Characterization of the Human Glucocorticoid Receptor Promoter†

Yoshitaka Nobukuni,*,‡ Catharine L. Smith,§ Gordon L. Hager,§ and Sevilla D. Detera-Wadleigh‡

Clinical Neurogenetics Branch, National Institute of Mental Health, National Institutes of Health, Bethesda, Maryland 20892, and Hormone Action and Oncogenesis Section, Laboratory of Molecular Virology, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20822

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ABSTRACT: To elucidate the functional elements that are involved in the regulation of the human glucocorticoid receptor (hGR) gene, transient expression, DNase I footprinting, and gel mobility shift analyses were conducted. We found that the hGR promoter region between −700 and +38 bp contained 11 footprinted sites. Deletion of the −374 to −183 bp region, which is highly conserved between human and mouse (93%), induced a 5−24-fold reduction in promoter activity in HeLa, NIH3T3, CV1, and HepG2 cells. Three footprints, FP5, FP6, and FP7, were shown to map to this region. In particular, the FP7 site was found to be within the −374 to −347 bp region. Deletion of this region triggered a significant decline in promoter activity in HeLa and NIH3T3 cells but not in HepG2 cells. AP2 was found to bind FP7. In HepG2 cells AP2 elicited transactivation of the hGR promoter activity. Transfection data revealed that the upstream GC box-rich fragment between −700 and −375 bp induced a 4−7-fold activation of the heterologous tk promoter in an orientation-independent manner. Our studies demonstrate that several transcription factors are involved in regulating GR expression and that AP2 could function as an important positive regulator of GR promoter activity.

The glucocorticoid receptor (GR)[1] is a transcription factor that belongs to the multimembered nuclear hormone receptor superfamily (Yamamoto, 1985; Evans, 1988; Green & Chambon, 1988; Beato, 1989; O'Malley, 1990; Laudet et al., 1992; Amero et al., 1992; Detera-Wadleigh & Fanning, 1994). It mediates the action of glucocorticoids in homeostasis, stress response, intermediary metabolism, development, differentiation, central nervous system function, and inflammatory/immune responses (Baxter & Rousseau, 1979).

Although widely distributed, the expression level of GR varies considerably among different tissues (Ballard et al., 1974) and within different areas of a single tissue (Herman et al., 1989). It has been established that cellular receptor number is important in determining the magnitude of the biological effects of glucocorticoids (Vanderbilt et al., 1987). Thus, to further understand the physiologic control of glucocorticoid action, it is important to elucidate the basic mechanisms involved in the regulation of GR gene expression. The regulation of GR at the level of transcription, however, is not well understood. So far, the cis elements that are involved in promoting GR gene activity have not been determined. The transcription factors that bind these elements also remain to be identified.

Recently human (Zong et al., 1990; Encio & Detera-Wadleigh, 1991; Leclerc et al., 1991) and mouse GR promoter regions (Strähle et al., 1992) have been cloned and characterized. In mouse three different first exons, derived from three different promoters, 1A, 1B, and 1C, are alternatively spliced onto a common exon 2. Promoter 1A is cell-type specific in its activity and has been found to be active in T-lymphocytes. The two downstream promoters, 1B and 1C, situated in a CpG island, have been shown to have varying activities in different cell lines and tissues (Strähle et al., 1992).

In human only one promoter has been reported so far (Zong et al., 1990; Encio & Detera-Wadleigh, 1991; Leclerc et al., 1991), and it corresponds in sequence to promoter 1C of the mouse GR (Strähle et al., 1992). The human GR promoter has several specific features in common with "TATA-less promoters" (Azizkhan et al., 1993). It lacks characteristic TATA and CAAT boxes and contains multiple transcription start sites (Zong et al., 1990). It is G+C rich with multiple putative GC-box consensus sequences. Furthermore, an untranslated exon 1 has been identified (Zong et al., 1990; Encio & Detera-Wadleigh, 1991; Leclerc et al., 1991). No consensus glucocorticoid response elements have been found in the −3.0 kbp of the 5′-flanking sequence (Zong et al., 1990).

As a first step toward elucidating the molecular mechanisms involved in the human GR (hGR) gene expression, we have analyzed the hGR promoter by transient transfection, DNase I footprinting, and gel retardation analyses. In this study, we identified important regions for GR promoter activity, characterized some of the transacting factors that interact with these regulatory elements, and showed that AP2 may be an important determining factor for the differences in hGR promoter activity among different cell lines.

