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Multiple Glucocorticoid Receptor Transcripts in Membrane Glucocorticoid Receptor-Enriched S-49 Mouse Lymphoma Cells

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Abstract A cDNA library from plasma membrane glucocorticoid receptor-enriched (mGR^{++}) S- 49 mouse T lymphoma cells was screened with full-length rat intracellular GR (iGR) cDNA, BUGR-2 antibody, and PCR amplimers to portions of the mouse GR cDNA. One or two single-base substitutions resulting in amino acid changes (which do not incapacitate the receptor) were found in all but one clone: Val437 → Gly (located in the first zinc finger), and Glu546 → Gly (in the steroid-binding domain). Two previously unidentified exon 1 variants (1D and 1E), and two of three previously reported variants (1A, 1B) were found to be spliced onto the common exon 2. Exon 1D- and 1E-containing transcripts were confirmed by direct sequencing of amplimers from reverse transcriptase-coupled PCR. RNase protection studies revealed that one of these transcripts was expressed in mGR^{++} cells only, but not in two mGR-less (mGR^{--} S-49, and AtT-20 mouse pituitary) cell lines. These studies suggest that at least four promoters may be responsible for the control of GR (iGR and mGR) types in mouse lymphoma cells. J. Cell. Biochem. 74:418–429, 1999.

Key words: membrane glucocorticoid receptor; alternative RNA splicing; polymerase chain reaction; leukemia

Steroid hormone action in most cellular systems studied so far involves hormone entry into the cell and binding to nuclear receptors with subsequent modulation of transcription. However, rapid steroid effects previously reported in several cell types are not compatible with this classical scheme and it has been suggested that these effects can be produced by steroids acting at a novel type of steroid receptor on the plasma membrane [reviewed in Watson and Gametchu, 1999]. Such descriptions include a membrane glucocorticoid receptor (mGR) linked to G-protein mediated signal transduction [Orchinik et al., 1992]. In our previous studies, we used sequential immuno-separation techniques (immunopanning, fluorescent cell sorting, and soft agar cloning) to produce stable mGR-enriched and -deficient cell lines from wild-type S-49 lymphoma cells [Gametchu, 1987; Gametchu et al., 1991a,b). Using direct immunofluorescence staining with a monoclonal antibody raised against purified intracellular GR (iGR) of the rat [Gametchu and Harrison, 1984], we found an iGR-like protein in the plasma membrane of lysis-sensitive S-49 mouse lymphoma cells. A combination of affinity labeling, Western blotting, and autoradiography, revealed heterogeneity in molecular size of this membrane protein ranging from 85 to 145 kilodaltons. The presence of this membrane protein appeared to correlate well with dexamethasone-mediated cytolysis [Gametchu, 1987; Gametchu et al., 1993, 1994]. We found that although mGR and iGR differ in cellular localization, molecular size, and a slightly altered ability of other steroids to compete for glucocorticoid binding, they share several epitopes and many proteolytic cleavage sites.

In the past 10–15 years, iGR has been characterized in detail: the ~87 kD (predicted from cDNA sequence) to 97 kD (apparent size on

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SDS gels) hormone-binding protein has been purified to near homogeneity. Monoclonal and polyclonal antibodies have been produced and used to characterize several iGRs, and corresponding cDNAs have been isolated and sequenced including those from the rat [Miesfeld et al., 1984], human [Hollenberg et al., 1985], and mouse [Danielsen et al., 1986]. These data, together with those for other steroid hormone receptors, have allowed detailed characterization of the structure and function of these proteins. Studies on the structure and organization of the GR gene (both for iGR or mGR according to our findings) indicate that it is a complex genomic unit capable of directing the synthesis of multiple transcripts [Encio and Deteral-Wadleigh, 1991; Gearing et al., 1993; Strähle et al., 1992]. At least three transcripts have been previously demonstrated for this gene; these differ in that their 5′ untranslated regions (UTRs) are encoded by different exon 1's. In order to examine the molecular mechanisms underlying the regulation of the GR gene in relationship to the expression of mGR, we screened a cDNA library made from mGR-enriched S-49 mouse lymphoma cells. Our assumption was that mGR would have similar characteristics to iGR based on our past biochemical and immunological characterizations. The present work demonstrates multiple transcripts differing mainly in the 5′UTR, some whose sequences are reported for the first time, two coding region variants, and association of one message type with the expression of mGR.

MATERIALS AND METHODS

Cell Lines and Animals and Antibodies

Mouse S-49 T lymphoma, and AtT-20 pituitary tumor cell lines were obtained from the American Type Culture Collection (Rockville, MD). S-49 cells were further selected and stabilized for the expression of mGR for these studies using additional sequential cell separation techniques. These cells now have undergone immunopanning three times, fluorescent cell sorting twice, and soft agar cloning twice [Gametchu et al., 1991b]. Balb/c mice were purchased from Jackson Laboratories (Bar Harbour, ME). Our BUGR-2 monoclonal Ab was prepared from culture supernatants [Gametchu and Harrison, 1984].

