Proc. Natl. Acad. Sci. USA
Vol. 89, pp. 6731–6735, August 1992
Biochemistry

At least three promoters direct expression of the mouse glucocorticoid receptor gene

(DNase I hypersensitive sites/T lymphocytes)

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Communicated by Elwood V. Jensen, March 23, 1992

ABSTRACT **We have characterized the gene for the mouse glucocorticoid receptor. The gene spans ≈110 kilobases, and glucocorticoid receptor transcripts are assembled from nine exons. Expression of the gene is controlled by at least three promoters, resulting in glucocorticoid receptor transcripts with different 5′ nontranslated exons. One promoter is cell-specific, found to be active only in T lymphocytes. The other two promoters are active to various degrees in all cell lines and tissues so far analyzed and are located in a CpG island. The promoter activities are accompanied by DNase I hypersensitivity sites in chromatin. In contrast to a conservation of exon–intron structure, differences in promoter organization suggest a divergence between the evolution of regulatory and coding regions among members of the steroid receptor superfamily.**

Glucocorticoid hormones control a variety of anabolic and catabolic processes in a wide range of cells (1). In addition to their function in intermediary metabolism (for example, gluconeogenesis), glucocorticoids are involved in developmental processes such as differentiation of the adrenal medulla (2), programmed cell death (apoptosis) of lymphocytes (3), and perinatal activation of liver-specific functions (4). These effects are mediated by the intracellular glucocorticoid receptor (GR) that is activated by the binding of the hormone to induce or repress expression of specific target genes (5, 6). The receptor binds to partially palindromic glucocorticoid response elements of ≈15 base pairs (bp) situated in the regulatory region of target genes (7–9). The GR was one of the first transcription factors whose cDNA was cloned from mammals (10–13). Subsequent cloning of the cDNAs for other receptor proteins showed that the GR is a member of a superfamily of structurally related ligand-activated regulators of transcription (6, 14).

As a reflection of the variety of processes controlled by glucocorticoids, the receptor is found in many cell types and tissues, but its level varies considerably among different tissues (15). In addition, *in situ* hybridization studies that have shown variations in the level of GR mRNA within different areas of the hippocampus (16) demonstrate a pronounced variation of GR gene expression even within a single tissue. Also, the expression of the GR gene has been found to be either autorepressed (17, 18) on the transcriptional level or autostimulated (19) in a cell-type-dependent manner by the ligand itself. To study this differential expression in various target tissues, we characterized the mouse GR gene.§ Three promoters with different cell specificities were identified that may be partly responsible for the independent control of the GR gene in different tissues.

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EXPERIMENTAL PROCEDURES

Isolation of the Mouse GR Gene and a Mouse GR cDNA. λ genomic libraries prepared from DNA of c^{ch}/c^{ch} and c^{14CoS}/c^{14CoS} mice, partially *Sau*3A-digested and cloned into the *Bam*HI site of λDashII, were screened with the three ^{32}P-labeled RNA probes described below. Overlaps between positive λ phage were established by partial DNA restriction mapping (20, 21) and Southern blot analysis (20). Phages λ4.15, λ2.9, λ5.13, and λ5.15 were isolated from the c^{14CoS}/c^{14CoS}-derived library (22), whereas λ11A and λMG21 were from separate c^{ch}/c^{ch} libraries (23). A mouse GR cDNA clone, designated mGR6, was isolated from a newborn mouse liver λZAP cDNA library screened with probe A (described below).

cDNA and Genomic DNA Probes for Library Screening. Probe A was a *Sal* I–*Hin*dIII fragment from position +419 to position +935; probe B was a *Hin*dIII fragment from position +1300 to position +2378 of a GR cDNA (12); and probe H was a 412-bp 5′-end *Eco*RI–*Dra* II fragment from a mouse lymphoma library (ref. 24 and R. Miesfeld, personal communication). Numbering is relative to the initiation ATG of the mouse GR cDNA, with the A designated +1. Three genomic probes derived from phage isolated in this work were used for Southern blot and pulsed-field gel electrophoresis (PFGE) analyses. Probe C was derived from an *Eco*RI fragment at the 5′ end of λ5.13. Probe F was derived from the *Eco*RI fragment of λ4.15 containing exons 1B, 1C, and 2. Probe G was derived from the 2.5-kilobase (kb) *Eco*RI–*Xba* I fragment from λMG21 that extends 3′ from the *Xba* I site within exon 1A. For the RNase protection analysis, RP1 was an 895-bp *Ssp* I–*Xba* I fragment from λMG21, RP2 was a 437-bp *Stu* I–*Sma* I fragment from λ4.15, and RP3 was a 328-bp *Not* I–*Apa* I fragment also from λ4.15.

