plasma membrane-associated receptors for steroid hormones have been hypothesized to mediate some membrane-initiated actions, and were described in a variety of tissues and cell lines [Szego, 1978]. Nevertheless, research progress has been slow in this area, primarily due to technical problems of

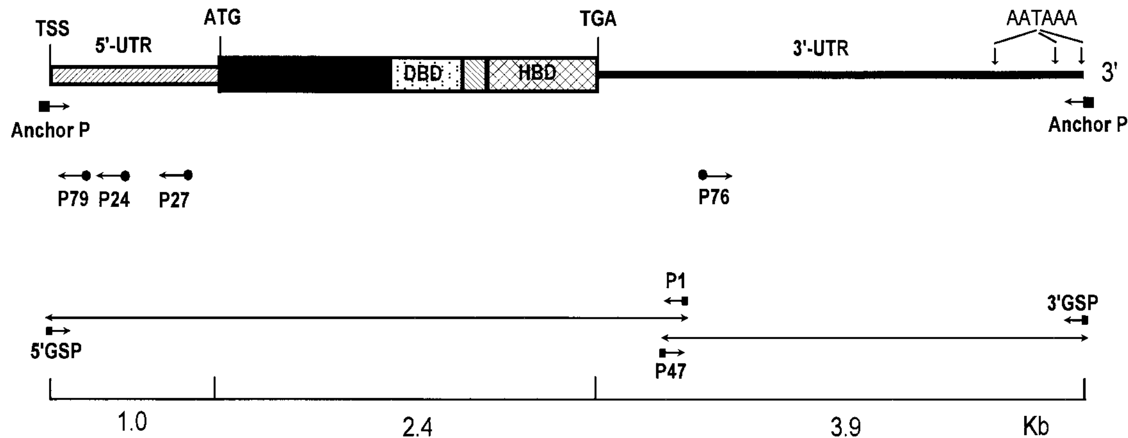

Fig. 5. Structure of mouse glucocorticoid receptor transcript 1A cDNA. A full- length GR transcript 1A was constructed by a combination of 5′RACE, 3′RACE, LD-PCR, and restriction enzyme site splicing. In this schematic diagram the coding regions are wider boxes, the filled region shows the N-terminus region while the DNA-binding (DBD), hinge, and hormone-binding (HBD) domains are represented by hatched and stipled regions. Narrower boxes delineate the untranslated (UTR) sequences. TSS, the transcription start site. The area of the cDNA arising from Exon 1A is the entirely of the 5′ UTR sequence plus 13 additional bases of Exon 2 up to the ATG translation start site shown. Three polyadenylation sites are shown near the 3′ end. Primers used for 5′RACE (5′ Anchor P, P27, and P24), 3′RACE (3′ Anchor P, P47, and P76) and primer extension (P79) are indicated. Primers P1 and P47 were paired with primers 5′GSP or 3′GSP, respectively, and used for the final 5′RACE or 3′ RACE to produce the overlapping 5′ end and 3′ end amplimers which were spliced together to create a full length cDNA.

working with and describing steroid binding to proteins anchored in a lipid substrate. As a result, some basic questions, including the relationship between the intracellular and membrane steroid receptor forms, receptor function, and molecular etiology of the membrane form were still unanswered. Our accompanying manuscript establishes that cells enriched for mGR expression predominantly produce the 1A GR alternative transcript, of the five available transcript forms. In hopes of determining if the 1A transcript was responsible for the membrane form of receptor, we then sought to clone, characterize, translate, and transfect its cDNA.

Since the coding region was essentially the same in all the GR alternative transcripts (with minor differences not being associated with changes in function for the lymphocytolytic response, see accompanying manuscript) we further focused our attention on the untranslated regions of the message. Four different methods were used to establish the 5′ end of this large message to insure that all relevant sequence could be considered for functional significance in producing the membrane form of GR. Results from primer extension and PCR experiments corroborated each other and indicated that the cloning techniques based on 5′ RACE and exon-trapping had not provided us with full-length clones. As further confirmation that we had indeed finally reached the transcription start site by these other methods, TATA (5′-TATAAT-3′ located 32 nucleotides upstream) and CAAT(5′-CCAAT-3′ at −183) sequences were found in the genomic clone. Thus, the position of the GR 1A RNA transcription start site is located 1026 bp upstream of the ATG codon and full-length 1A clones were constructed from this information.