† The GenBank Accession Number for the sequence of the human glucocorticoid receptor promoter in Figure 3 is U10430.

* Correspondence should be addressed to this author at the Unit on Gene Mapping and Expression, Clinical Neurogenetics Branch, National Institute of Mental Health, National Institutes of Health, Building 10, Room 3N218, 9000 Rockville Pike, Bethesda, MD 20892 [telephone, (301) 496-1641; FAX, (301) 402-0859].

‡ National Institute of Mental Health.

§ National Cancer Institute.

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[1] Abbreviations: AP2, activator protein-2; GR, glucocorticoid receptor; GRE, glucocorticoid responsive element; tk, thymidine kinase; GMCSF, granulocyte−macrophage colony stimulating factor; HSV, herpes simplex virus; IL-4 and -5, interleukin-4 and -5.

EXPERIMENTAL PROCEDURES

Construction of Promoter–Luciferase Fusion Genes. The promoterless vector, pXP2, containing the luciferase reporter gene and pT109-luc, which contains the HSV tk promoter and the luciferase reporter gene (Nordeen, 1988), were kindly supplied by Dr. S. K. Nordeen. The 2.9 kbp *Bgl*II–*Nco*I fragment of the hGR 5′-flanking region was subcloned into pXP2 upstream of the luciferase gene (Encio and Detera-Wadleigh, unpublished data), and serial deletion mutants were prepared using the exonuclease III/mung bean nuclease digestion (Figure 1A). The *Sac*I (−700 to −183 bp), *Not*I–*Sac*I (−374 to −183 bp), and *Sac*I–*Not*I (−700 to −375 bp) fragments were subcloned into pT109-luc upstream of the tk promoter (Figure 2). Plasmid DNAs used for transfection experiments were purified by CsCl density gradient centrifugation (Maniatis et al., 1989).

Transient Expression Assay. Transfections were done using the calcium phosphate precipitation method (Graham & van der Eb, 1973; Wigler et al., 1979) in six-well plates into either HeLa, NIH3T3, CV1, or HepG2 cells, grown in phenol red-free DMEM media containing 10% charcoal-treated fetal calf serum. Each well received 2 mL of cell suspension containing between 2×10^5 and 3×10^5 cells. A total of 6 μg of DNA consisting of 2 μg of promoter–luciferase chimeric plasmid, 2 μg of the β-galactosidase expression plasmid pCH110 (Pharmacia Biotech Inc., Piscataway, NJ), and 2 μg of carrier pUC18 plasmid DNA was transfected into each well after an overnight seeding. Transfections were done in triplicates, and each experiment was repeated at least three times. Promoter activities were determined by measuring the luciferase activity as described elsewhere (De Wet et al., 1987), and corrections for variations in transfection efficiency were done using β-galactosidase activity.

Preparation of Nuclear Extracts. Nuclear extracts were prepared essentially as previously described (Dignam et al., 1983). Cells were washed twice with phosphate-buffered saline (PBS) and then scraped off with a rubber policeman. All procedures after this step were done at 4 °C. After centrifugation at 2000*g* for 5 min, the cell pellet was suspended in five times its volume of cold buffer A [10 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, and 0.5 mM dithiothreitol (DTT)] and incubated on ice for 10 min. After centrifugation, the cell pellet was resuspended in 2 volumes of buffer A and homogenized by 10 strokes using a glass Dounce homogenizer (pestle B). The homogenate was centrifuged at 2000*g* for 10 min to separate the cytoplasmic extract. The pellet was recentrifuged at 25000*g* for 20 min and resuspended in ice-cold buffer C [20 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 0.6 M NaCl, 0.2 mM EDTA, 0.5 mM phenylmethanesulfonyl fluoride (PMSF), 0.5 mM DTT, and 25% glycerol] (3 mL for 10^9 cells) and homogenized by 10 strokes with a glass Dounce homogenizer. After being gently stirred for 30 min, the homogenate was centrifuged at 25000*g* for 30 min, and the supernatant was dialyzed for 8–12 h against 50 volumes of buffer D (20 mM HEPES, pH 7.9, 0.1 M KCl, 0.2 mM EDTA, 0.5 mM PMSF, 0.5 mM DTT, and 20% glycerol). The dialysate was centrifuged at 25000*g* for 20 min, and aliquots of the supernatant were frozen and stored in −80 °C.