DNA Sequencing. DNA sequences were determined by the dideoxynucleotide chain termination method using deoxyadenosine 5′-[α-[^{35}S]thio]triphosphate and T7 DNA polymerase (25).

PCR. Polyadenylylated RNA (5 μg) was transcribed by avian myeloblastosis virus reverse transcriptase (40 units; Boehringer) into cDNA by using random hexanucleotides (1 μg) as primers and the cDNA was then used for a PCR (26). PCR primers were P2 (5′-CTGCTGGGGACTTCGTCTCT-3′) with P1A (5′-GAGCTACTCTGCGTAAGAAT-3′), P1B (5′-GGGGACCTGGCAGCACGCGAGT-3′), or P1C (5′-

Abbreviations: GR, glucocorticoid receptor; PFGE, pulsed-field gel electrophoresis; nt, nucleotide(s).

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§The sequence reported in this paper has been deposited in the GenBank data base (accession no. X66367).

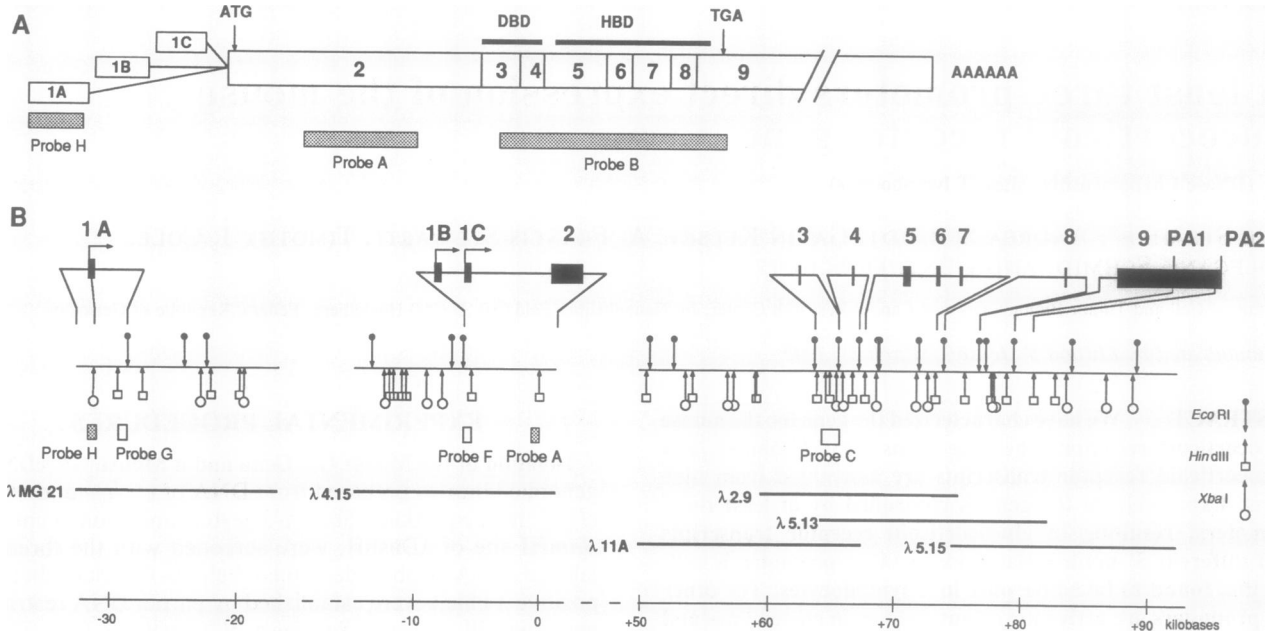

FIG. 1. Structure of the mouse GR gene. (*A*) A schematic representation of the mouse GR mRNA and the probes used to isolate the chromosomal gene. Derivation of the eight coding exons (exons 2–9) and the three alternative 5′ exons (1A–1C) is based upon data in Table 1. Regions encoding the DNA binding (DBD) and hormone binding (HBD) domains, and locations of the translational start and stop codons are indicated. Probes used are indicated below by stippled bars. (*B*) Genomic organization of the eight coding exons (exons 2–9) and three alternative 5′ exons (1A–1C) of the mouse GR gene. Six phage isolated with the GR cDNA are indicated by heavy lines and labeled λMG21 to λ5.15. Restriction map for *Eco*RI, *Hin*dIII, and *Xba* I is indicated, above which are shown the subcloned *Eco*RI restriction fragments used to localize exons (depicted as solid bars). Horizontal arrows indicate start sites for transcription and PA1 and PA2, two putative polyadenylylation signals. Probes C, F, and G (open bars) are single-copy genomic fragments used elsewhere in this work; the location of two cDNA probes, A and H, is also shown (stippled boxes). The scale (kb) at the bottom is based upon the restriction map and the PFGE analysis given in Fig. 2.