Construction and Screening of cDNA Library

A cDNA library was constructed by InVitrogen (San Diego, CA) in the pcDNA II bacterial expression vector system using 5 μg of polyadenylated RNA from mGR-enriched S-49 mouse lymphoma cells [Gubler and Hoffman, 1983]. Both oligo-dT and random primers were used to initiate reverse transcription. The resulting library contained 1.8×10^6 independent recombinants with an average insert size of 1 Kb. Screening of the cDNA expression library was done with BuGR-2 hybridoma supernatant [Gametchu and Harrison, 1984] using an alkaline phosphatase-conjugate substrate kit (Bio-Rad, Richmond, VA). The antibody screening strategy was done to ensure that the cDNAs encoding the BUGR epitope, which recognizes both iGR and mGR, were not missed. This was to recover mGR clones in case the BUGR epitope also resided on a protein other than the known GR. For screening with the rat GR coding region a full-length coding region cDNA was provided by Dr. K. Yamamoto [Miesfeld et al., 1984]. Mouse cDNA fragments for hybridization screening were produced by PCR using clones from our library as template [see accompanying paper, Chen et al., this issue, 74:430–446.) Probes were ^{32}P-labeled by random-priming and stringent (68°C) hybridization conditions were used [Miesfeld et al., 1984; Northrop et al., 1986]. To confirm the existence of two suspected new alternatives for exon 1, the library was rescreened with a 242 bp probe generated by PCR with primers oligo P2 and oligo P3 (consisting of 41 bp of exon 1D sequence plus 201 bp of exon 2, Fig. 2), and moderate stringency hybridization conditions (58°C) followed by clone purification and rescreening with the more stringent conditions. Plasmid minipreps were sequenced in both directions (U.S. Biochemical Corp., Cleveland, OH, Sequenase Version 2.0 kit) using T7 gene 6 exonuclease for production of single-stranded templates [Straus and Zagursky, 1991]. Two alternative reverse primers, 5′-AAGGTAATTGTGCTGTCCTTCC (22-mer) and 5′-CACTGCGGCAATCACTTG (18-mer), were designed to confirm base changes at nucleotides 1310 and 1637, respectively. GCG (Madison, WI) software was used to analyze sequences.

Reverse Transcriptase-Coupled Polymerase Chain Reaction (RT-PCR)

Five different oligonucleotides were used (Figs. 2 and 3). Oligo P1 (5′-TGAACTCTTGGGATTCTC) and oligo P2 (5′-AGAATCCTCTGCTGCTTGG) corresponded to the nucleotide

sequences +436 to +453 and +170 to +188 downstream from the GR translation start site, respectively; oligo P3 (5′-CGCTAAGAGGG-TTTTGCATTCG) and oligo P4 (5′-CAGGA-CAGCCAGCTTTTTCC) contained the 22 nucleotides complementary to the specific 1D and 1E sequence of the 5′-UTR. Oligo P5 (5′-CGGATTC-TAAGTGGGTGGAACA) corresponded to the sequence between nucleotides -2515 and -2494 upstream of the initiator ATG of the mouse GR genomic sequence and was used for amplifying exon 1C. Total RNA was extracted from the mGR-enriched S-49 cells according to the acid-guanidinium-thiocyanate-phenol-chloroform method [Chomczynski and Sacchi, 1987]. Aliquots of this RNA (1–2 µg) were reverse transcribed using a kit supplied by Perkin-Elmer Cetus Corp. (Emeryville, CA) and 10 pmol of antisense primer (oligo P1), and then PCR amplified (40 cycles). For amplification of exon 1E, after 40 cycles of PCR amplification with oligo P1, the product was diluted and re-amplified using another nested GR-specific oligo, P2. The amplified DNA was analyzed on a 1.5% agarose gel, and directly sequenced as described above.

Isolation of cDNA by 5′ RACE

To obtain more sequence information of the 1D specific transcript in the 5′-UTR region we isolated poly(A)$^+$ RNAs prepared from S-49^{++} cells with Qiagen's Oligotex Direct mRNA kit (Chatsworth, CA) and then we synthesized cDNAs using Clontech's Marathon cDNA amplification kit . Briefly, adaptor ligated cDNAs were used as template for PCR amplification, using a primer complementary to the adaptor and the 1D specific primer (position is given relative to the translation initiation site ATG): P75 (5′-CAU CAU CAU CAU TTC GGA GGA AGT TGC ACG GCG AAT G-3′, -14/-38). Then long-distance PCR was performed in a standard mixture [Sanchez et al., 1998]. The 5′RACE products were cloned into pAMP1 vector following the manufacturer's procedures (Gibco BRL, Gaithersburg, MD) and screened by hybridization with the PCR-generated 242 bp exon 1D-exon 2 fragment (P2 → P3, described above). More than 20 positive clones were isolated for further PCR and size determination analyses. The clone with the longest insert was sequenced.

Northern Analysis

RNA was isolated from mGR-enriched S-49 cells using the LiCl-urea precipitation technique of Auffray and Rougeon [1980] and mRNA was isolated on an oligo-dT cellulose column [Aviv and Leder, 1972]. Samples (3 and 6 µg) were electrophoresed on a 1% formaldehyde gel in MOPS buffer [Thomas, 1980], followed by capillary blot transfer to Gene Screen Plus (DuPont-NEN, Boston, MA). Samples from oligo dT column flow-through were resolved in a separate lane to serve as negative control for hybridization. The gel was stained with ethidium bromide to view size markers (both *E. coli* and sample residual ribosomal bands) and to inspect the total amount of RNA in each lane to assure accurate loading. We also visualized the amount of total RNA present after capillary transfer (ethidium bromide staining of the hybridization filter to which the RNA has been transferred). The blot was prehybridized and then hybridized (according to the Gene Screen Plus manufacturer's directions) with nick translated, ^{32}P-labeled [Leroy et al., 1996] full-length rat GR coding sequence cDNA provided by Dr. K. Yamamoto [Miesfeld et al., 1984].

RNase Protection Assay

The representation of variant transcripts in these cell lines and tissues was examined using an RNase protection assay [Cheifetz, 1998]. Tissues were harvested from Balb/c mice (adrenal gland, brain, heart, kidney, liver, lung, skeletal muscle of the limb, pancreas, spleen, and thymus) and total RNA was prepared by the single-step acid guanidine thiocyanate phenol-chloroform extraction method [Chomczynski and Sacchi, 1987]. Labeled RNA probes complementary to part of each target RNA (including a unique portion) were synthesized by creating PCR-generated templates. To do this, transcript-specific primers in the 5′UTR were paired with a single downstream primer in the coding region (oligo P2, Figs. 2 and 6, panel I). The SP6 phage promoter sequence was appended to the downstream PCR primers and thus incorporated into the PCR product [Stoflet et al., 1988]. The antisense RNA probes were generated by in vitro transcription of PCR products using SP6 polymerase in the presence of [^{32}P] UTP (3000 Ci/mmole, Amersham, Arlington Heights, IL). Then these ^{32}P-labeled RNA probes were mixed with the sample RNA and incubated and assayed according to the protocol supplied by the manufacturer of the kit (Ambion, Austin, TX) on a 5% denaturing urea-polyacrylamide gel, visualized by autoradiography [Stoflet et al., 1988].