DNase I Footprinting Analysis. To prepare probes for footprinting analyses, the plasmids N1, N5, N11, and N24 (Figures 1 and 2) were linearized with *Bam*HI and labeled by a fill-in reaction with Klenow fragment and [α-^{32}P]dATP and then digested using either *Xba*I or *Not*I. The labeled GR promoter fragments were purified on a 6% polyacrylamide gel. DNase I footprinting analysis was performed as described elsewhere (Galas & Schmitz, 1978). Briefly, 1–2 ng of end-labeled DNA fragment was incubated in 50 μL of binding buffer (10 mM Tris-HCl, pH 7.5, 50 mM NaCl, 0.05% Nonidet P-40, 1 mM EDTA, 1 mM DTT, and 10% glycerol) for 30 min at room temperature in the presence of 50–100 μg of nuclear extract. $MgCl_2$ and $CaCl_2$ were added to final concentrations of 1 and 0.5 mM, respectively, and incubation was continued for 1 min at room temperature. The reaction mixture was digested with 0.5–5 units of DNase I for 1 min at room temperature and stopped with 140 μL of a solution containing 192 mM sodium acetate, 32 mM EDTA, 0.14% SDS, and 64 μg/mL yeast tRNA. After phenol–chloroform extraction, the pellet was resuspended in a formamide dye buffer, denatured by heating at 94 °C for 5 min, and analyzed on a 6% polyacrylamide–urea sequencing gel. The gel was dried and autoradiography was done at −80 °C.

Table 1: Oligonucleotides Used for Gel Mobility Shift Experiment

Name	Sequence
FP5	-208 -179 5′-CCGCCGCCTCCTCCATTTTGCGAGCTCGTG-3′ 3′-GGCGGCGGAGGAGGTAAAACGCTCGAGCAC-5′
FP6	-307 -276 5′-GTGCGCGCCGTGGCGCCGCCTCCACCCGCTCC-3′ 3′-CACGCGCGGCACCGCGGCGGAGGTGGGCGAGG-5′
FP7	-375 -341 5′-AGTCCGGCCGCCGCGCGGCCCCTCGGGCGGGGAGCGGCC-3′ 3′-GCCGGCGGCGCGCCGGGGAGCCCGCCCCTCGCCGGCTGA-5′
FP8	-527 -501 5′-AGTCGGCGCACCGGGCGGGGCGGCCACGCCA-3′ 3′-CCGCGTGGCCCGCCCCGCCGGTGCGGTCTGA-5′
FP9	-556 -530 5′-AGTCTGGGGAGTTGGGGGCGGGGGGCGAAGC-3′ 3′-ACCCCTCAACCCCCGCCCCCCGCTTCGCTGA-5′
FP10	-618 -597 5′-AGTCTCTTAACGCCGCCCCAGAGAGA-3′ 3′-AGAATTGCGGCGGGGTCTCTCTCTGA-5′
FP11	-681 -654 5′-AGTCGCGGAGCTGGGCGGGGGCGGGAAGGAGG-3′ 3′-CGCCTCGACCCGCCCCCGCCCTTCCTCCCTGA-5′
Sp1	5′-ATTCGATCGGGGCGGGGCGAGC-3′ 3′-TAAGCTAGCCCCGCCCCGCTCG-5′
AP2	5′-GATCGAACTGACCGCCCGCGGCCCGT-3′ 3′-CTAGCTTGACTGGCGGGCGCCGGGCA-5′

Gel Mobility Shift Assay. The deoxyoligonucleotides used in the gel mobility shift experiments (Garner & Revzin, 1981; Fried & Crothers, 1981) are listed in Table 1. Deoxyoligonucleotides were labeled either by a fill-in reaction using DNA polymerase I Klenow fragment and [α-^{32}P]dCTP or by end-labeling using T4 polynucleotide kinase and [γ-^{32}P]-ATP. The binding reaction was conducted by incubating the end-labeled oligonucleotide probes [$(1.0–2.0) \times 10^4$ cpm] with 5 μg of nuclear extract and 1 μg of poly(dI-dC) in a binding buffer (10 mM Tris-HCl, pH 7.5, 50–150 mM NaCl, 0.05% NP-40, 1 mM EDTA, 1 mM DTT, and 10% glycerol), in a final volume of 20 μl for 30 min at room temperature. Following binding, the DNA–protein complexes were separated by electrophoresis on a 6% nonde-