ACTGTTGACTTCCTTCT-3′). Fragments were amplified and analyzed as described (20).

RNase Protection. Poly(A)$^+$ RNA (20 μg) was hybridized with *in vitro*-transcribed uniformly labeled antisense RNA probes and analyzed as described (20).

PFGE. PFGE was performed on DNA from livers of c^{ch}/c^{ch} mice as described (22).

DNase I Hypersensitive Site Analysis. DNase I hypersensitive site mapping was performed as described (27).

RESULTS

Structural Organization of the Mouse GR Gene. Two unamplified libraries were screened with three probes derived from mouse GR cDNAs (Fig. 1*A*). Four overlapping phages, λ11A, λ2.9, λ5.13, and λ5.15, were isolated with probe B, and a single phage, λ4.15, was isolated with probe A. Three nearly identical phage were isolated with probe H, of which a single representative, λMG21, was further characterized

Table 1. Exon–intron boundaries of the mouse GR gene

Splice position, kb	Exon	Sequence			Exon
		Exon	Intron	Exon	
* /−13	1A	AGGAAG	**GTAAGT**		
−3327/−13	1B	CTCACA	**GTATGT..TTTGTAG**	TTA..CA**ATG**	2
−2238/−13	1C	GCTGAG	**GTGAGC**		
1208/1209	2	TCA AG	**GTAAGT..CCTATAG**	C CCT GGA	3
(402)		Ser-Ser		Pro-Gly	
1372/1373	3	GAA G	**GTAGTG..CTTATAG**	GA CAG	4
(457)		Glu		Gly-Gln	
1489/1490	4	GAA G	**GTAATG..TTCACAG**	CT CGA	5
(496)		Glu		Ala-Arg	
1765/1766	5	CCA G	**GTAAGG..CATCTAG**	GA TTC	6
(588)		Pro		Gly-Phe	
1910/1911	6	AAT GA	**GTAAGT..CTCCCAG**	G CAG AGA	7
(636)		Asn-Glu		Gln-Arg	
2041/2042	7	TCA G	**GTTGGT..CTTTTAG**	TT CCT	8
(680)		Ser		Val-Pro	
2199/2200	8	CAT GAT	**GTAAGT..CTTGCAG**	GTG GTT	9
(732)		His-Asp		Val-Val	

Exon–intron boundaries were determined by sequencing genomic subclones. Splice positions of exons 1A, 1B, and 1C are relative to genomic sequences, and splice positions of exons 2–9 are relative to the cDNA. Numbering of nucleotides and amino acids is relative to the mouse GR cDNA with the A of the start codon ATG in exon 2 designated as nt +1. The conserved intronic GT/AG oligonucleotide is shown in boldface type; the ATG start codon is underlined.
*Approximately −32 kb; see Fig. 2.