We subsequently analyzed the sequence of the full-length 5′ and 3′ untranslated regions. Although the 3′ end of the transcript did not vary from known GR sequences [Miesfeld et al., 1984], the entire 5′ UTR and its proximal promoter region proved to be unique. Therefore, it could be contributing to the expression of an alternative receptor form in the membrane, although the mechanism by which the 5′ UTR can affect translational control in such a way that the protein product is modified in size and subcellular location remains to be elucidated. Since the membrane form of the receptor had been shown to be larger [Gametchu, 1987; Gametchu et al., 1991a; Sackey et al., 1997], we transcribed and translated the expression vector products in vitro to see if this size difference could be reproduced. We found that 1A GR cDNA could produce the high molecular weight species of GR that we had previously associated with the mGR, matching the membrane receptor extracted from cells for comparison in these studies. It is unlikely that this higher M_r pro-

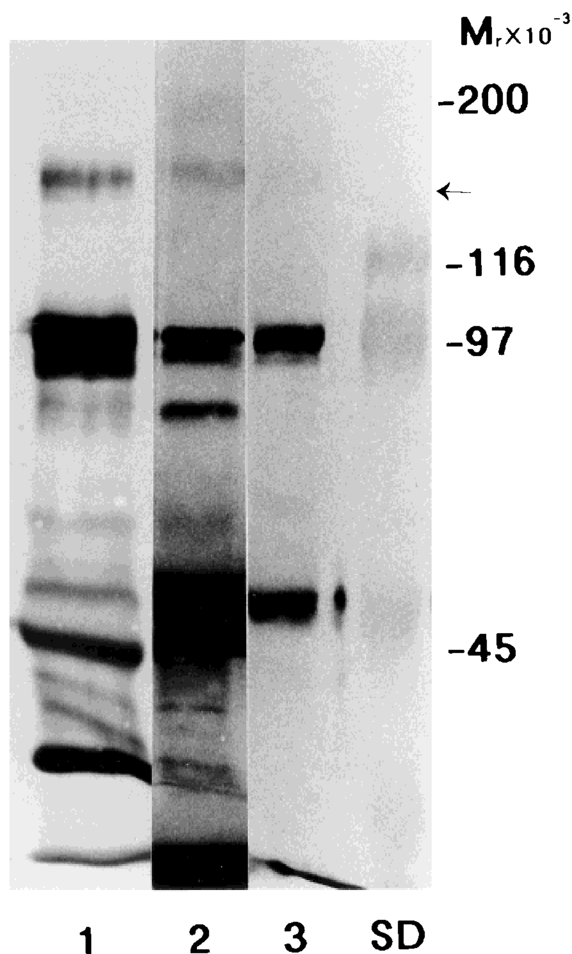

Fig. 6. Western blot analysis of in vitro transcription/translation products of the full-length 1A cDNA containing plasmid. Receptors were immunoprecipitated, separated on 7.5% SDS-PAGE, and transferred onto a nitrocellulose membrane for visulaization with BUGR-2 anti-mouse GR antibody followed by alkaline phosphatase-conjugated anti-mouse IgG antibody and color development. **Lane 1:** Membrane extracted proteins; **Lane 2:** In vitro translation products; **Lane 3:** Cytosol proteins; **Lane SD:** Molecular weight standards. The large 150 kD band often associated with mGR is shown at the arrow and other major receptor bands are size-marked. The membrane extract preparations (representative of three experiments) and the in vitro translation products (representative of four experiments) were run on separate gels due to their susceptibility to cleavage when frozen and thawed.

tein results from a further upstream translation start site, since stop codons follow any translation start sites seen in the 5'UTR. We noted that the 94–97 KD intracellular receptor form was also present as a 1A cDNA in in vitro translation product. The 1A transcript may also produce the lower size species seen intracellularly, or the membrane form may be susceptible to proteolytic cleavage, as we have demonstrated previously [Gametchu et al., 1995, 1991b, 1994) and thus fortuitously give rise to a fragment the size of the intracellular receptor which still retains the BUGR-2 epitope located in the middle of the protein [Eisen et al., 1985]. From the structural point of view, the size difference in mGR could well be attributed to post-translational modifications, since our analysis of the coding region of the 1A transcript did not provide any additional sequence to account for a higher molecular size. Some post-translational modifications of steroid receptors have been reported [Gametchu et al., 1995; Housley and Pratt, 1983; Mirghani et al., 1993] and we have reviewed their occurrence in steroid receptor structure previously [Gametchu et al., 1995, 1991a,b]. Since our higher M_r GR species was produced in a rabbit reticulocyte lysate, it is important to consider the synthetic capabilities provided by that preparation. Although it has been widely held that such a preparation is lacking in microsomes (the biochemical equivalent of the cells' endoplasmic reticular protein synthetic pathway) and therefore cannot produce many of the lipid and N-linked carbohydrate modifications expected to occur in that compartment, there is other evidence that such preparations do contain some of the capacity of this protein targeting pathway, and therefore may allow such modifications [Zelenka et al., 1997]. Covalent protein modification by lipid attachments is a rapidly evolving field, and perhaps the currently known forms of lipid modifications and their reported consensus sequences should not be considered the only possible ones.