RESULTS

Point Mutations in the Coding Region of GR

Our cDNA library prepared from mRNA of mGR-enriched mouse S-49 cells and screened under high-stringency conditions with GR cDNAs resulted in a total of 77 cDNA clones that we sequenced in both directions (Fig. 1). Since the cDNA library was amplified before screening, several identical clones were isolated, as shown. Overlapping clones spanned the open reading frame of 2349 bp previously reported [Danielsen et al., 1986]. Comparison with the published mouse GR sequence [Danielsen et al., 1986] revealed that the sequence in the open reading frame of GR cDNA was exactly the same as that reported for mouse lymphoma cells, except that there were two single base changes from the wild-type sequence. All 15 clones (excluding duplicates) spanning the region around nt 1310 showed a T → G transversion. This mutation codes for a glycine instead of a valine at amino acid position 437 of the protein, in the first zinc finger region of the DNA-binding domain. Five out of six independent clones had a second mutation (A → G transition at residue 1637); this change resulted in the replacement of glutamic acid by glycine at amino acid position 546 in the steroid-binding domain. Only one clone had the wild-type sequence at this latter position. Thus mGR-enriched S-49 cells express two different types of GR gene products and this perhaps relates to heterozygosity at this locus. Epitope selection was also used to identify GR clones and corroborate cDNA selection methods. Five independent clones were isolated. The BuGR-2 epitope maps to the 3′ end of exon 2 (at ~nt 1183–1231), coding for mouse GR amino acid residues 395–411 (407–423 in the rat GR sequence) at the amino-terminal side of the DNA-binding region [Rusconi and Yamamoto, 1987]. Nucleotide sequencing of all of these cDNA clones confirmed the same base change at nucleotide 1310.

Transcript Heterogeneity at the 5′ Nontranslated Region of GR

To examine the structure of the 5′ noncoding region of GR, we further screened the cDNA

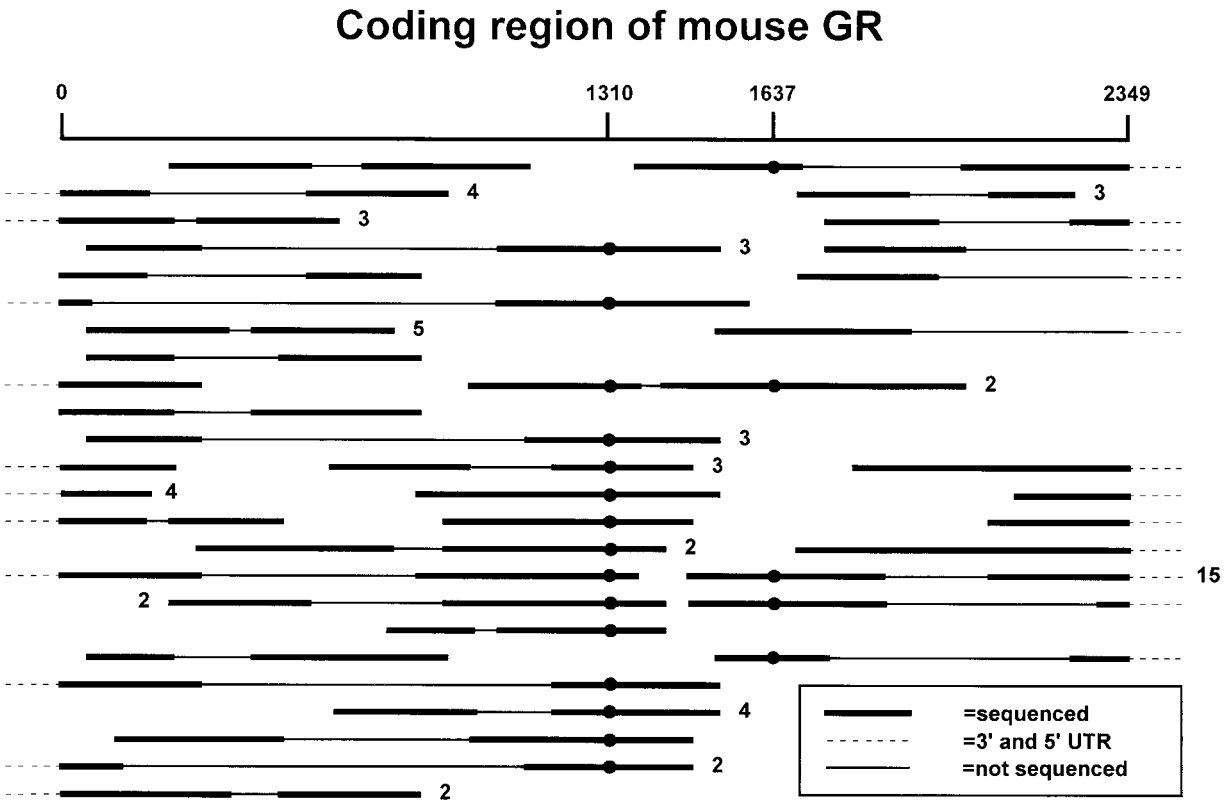

Fig. 1. Point mutations in the coding regions of GR cDNAs isolated from a mGR-enriched S-49 cell cDNA library. A diagram of 82 clones and their relative positions on a composite map. Closed circles represent positions of single nucleotide substitutions (the same in each case, see text). Numbers at the top refer to the base substitution positions. The numbers at the right of each sequence represent the number of identical clones isolated.

libraries with the PCR-generated 242 bp fragment containing 41 bp of exon 1D sequence and 201 bp of adjacent downstream exon 2 sequence. Eleven unique clones containing 5′-UTR GR sequence were isolated; these transcripts were not identical, as shown by the sequence in Table I. All 11 clones had the 13 nt identical sequence upstream of the translation initiation codon, but diverged in sequence further upstream. The divergence corresponds to the position of an intron-exon junction in both the human and mouse GR gene [Encio and Deteral-Wadleigh, 1991; Zong et al., 1990]. Comparison with the published genomic sequence of the mouse GR gene [Strähle et al., 1992] showed that two of our sequences were identical to the published sequence (nt −3496 to −3327 upstream from the ATG designated exon 1B, and sequence −32 kb upstream of the ATG termed exon 1A [Strähle et al., 1992]). Interestingly, two of our cDNA clones contained sequence identical to the published flanking genomic sequence at position −3643 to −3628. This sequence was also alternatively spliced onto the common exon 2 in our transcripts (Figs. 2,3). The clones representing this area contained 39 bp (containing the 13 nt sequence which extends from the splice site to the translation