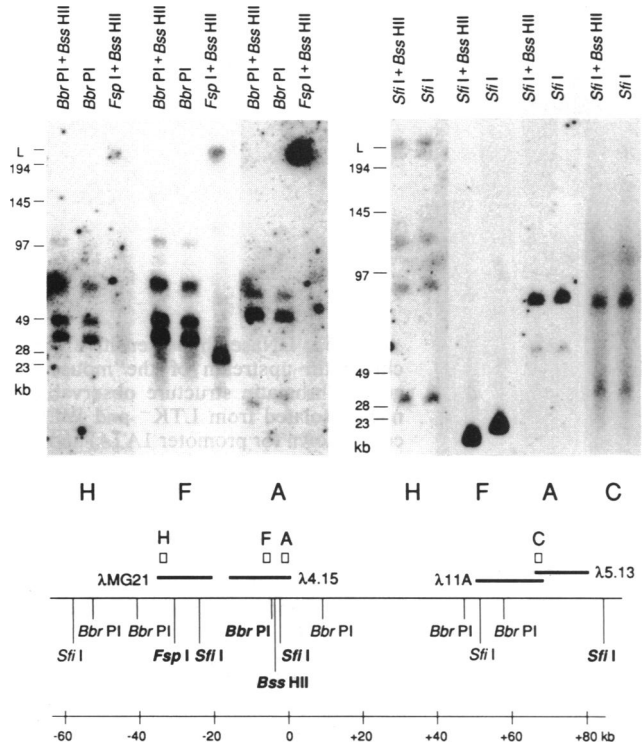

FIG. 2. Physical mapping of the mouse GR gene by PFGE. Probes H, F, A, and C were hybridized to filters bearing digests with rare-cutting restriction endonucleases. *Bss*HII was used in double digests since this enzyme cuts to completion in the CpG island. Size markers were multimers of wild-type phage λ and phage λ digested with *Hin*dIII. Fully cleaved sites are indicated in boldface type. The location of four of the GR phages is indicated by the horizontal bars; probes H, F, A, and C are indicated by the open boxes.

(Fig. 1*B*). DNA fragments that hybridized with the GR cDNA probes were subcloned and oligonucleotides derived from the GR cDNA sequence were used as sequencing primers to identify exon–intron boundaries. The translated part of the cDNA was distributed among eight exons, and all splice junctions conformed to the GT/AG rule (Table 1 and Fig. 1*B*). The N-terminal region is situated in a single exon of 1230 nucleotides (nt), designated exon 2 in Fig. 1. The two zinc fingers of the DNA binding domain are encoded by separate exons (exons 3 and 4) of 183 and 115 nt, respectively. The

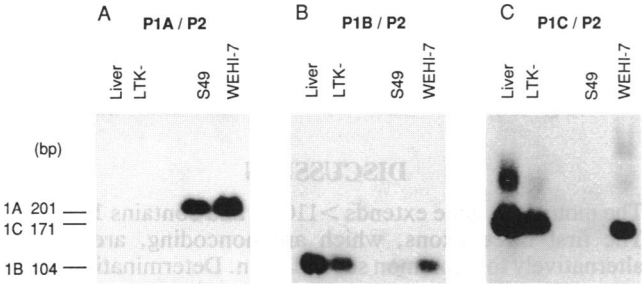

FIG. 3. PCR analysis of expression from the mouse GR promoters. Amplification products from liver, LTK$^-$, S49 T-lymphocyte, and WEHI-7 T-lymphocyte cDNAs using the primer pairs P1A/P2 (201-bp fragment), P1B/P2 (104-bp fragment), and P1C/P2 (171-bp fragment) (see Fig. 4*D*) were examined by separation in agarose gels and subsequently by Southern blot analysis (*A–C*, respectively). Primer P2 is complementary to the genomic sequences between nt +34 and +53 relative to the translation initiation site whereas primers P1B and P1C bind sequences at nt −3365/−3344 and nt −2508/−2492, respectively (unpublished results). Primer P1A binds 139 bp upstream of the splice site in exon 1A.

C-terminal part of the receptor, containing the hormone binding domain, is divided among five exons.

The 5′ splice acceptor site of exon 2 lies 12 nt upstream of the translation initiation codon. Analysis of GR cDNAs that extend further 5′ shows a divergence in sequence beyond this point. These include the 5′ ends of cDNAs isolated from T-lymphoma cells (ref. 24 and R. Miesfeld, personal communication), the 5′ end of a mouse GR cDNA isolated here from a mouse liver cDNA library (mGR6, see Table 1), and the 5′ end of GR cDNAs isolated from rat liver (10). This suggests the possibility that three first exons are alternatively spliced onto a common exon 2. Sequencing of 5 kb of genomic sequence upstream of exon 2 identified two of the 5′ ends within a large CpG island (sequence to be published elsewhere). Sequences identical to the mouse liver cDNA mGR6 reside at positions −2238 to −2571 from the ATG (designated exon 1C) and sequences homologous to the rat liver cDNA reside at positions −3380 to −3327 (exon 1B). The 5′ end of the T-lymphoma cell cDNA, in contrast, was mapped ≈32 kb upstream of the ATG and was termed exon 1A (Figs. 1 and 2). There was no overlap between λMG21 and λ4.15 and