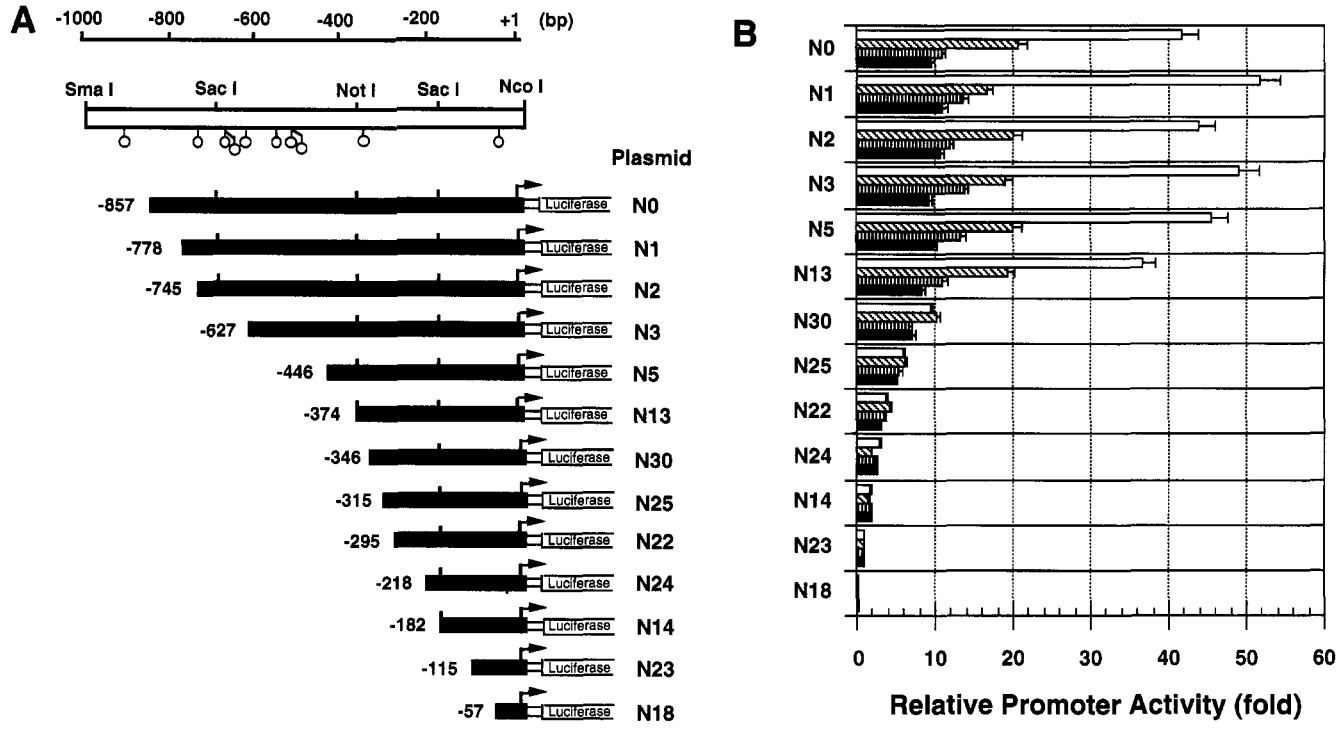


FIGURE 1: (A) Structure of the human glucocorticoid receptor (hGR) promoter chimeras. A series of promoter deletion mutant−luciferase gene chimeric plasmids with variable 5′ ends (from −857 to −57 bp) and a common 3′ end at +38 bp were constructed (see Materials and Methods). A schematic diagram depicting the 1.0 kbp *Sma*I−*Nco*I fragment of the hGR gene promoter is shown above the constructs. The bent arrow indicates the major transcription start site for hGR in IM9 cells (Encio & Detera-Wadleigh, 1991) and the direction of transcription. The circles are GC boxes (Briggs et al., 1986) with the hexanucleotide sequence of either 5′-GGGCGG-3′ or 5′-CCGCCC-3′. (B) Promoter activity of the hGR 5′-flanking region. A series of promoter chimeras (panel A) were transfected into HeLa (open bars), NIH3T3 (slanted hatched bars), CV1 (vertically hatched bars), and HepG2 cells (solid bars). Promoter activity is expressed relative to the N23 plasmid which contains the −115 to +38 bp promoter fragment and is corrected for variations in transfection efficiency using β-galactosidase activity. Data represented in bars are the mean of at least three independent experiments, and the standard errors (SE) are shown as horizontal lines on sides of the bars.