TABLE I. Summary of Cloned cDNA Sequence From 5′ Untranslated Region[a]

cDNA Clone#	Splice position, bp		Nucleotide sequence	
			Exon 1	Exon 2
R7AB	~32 kb/−13	1A	...TGGGAGGAAG	TTAA...CAATG...
F29	~32 kb/−13	1A	...TGGGAGGAAG	TTAA...CAATG...
F79	~32 kb/−13	1A	...TGGGAGGAAG	TTAA...CAATG...
31892	~32 kb/−13	1A	...TGGGAGGAAG	TTAA...CAATG...
R60	~32 kb/−13	1A	...TGGGAGGAAG	TTAA...CAATG...
M5P1	~32 kb/−13	1A	...TGGGAGGAAG	TTAA...CAATG...
M5P2	~32 kb/−13	1A	...TGGGAGGAAG	TTAA...CAATG...
R34B	−3628/−13	1D	...TCCTCCGAAT	TTAA...CAATG...
R56	−3628/−13	1D	...TCCTCCGAAT	TTAA...CAATG...
AB5	−3327/−13	1B	...CGGGCTCACA	TTAA...CAATG...
E17	−1705/−13	1E	...AACTCAACAG	TTAA...CAATG...

[a]Exon border sequences of 11 independently isolated cDNA clones from the cDNA library of mGR-enriched S-49 cells. Splice positions of exons 1A, 1B, 1D, and 1E are relative to genomic sequences. Dotted lines indicate upstream and downstream sequences not shown. Numbering of nucleotides is relative to the mouse GR cDNA with the A of the start codon ATG in exon 2 designated as nt +1. The ATG start codon is underlined.

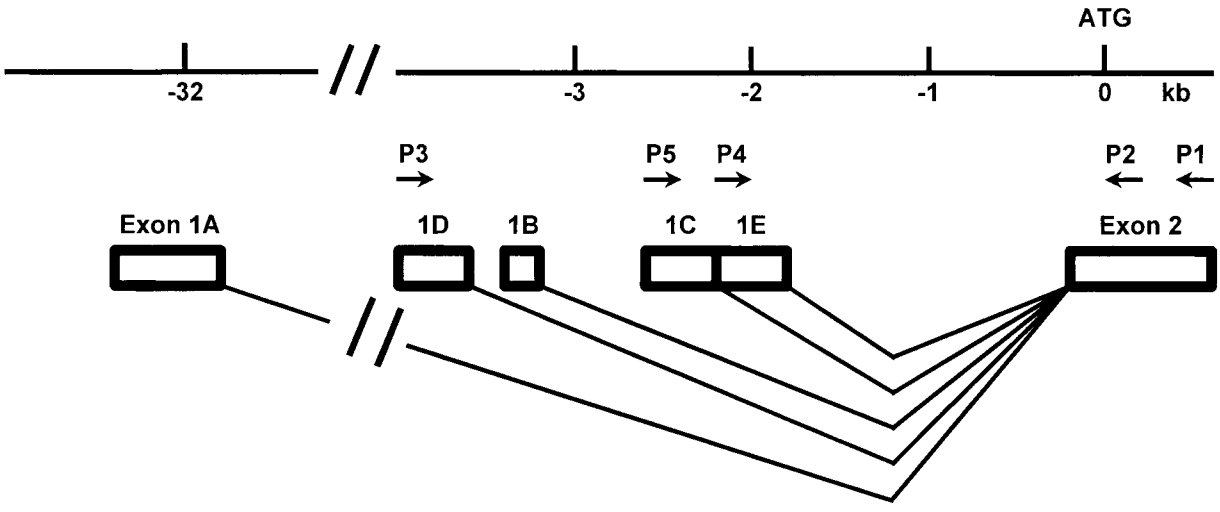

Fig. 2. Schematic representation of the mouse GR transcriptional start site region. Exons 1A-E, and exon 2 are boxed. The exons are spliced onto the same exon 2 at position -13 with respect to the translation start site (+1). The position and orientation of RT/PCR primers used (P1–P5) are indicated by arrows. Four of these 5′ UTR cDNA variants (A,B,D,E) are represented in our library. The portions of the peptide-coding domains depicted by this cartoon (exon 2) are identical in all four RNA species found in our library.