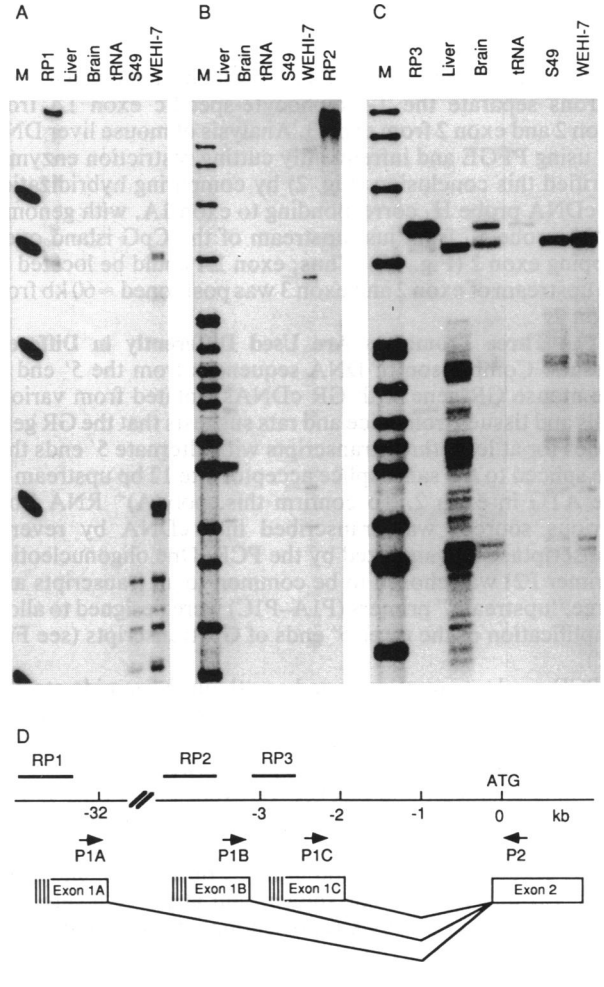

FIG. 4. Characterization of the mouse GR promoters by RNase protection. Poly(A)$^+$ RNA (20 μg), prepared from mouse liver and brain and WEHI-7 and S49 T-lymphocyte cells, was hybridized to labeled antisense RNA probes. These probes were RP1 (*A*), RP2 (*B*), and RP3 (*C*) (see *D* for their relative position). Molecular size markers (lanes M) are from plasmid pBR322 digested with *Msp* I. (*D*) Schematic representation of the mouse GR transcriptional start site region. Exons 1A, 1B, 1C, and 2 are boxed. The position of the RNA probes is indicated by solid bars. Multiple start sites are indicated by several vertical lines in exons 1A–1C. The position and orientation of PCR primers used in Fig. 3 are indicated by arrows.