The targeting of mGR to a plasma membrane location was studied by transfection and FACS analyses of two different cell lines. While control groups (cells transfected with empty vector) produced no significant GR specific fluorescence, high level of mGR expression was seen in cells transfected with 1A cDNA, suggesting an association of 1A cDNA with the expression and plasma membrane localization of mGR. The process by which proteins are targeted to the plasma membrane is complex and involves a variety of mechanisms [van Adelsberg, 1998], apparently some as yet to be discovered. Very recently, interest in the function of 5′UTRs has increased, and we are just beginning to understand the translational control mechanisms evidently mediated by these sequences [Geballe, 1996]. We can only speculate that alteration of translation rates and locations may alter asso-

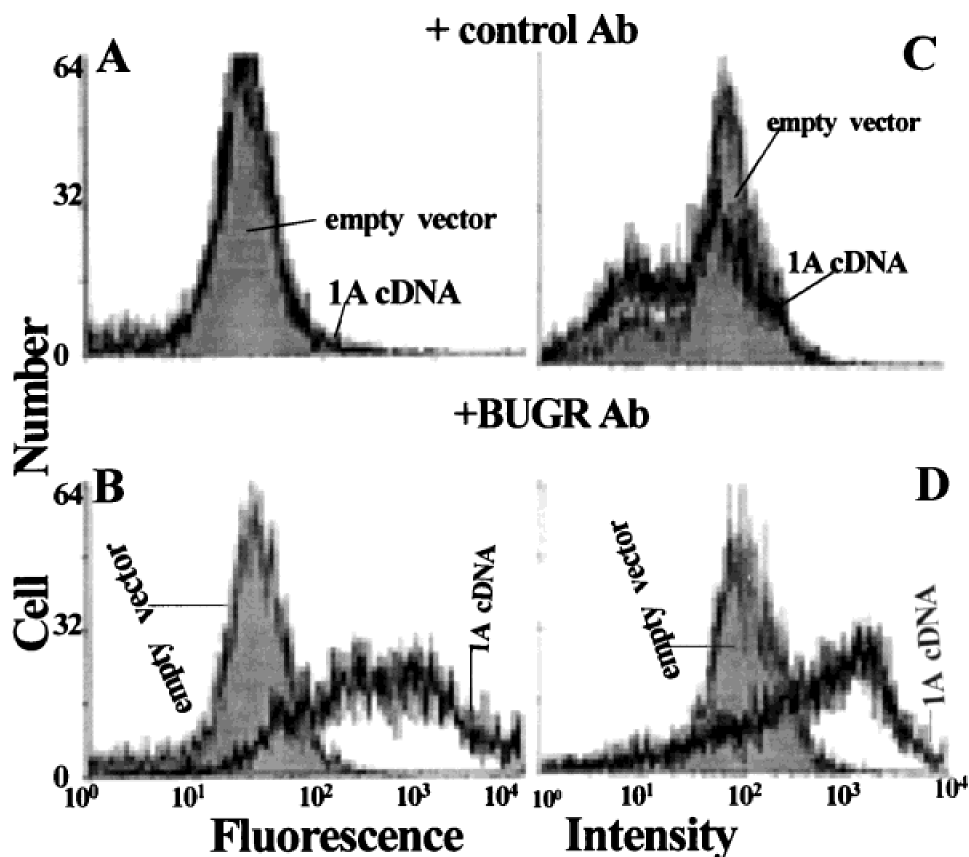

Fig. 7. FACS analysis of mGR in live, stably-transfected cells. AtT-20 **(A,B)** and Cos-7 **(C,D)** cells live-labeled with BUGR-2 FITC-conjugated anti-GR Ab (B,D) or non-specific (controls, A,C) Ab (FITC-conjugated goat anti-rabbit IgG). In each, cells were transfected with either empty vector or 1A GR cDNA. Cells receiving 1A cDNA and stained with BUGR Ab exhibited significant membrane staining as evidenced by the higher fluorescence intensity of that population of cells.