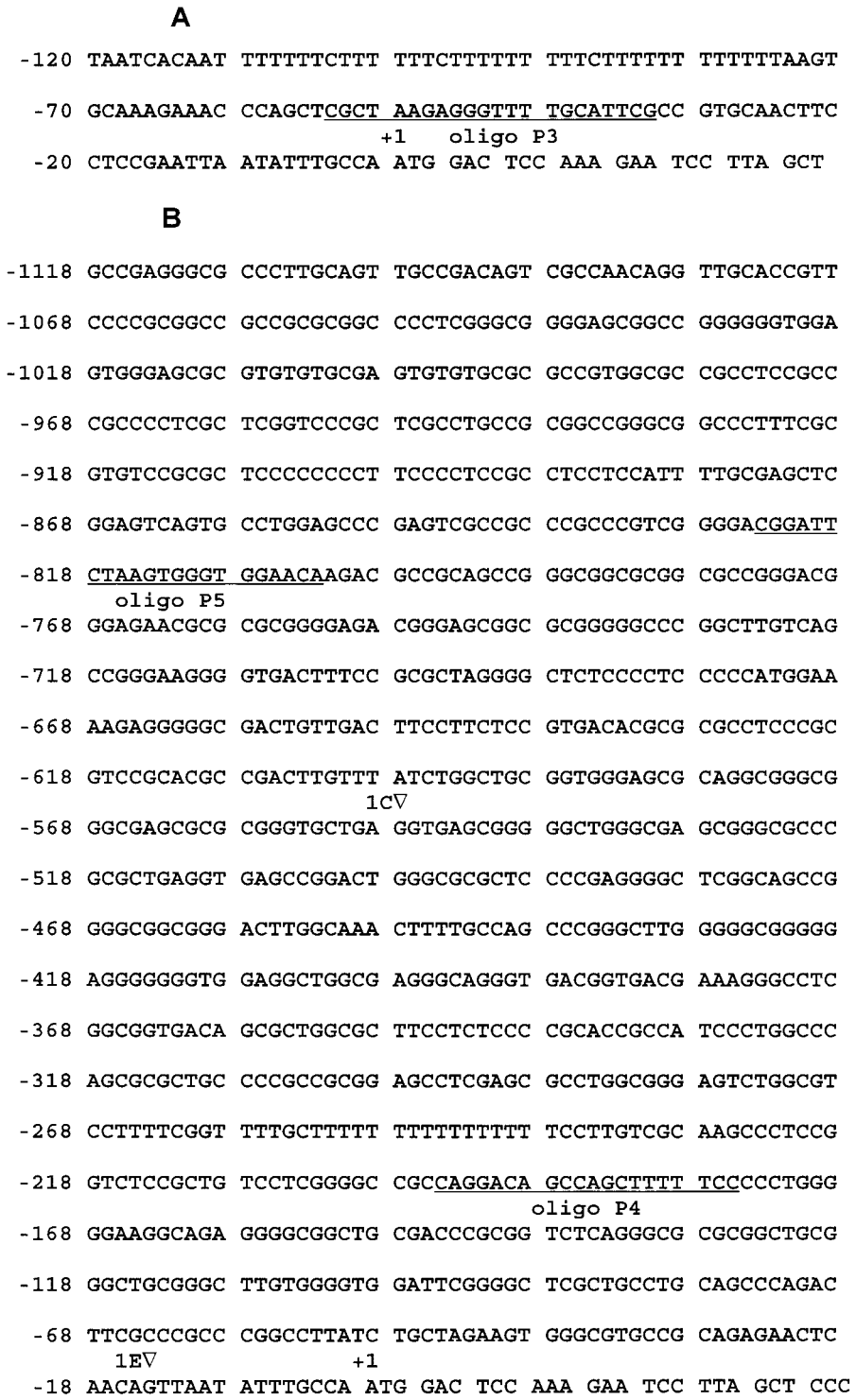

Fig. 3. Nucleic acid sequence of cDNA encoding mouse GR exons 1D and 1E 5′ UTR. **A:** 1D cDNA sequence. **B:** 1E cDNA sequence. The nucleotides are numbered in the 5′ to 3′ direction with the A of the translation initiation start codon ATG designated as nt +1. The oligonucleotides (P3, P4, and P5) used for RT/PCR are underlined. The open triangle indicates the splicing sites of exons 1D, 1E, and previously reported exon 1C.

start site) of the 5′-UTR and 827 bp of the coding region. Using 5′RACE, this sequence was further extended by 81 nucleotides. We named this previously unidentified exon 1D (Figs. 2,3A).

Another clone (E17, Table I) contained 302 bp of mouse GR coding region flanked by 1118 bp of 5′ UTR (Fig. 3B). The protein-coding portion of this cDNA clone was identical to the reported mouse GR cDNA sequence [Danielsen et al., 1986]. The 5′-UTR portion contained previously reported GR genomic sequence spanning nt −2809 to −1705. There was an intron of 1.7 kb between this 5′ UTR and exon 2 (data not

shown). We believe that this 5′-terminal sequence represents a portion of an alternatively spliced GR mRNA because the 3′-end of the sequence also terminates at the known splice junction site of the common exon 2 with the elimination of the 1691 bp intronic sequence. We named the sequence present in this new transcript exon 1E. This clone also contained a portion of the previously reported exon 1C cDNA sequence contiguously followed by genomic sequence nt −2238 to −1705; this suggests that the splicing of exon 1C did not occur in mGR-enriched S-49 cells.

In order to confirm the presence of the new 1D and 1E sequences, total RNA from S-49 mGR-enriched cells was isolated, reverse transcribed, and then amplified by PCR using four different oligonucleotides (Figs. 2,3). An amplification product of 508 bp was detected with exon 1D-specific oligos P3 and P1 primer pairs, and a DNA fragment of 383 bp was generated with oligo P2 and 1E-specific oligo P4 (Fig. 4). This resulted in the 5′ end extension of the 1D sequence to 41 bases, and then sequence of selected clones from 5′RACE further extended this sequence to 108 bases. Both amplimers corresponded to the expected fragment sizes. These results confirmed the cDNA library data, demonstrating that splicing of the newly described exons 1D and 1E to exon 2 occurs in mGR-enriched S-49 cells. RT/PCR analysis performed with a primer specific for exon 1C (oligo P5), did not yield any expected amplification products (data not shown), confirming the absence of this sequence in the RNA mGR-enriched S-49 cell population.

The existence of these different upstream transcripts was further investigated by Northern blot analysis of mRNA from our current mGR-enriched S-49 cells that have now been selected by sequential techniques multiple times. A representative blot hybridized with ^{32}P-labeled rat GR coding region cDNA is shown in Figure 5. Two broad size ranges of GR mRNA transcripts (centering on approximately 6 and 8 kb) were found as previously reported [Gametchu et al., 1994; Miesfeld et al., 1984; Northrop et al., 1986] for other cell preparations.