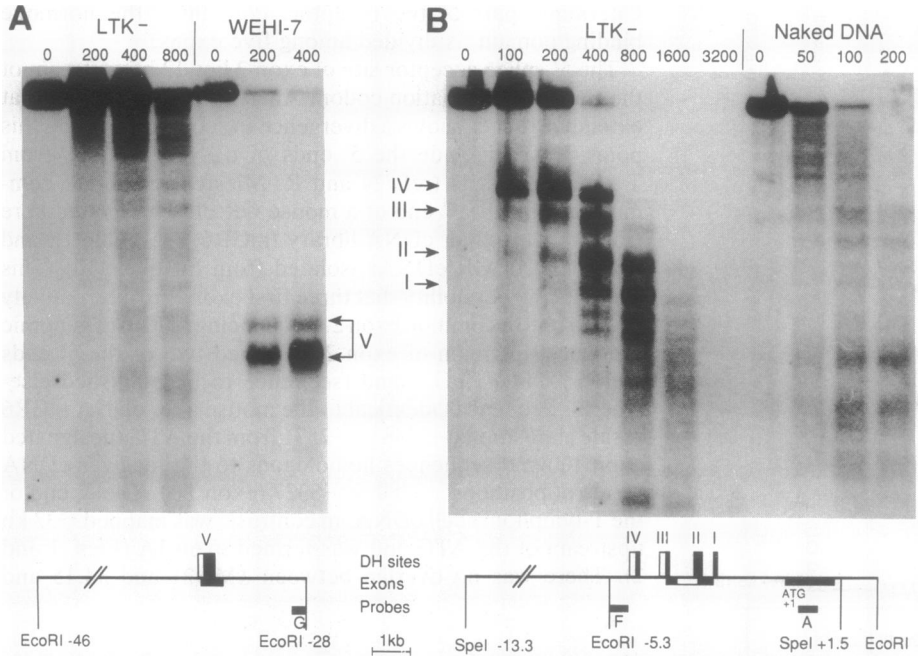

FIG. 5. DNase I hypersensitive sites in chromatin upstream of the mouse GR gene. Chromatin structure observable in nuclei isolated from LTK$^-$ and WEHI-7 cells, shown for promoter 1A (*A*) and promoters 1B and 1C (*B*). Nuclei and genomic DNAs purified from LTK$^-$ cells were digested with increasing amounts of DNase I (units/ml) as indicated. Purified DNA was digested with *Eco*RI and indirectly end-labeled with probe G (*A*) or digested with *Spe* I and indirectly end-labeled with probe A (*B*).

between λ4.15 and λ11A (Fig. 1), which implied that large introns separate the T-lymphocyte-specific exon 1A from exon 2 and exon 2 from exon 3. Analysis of mouse liver DNA by using PFGE and infrequently cutting restriction enzymes verified this conclusion (Fig. 2) by comparing hybridization of cDNA probe H, corresponding to exon 1A, with genomic DNA probe F, from just upstream of the CpG island overlapping exon 2 (Fig. 1*B*). Thus, exon 1A could be located 32 kb upstream of exon 2 and exon 3 was positioned ≈60 kb from exon 2.

The Three Promoters Are Used Differently in Different Tissues. Comparison of DNA sequences from the 5′ end of the mouse GR gene with GR cDNAs isolated from various cells and tissues from mice and rats suggests that the GR gene codes for at least three transcripts with alternate 5′ ends that are spliced to the same splice acceptor site 12 bp upstream of the ATG in exon 2. To confirm this, poly(A)$^+$ RNA from various sources was transcribed into cDNA by reverse transcriptase and analyzed by the PCR. One oligonucleotide (primer P2) was chosen to be common to all transcripts and three "upstream" primers (P1A–P1C) were designed to allow amplification of the three 5′ ends of GR transcripts (see Fig. 4*C*).

PCR products were analyzed on ethidium bromide-stained agarose gels and, after blotting to nylon filters, by hybridization to probes specific for the individual transcripts (see Fig. 4*B*). The autoradiographs are shown in Fig. 3. PCR products characteristic of exon 1A were found only in S49 and WEHI-7 cells (Fig. 3*A*) but not in liver, fibroblasts, or brain (Fig. 3 *B* and *C*; data not shown). Transcripts containing exons 1B and 1C were found in liver, fibroblasts, and brain (Fig. 3 *B* and *C*; data not shown) and at lower levels in T lymphocytes. As a cDNA representing a transcript containing exon 1B has yet to be isolated from the mouse, the 104-nt P1B/P2 PCR product was subcloned and sequenced. The DNA sequence was identical to that predicted for a transcript containing exon 1B from the mouse GR gene (Table 1 and data not shown).

To compare the activities of these three promoters and to assign potential transcriptional start sites, RNase protection assays were performed using poly(A)$^+$ RNA prepared from several mouse tissues and T-cell lines (Fig. 4). All three promoters showed heterogeneity in the start site of transcription. With a probe specific for exon 1A RNase-resistant products were detected only with RNA from T-cell lines (Fig. 4*A*), consistent with the result of the PCR in which amplification products corresponding to exon 1A were only found in T cells (Fig. 3). In thymus RNA prepared from 4-week-old mice, protected fragments of similar size were observed upon long exposures (data not shown). The RNA probe specific for promoter 1B detected a comparatively strong signal with liver RNA, but only weak signals with RNA from other tissues and cells (Fig. 4*B*). Common start sites of variable strength originating from promoter 1C were detected in all RNA samples tested (Fig. 4*C*).