ciation of protein partners for emerging proteins and that perhaps this could affect subcellular localization. One such mechanism is studied by employing trafficking disruptor chemicals such as monensin and brefeldin [Ghosh-Choudhury et al., 1994; Ulmer, 1991]. Previously, we have successfully employed these chemicals to block replenishment of mGR in the plasma membrane of protein-stripped CCRF-CEM ALL cells [Gametchu et al., 1995]. Although the transfection experiments confirm these results, the overall mechanism of mGR trafficking awaits further investigation.

Finally, we tested the hypothesis that mGR encodes transcript 1A and participates in the lymphocytolytic effect of GCs. These studies suggest that transfection with transcript 1A imparts GC signaling leading to cell death. The partial sensitivity of transfectants is likely due to the fact that transfected cells which normally do not undergo hormone-induced apoptosis may lack or have limiting amounts of other cellular factors necessary for GC-signaling or the apoptotic response. Alternatively, these unsynchronized cells probably were not uniformly in the cell cycle stage where apoptosis can occur [Sackey et al., 1997].

In summary, through characterization of the 1A GR transcript, including its large 5′and 3′ untranslated segments, we have shown that only the 5′ untranslated end is unique. Expression of this transcript from an expression vector demonstrates that it contains the information necessary to encode a 150 KD protein which is identical in size and epitope recognition to that isolated from cellular plasma membranes. Transfection of mGR-less and GC-lysis resistant cells with 1A GR cDNA provides mGR expression and GC-sensitivity to these cells. The explanation of the protein's larger size and how this modifies its cellular location, leading to cell death, is still lacking and will be the

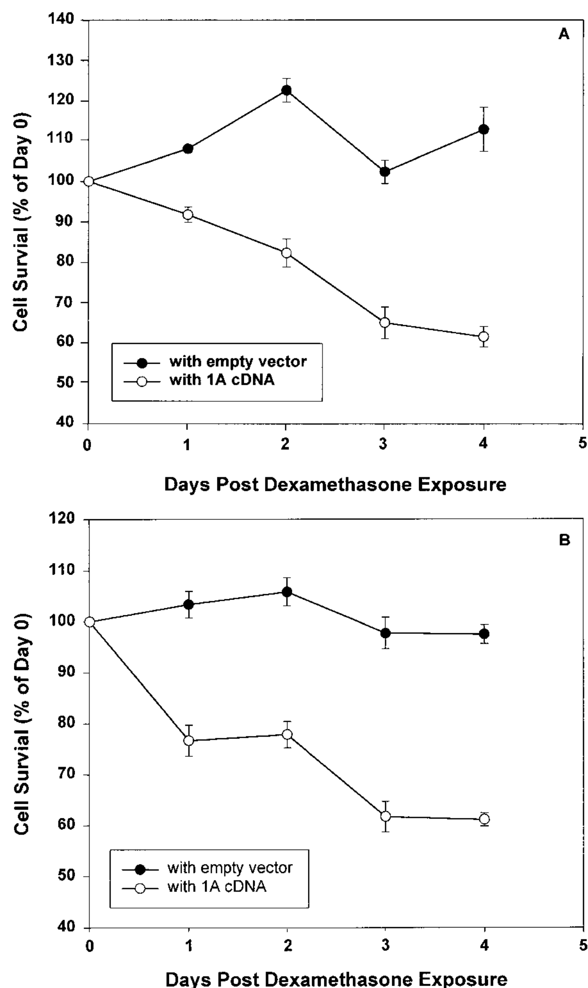

Fig. 8. Glucocorticoid sensitivity assay of transfected cells. For stably-transfected AtT-20 **(A)** and transiently transfected HL-60 cells **(B)** cell viability as a result of dexamethasone treatment was expressed as % of the day 0 cell number (cell survival). This result represents mean+SEM of six experiments with triplicate samples for each value assessed. (○) Cells transfected with full-length GR 1A cDNA. (●) Cell transfected with empty vector.

subject of future studies with the cloned 1A sequence.

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