RNase Protection Studies

RNase protection assays were used to study the representation of the four variant transcripts in mGR^{++}, mGR^{--}, mouse pituitary tumor (AtT-20 cells, previously shown to be lacking mGR despite high iGR content [Gametchu, 1987]) cells, as well as several Balb/c mouse tissues (adrenal gland, brain, heart, kidney, liver, lung, muscle, pancreas, spleen, and thymus). Figure 6, panel I, shows a schematic depiction of the four different probes (for 1A, 1B, 1D, and 1E). All transcripts contained an

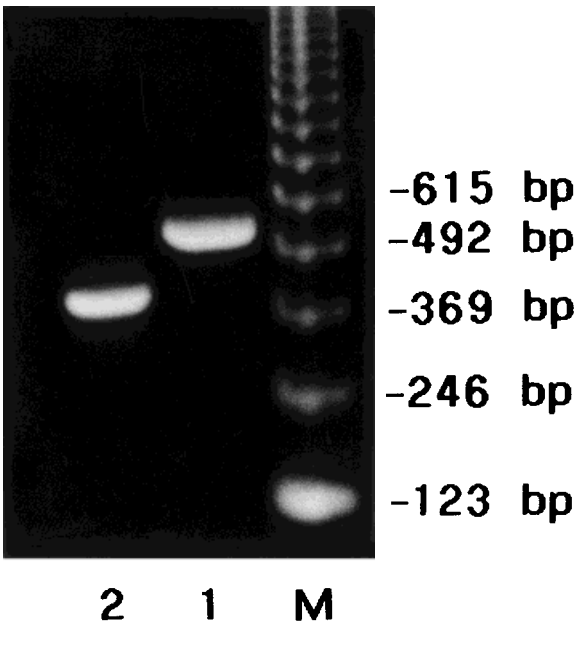

Fig. 4. PCR amplification products. DNA fragments were generated by RT/PCR from total RNA prepared from mGR-enriched S-49 cells: **lane 1** amplified with oligos P1/P3; **lane 2** amplified with oligos P2/P4. Size markers are 123 bp ladders.

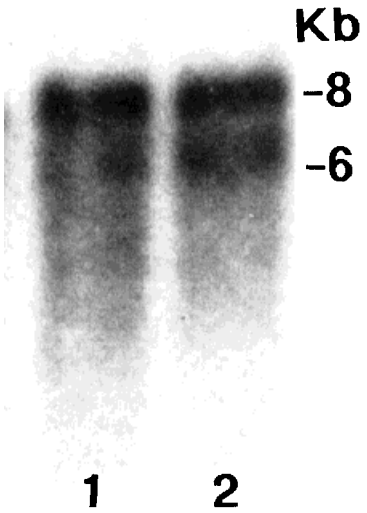

Fig. 5. Northern analysis of GR mRNA from mGR-enriched S-49 cells. **Lane 1:** 3 µg; **lane 2:** 6 µg. Poly(A) RNA was probed with ^{32}P-labeled rat GR cDNA and exposed to film for 3 days.

identical 201 base pairs towards the 5′ end, but diverged in sequence further upstream starting from the common splice site (at −13 nt). This analysis revealed that transcripts 1B, 1D, and 1E were expressed in mGR^{++}, mGR^{--}, and AtT-20 cells at varying levels, with the mGR^{--} cells containing higher RNA concentrations of these three transcripts (panel II). However, transcript 1A was predominantly expressed in mGR^{++} cells, and barely detectable in AtT-20 and mGR^{--} cells. Panel III shows similar studies in various mouse tissues. All transcript variants were expressed at varying levels in all tissues, with the liver and muscle containing the highest concentrations. Except for these two tissues, transcript 1E is the least expressed, and barely detectable in the adrenal gland.

DISCUSSION

To study sequences encoding GR proteins in cells enriched for mGR [Gametchu, 1987], we constructed a cDNA library from these cells. The library was screened with iGR cDNAs, PCR-generated probes, and an antibody recognizing an epitope on the iGR. Comparison with the published mouse GR coding sequence reveals an exact nucleotide sequence match [Danielsen et al., 1986], except that there are two single base changes from the wild-type sequence. The first T → G substitution at residue 1310 (which changes a Val to a Gly at position 437 of the protein), is observed in all the clones spanning this point. Because it is also present in other cell lines [Kasai, 1990], this position appears to be polymorphic. Analysis of the transcriptional activity of GR by Danielsen et al. [1986] reveals that the variant receptor containing the valine to glycine substitution (pSV2NA5′rec) supports a dexamethasone-inducible response, and has no effect on the receptor's ability to bind hormone. Extensive mutagenesis studies of the DNA-binding domains of the GR have indicated that changing amino acids that are conserved across species and receptor class lines do not necessarily result in inactive receptors. For instance, a His at position 439 of the mouse GR is present at the equivalent position of all steroid/thyroid hormone receptors cloned, yet mutation to Tyr results in a receptor with apparently normal transcriptional activity [Severne et al., 1988]. These results are consistent with our previous observations that the GR in mGR-enriched S-49 cells is functional and has the ability to mediate the lytic response to glucocorticoids [Gametchu, 1987].

The second single base substitution is an A → G transition at residue 1637, which results in the replacement of Glu by Gly at position 546. This change in the steroid binding domain of the GR exists in all the clones but one that include this region. Previous expression analysis of mutant clones indicated that this substitution eliminates the receptor's hormone binding activity and function [Danielsen et al., 1986]. This finding disagrees with our results on function [Gametchu, 1987] and binding [Gametchu et al., 1991a,b, 1994]. A possible explanation for this discrepancy could be that the mutation leads to a small alteration in receptor activity due to poor translation efficiency or rapid receptor degradation in certain systems. The transfected sequence may not have produced sufficient receptor in the other system to mediate hormonal induction of CAT activity from the mouse mammary tumor virus-long terminal repeat. Another possibility is that the correct amino acid at this position might be important to maintain iGR function but less important for the mGR. Finally, it is possible that a mutant GR allele paired with a wild-type allele may still produce enough functional protein for some activities and appear phenotypically normal.

Computer analysis of these alternative coding sequences produced no obvious explanation for why mGR is larger in apparent size (several species of 112–150 KD vs. 97 KD for wild-type iGR [Gametchu, 1987]). The two amino acid substitutions and their surrounding sequences code for no known plasma membrane-targeting signals. However, the field of protein targeting is rather young, and the mechanisms by which cells target their proteins to cellular compartments are complex. In some cases multi-compartmentalized isoforms of peptide hormones are made from the same gene yielding products with specific targeting sequence [Sotiropoulos et al., 1994]. Several other mechanisms exist to encode and differentially express multiple types of site-specific information, including alternative forms of transcription initiation, translation initiation, splicing, and post-translational modification. In one example [Rogers et al., 1980], IgM antibodies, synthesized by B lymphocytes, are found either anchored on the plasma membrane or secreted due to a combination of alternative pre mRNA cleavages and splicing.