GR Promoter Activity Correlates with Chromatin Structure. We examined the chromatin of the 5′ region, including the presumptive start sites of the GR gene, for DNase I hypersensitivity (28). With probe A (Fig. 1*B*), four strong hypersensitive sites (HSI to HSIV) are visible (Fig. 5*B*) after digestion of nuclei from mouse LTK$^-$ cells, but not after digestion of genomic DNA. All four reside within the CpG island, indicating a functional significance of this region. The results, summarized in the lower parts of Fig. 5, show that HSII and HSIII map to exon 1B and exon 1C, respectively, as determined above. Consistent with the ubiquitous expression inferred from PCR and RNase protection experiments, a very similar pattern of DNase I cleavages in chromatin was observed in WEHI-7 cells (data not shown). Similar chromatin analysis was also performed at the T-lymphocyte-specific promoter of exon 1A. This promoter is marked by DNase I hypersensitivity in WEHI-7 cells but not in LTK$^-$ cells (Fig. 5*A*) or in naked DNA (data not shown).

DISCUSSION

The mouse GR gene extends >110 kb and contains 11 exons. The first three exons, which are noncoding, are spliced alternatively to a common second exon. Determination of the exon–intron organization of the translated region revealed a gene structure very similar to the characterized chicken progesterone (29), human estrogen (30), and human androgen (31) receptor genes. In each case the N-terminal domain is encoded by one large exon. The DNA-binding domain is composed of two zinc-finger motifs each of which is encoded separately by the next two exons. The position of the splice site between these two zinc fingers (splice site, 1372/1373) is conserved as opposed to the more distantly related thyroid hormone and retinoic acid receptor genes in which a different splice position is used (30, 32–36). The C-terminal hormone

binding domain is encoded, in each case, by five exons. The similarity in sequence, the common domain structure, and common modes of action are additional proof that the various steroid receptors are related evolutionarily. The human GR gene has been described (37) and has an identical organization to the mouse gene encoding exons 2–9. However, only one promoter has been characterized for the human GR gene (37, 38), similar in sequence to exon 1C of the mouse GR gene characterized here.

Transcripts arising from the three promoters differ in their expression in various tissues and cell lines. Promoters 1B and 1C were active in all RNA samples tested, albeit at different intensities. Transcripts from promoter 1A were present in the T-lymphocyte cell lines S49 and WEHI-7 and were detectable in thymus but were not detectable in liver and brain poly(A)$^+$ RNA. The high activity of promoter 1A in T-lymphoma cells is in agreement with the abundance of GR cDNAs isolated from glucocorticoid-sensitive and glucocorticoid-resistant S49 T-lymphoma cells (24). The activity of this promoter correlates with the specialized function of apoptosis inducible by glucocorticoids in thymus and T-cell lines. Whether the activity of this promoter is developmentally regulated or connected with glucocorticoid-induced apoptosis remains to be determined. The specific nature of promoter 1A is strengthened by the finding that a DNase I hypersensitive site, situated over the promoter, was only detected in the T-lymphoma cell lines.

In contrast to the conservation of receptor gene structure, the promoter organization seems not to be conserved among various receptors. Expression of the human estrogen receptor gene is controlled by a single promoter (39). Two promoters control the expression of the closely related human and chicken progesterone receptor (40, 41). These promoters, in contrast to the promoters of the GR gene, give rise to transcripts coding for two forms of the progesterone receptor found in all the cell types and tissues analyzed. Unlike the GR most other steroid hormone receptors are expressed in a more cell-type-restricted manner. We believe that promoter sampling has been an important mechanism in the diversification of function of the GR.

We are grateful to Drs. R. Miesfeld and E. S. Dieken for communication of results prior to publication and a mouse cDNA clone; Thomas Dietrich and Werner Fleischer for synthesis of oligonucleotides and art work; Dr. S. Ruppert for his c^{ch}/c^{ch} genomic library and his mouse liver cDNA library; and Cornelia Schneider for excellent secretarial assistance. This work was supported by the Deutsche Forschungsgemeinschaft through the Leibniz-Program and SFB 229 and the Fonds der chemischen Industrie.

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