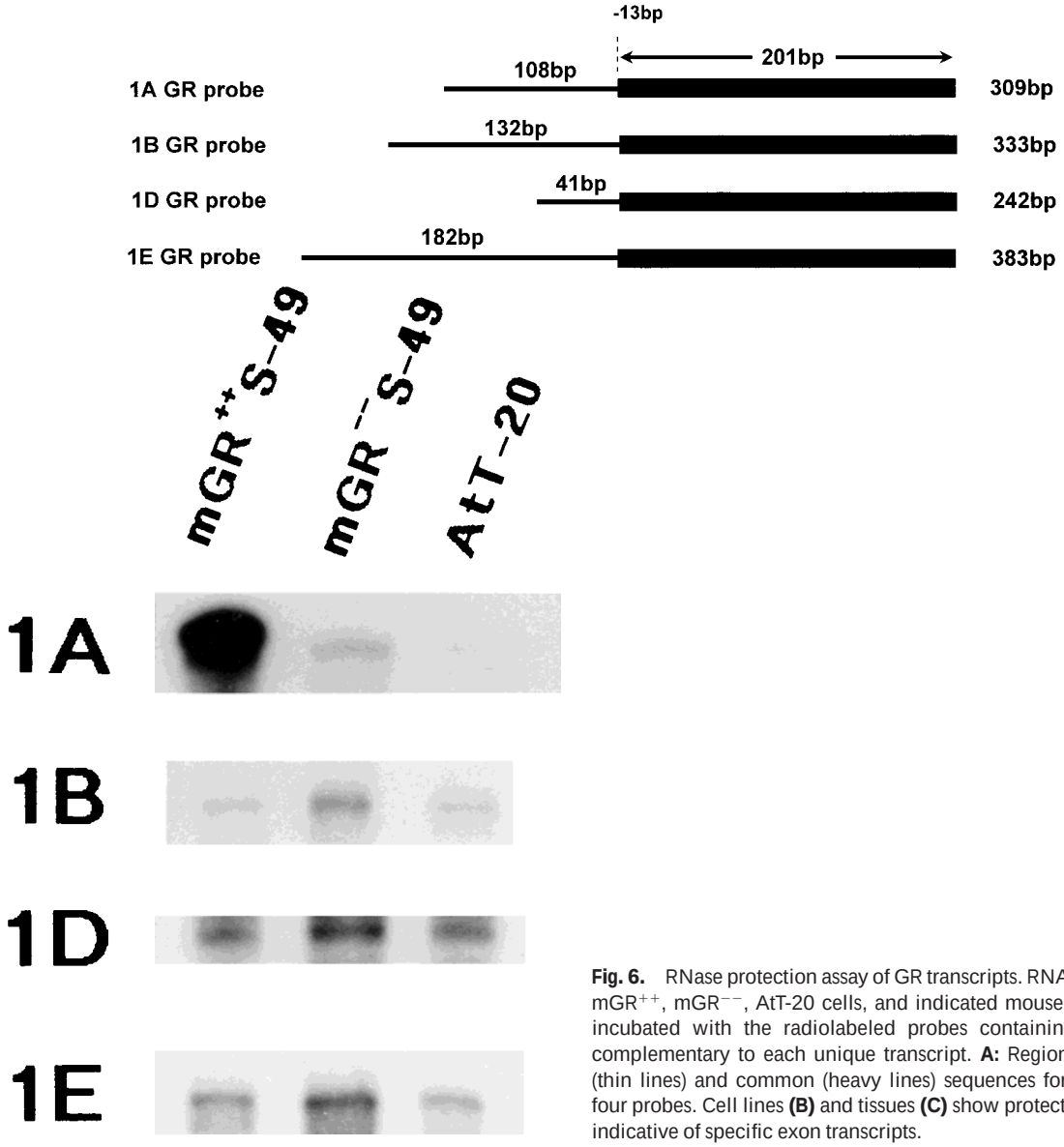

Fig. 6. RNase protection assay of GR transcripts. RNA (25 μg) from mGR^{++}, mGR^{--}, AtT-20 cells, and indicated mouse tissues were incubated with the radiolabeled probes containing sequences complementary to each unique transcript. **A:** Regions of specific (thin lines) and common (heavy lines) sequences for each of the four probes. Cell lines **(B)** and tissues **(C)** show protected fragments indicative of specific exon transcripts.

In another example, a novel growth factor [Decker, 1990] is created when a splice failure gives the membrane version an entirely new peptide sequence at the C-terminus, making this protein resistant to proteolytic cleavage and thus membrane-anchored. We shall continue to review this literature to see if any new examples explain the localization of mGR.

Expression differences between mGR and iGR could be generated by alternative promoters for the same gene or by gene multiplicity. Several members of the steroid/thyroid/retinoic acid receptor gene family are reported to be transcribed from multiple promoters. For instance, two distinct promoters generate transcripts encoding the two functionally different human progesterone receptor forms A and B, which differ by 128 amino acids at the amino terminus [Kastner et al., 1990]. Likewise, the human estrogen [Grandien et al., 1997] and mineralocorticoid [Kwak et al., 1993; Massaad et al., 1997; Zennaro et al., 1996] receptors are transcribed from multiple promoters in a tissue specific manner. For both retinoic acid receptors and thyroid hormone receptors diversity resides in a combination of multiple genes and alternative splicing of mRNA [Lazar et al., 1990; Mangelsdorf et al., 1990; Murragy et al., 1989; Zelent et al., 1991]. The human GR gene has previously been reported to encode two isoforms of iGRs generated by alternative splicing [Encio and Deteral-Wadleigh, 1991], with only

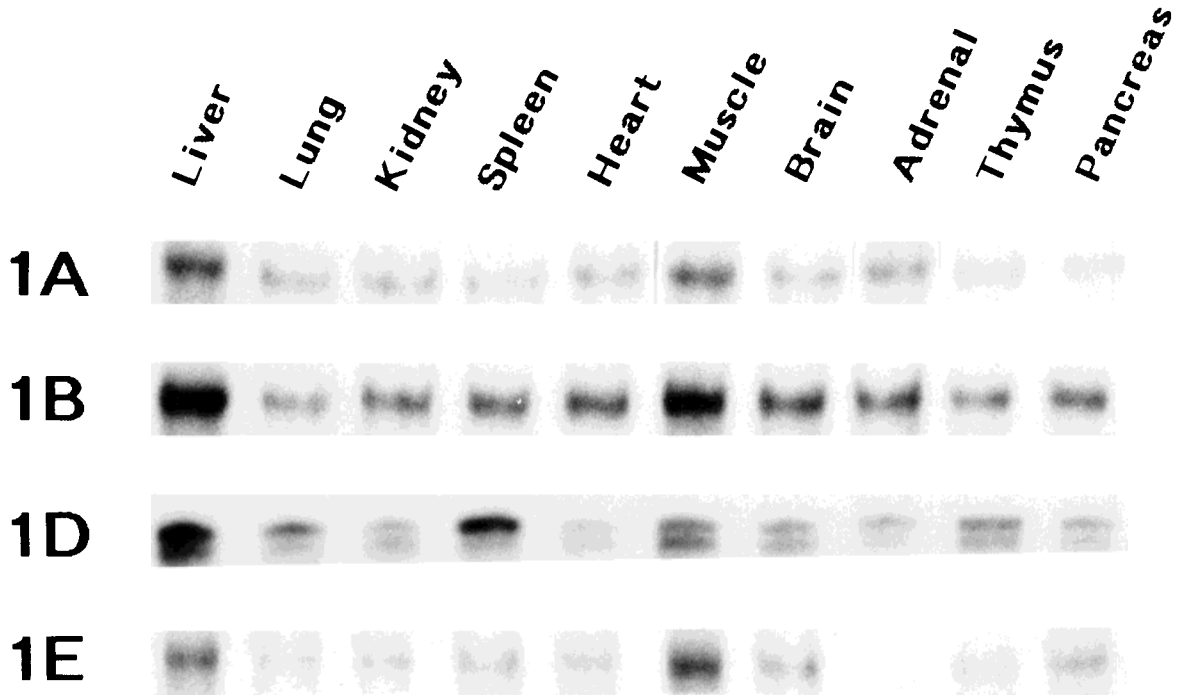

Figure 6. (Continued.)

the α form showing ligand-binding activity [Hollenberg et al., 1985]. Strähle et al. [1992] have found that the mouse GR gene encodes at least three transcripts (named 1A, 1B, and 1C, respectively) with different 5′ ends that are alternatively spliced onto a single splice acceptor site 13 bp upstream of the ATG in exon 2. A more recent report by Gearing et al. [1993] also showed that all three transcript variants (1A, 1B, and 1C) are present in the rat, indicating that its genomic organization is similar to that of the mouse. In our study, 1A and 1B were also found in the GR cDNAs isolated from mGR-enriched S-49 cells. In addition, cDNAs representing two previously unidentified exons, 1D and 1E, were found. Both are also alternatively spliced onto a common exon 2 conforming to the GT/AG rule. However, the exon 1C form characterized for the mouse and rat GR gene [Strähle et al., 1992], and previously reported to correspond to the human GR exon 1 [Zong et al., 1990], was not represented in our library. In the rodent study, it was found that transcripts arising from the three promoters for 1A, 1B, and 1C differed in their expression in various tissues and cells; promoters 1B and 1C were active in all tissues and cells tested, albeit at different intensities. Transcripts from promoter 1A were present in the T-lymphoma cell lines S-49 and WEHI-7 and were detectable in thymus, but were not detectable in liver and brain. The high activity of promoter 1A in these T-lymphoma cells is in agreement with the abundance of 1A-containing GR cDNAs (seven out of 11) isolated from our mGR-enriched, S-49 T-lymphoma cells. At present it is not known if these changes in exon usage are due to promoter usage, alterations in the concentration or activity of splicing factors, or differences in stability of the respective mRNAs.

The functional significance of transcript heterogeneity at the 5′end of these mRNAs is not known. The relationship between the structure and activity of these promoters (1A through 1E) and the specific functions of iGR and mGR are still unclear. However, differential expression of the four variant transcripts observed in our three cell groups (selected for the presence or lack of mGR, and sensitivity to GC-mediated cell lysis) suggests a role in mGR/iGR regulation. While transcripts 1B, 1D, and 1E were expressed in all cell groups (mGR^{++}, mGR^{--}, and AtT-20), transcript 1A was expressed predominantly in mGR^{++} cells, an established cell line for elevated mGR content and enhanced sensitivity to the killing effects of GC. These data lead to the more complete description of this particular alternate form of RNA [see ac-

companying paper, Chen et al., this issue, 74: 430–446].

To offer evidence of the different GR transcripts in our now highly-selected mGR^{++} cell line, we analyzed GR message sizes by Northern analysis. The sizes of the hybridizing bands were broad and probably include several comigrating GR mRNA species. The products of all alternative splicing events could be of approximately equal (exons 2–9 are the same), but not identical, size. Since the exact sizes of all alternative exon sequences are not known, we cannot determine if these alternative transcripts explain the difference between the 6 and 8 kb bands, or the broadness of the bands in these or other published blots [Gametchu et al., 1994; Miesfeld et al., 1984; Northrop et al., 1986]. The 7.3 kb full-length 1A transcript [see accompanying paper, Chen et al., this issue, 74:430–446] must be one of the species present in the population of sequences represented by these broad bands.

In conclusion, we have shown that mGR-enriched S-49 cells contain a heterogeneous population of GR RNAs. The 5′ UTRs of the mouse GR RNAs in these cells are composed of at least five alternative exons that undergo four or more alternative splicing events, giving rise to at least four transcripts which may encode the iGR and/or mGR. The functions of these alternative transcripts and their specific relationship to iGR vs. mGR are continuing subjects of our investigations.

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