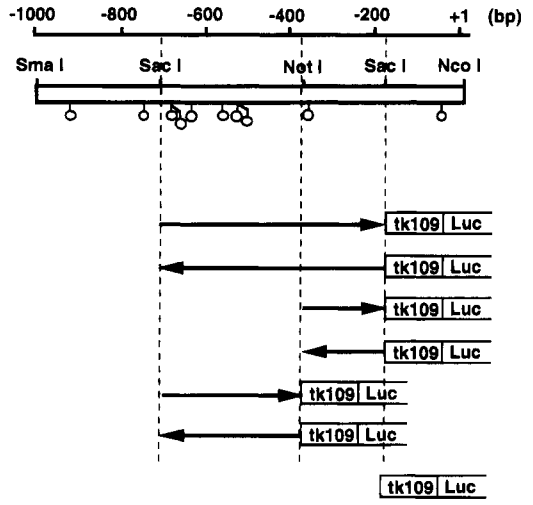
naturing polyacrylamide gel in 0.25 × TBE buffer for 3 h at 200 V in the cold room and visualized by autoradiography. For competition experiments, between a 20- and 200-fold molar excess of competitor DNA was incubated in the mixture prior to the addition of nuclear extract. For immunologic detection of Sp1 and AP2, anti-Sp1 and anti-AP2 antibodies (Santa Cruz Biotechnology Inc., Santa Cruz, CA) were used at 0.5 μg each in each binding reaction.

RESULTS

Analysis of hGR Promoter Activity. To define regions that are involved in GR gene expression, we transfected into HeLa, NIH3T3, CV-1, and HepG2 cells a series of deletion mutants of the hGR promoter having variable 5′ ends (from −857 to −57 bp) and a common 3′ end at +38 bp (Figure 1A). Transfection data indicated that differences exist in the relative levels of GR promoter activity in HeLa, NIH3T3, CV-1, and HepG2 cells (Figure 1B). The differences were prominent in the activity of promoter chimeras that contained longer upstream sequences, from N13 to N0. These constructs exhibited the highest level of activity in HeLa cells. With the same chimeras HepG2 and CV-1 cells appeared to support lower levels of transcription whereas in NIH3T3, the activity was greater than in either HepG2 and CV-1 but was about 2-fold lower than in HeLa. The region spanning −374 to +38 bp yielded levels of activity similar to those with longer 5′ extensions in all cell lines studied here. By contrast, removal of the region between −374 and −183 bp produced a large decline in promoter activity from 5- to 24-fold. In particular, a finer deletion of 28 bp within this region, i.e., between −374 and −347 bp, triggered a significant drop in activity of 74% in HeLa and 48% in NIH3T3, but in HepG2 the decline was only 14%. The activity of promoter chimeras with 5′ ends extending from −57 to −346 bp did not elicit any cellular differences. Further excisions of the 5′ end from −182 bp caused a gradual loss in promoter activity (Figure 1B).

Two Highly Conserved Regions Can Stimulate the Heterologous tk Promoter. We examined heterologous transcriptional enhancement mediated by the region between −374 and −183 bp since deletion analysis showed that this region is important for maximal GR expression. This promoter segment is 93% conserved between human and mouse (Figure 3). We also analyzed the upstream GC box-rich region between −700 and −375 bp since a previous study (Leclerc et al., 1991) has shown that the deletion of this region caused a significant decline in promoter activity. We subcloned three different fragments located between −700 and −183 bp, −700 and −375 bp, and −374 and −183 bp, in both forward and reverse orientations, upstream of the tk promoter. These constructs produced about a 4−7-fold activation of the tk-luc basal activity in HeLa, NIH3T3, and CV1 cells (Figure 2), suggesting that in the heterologous context both regions contain activating elements that function in these cell lines.

Nuclear Factor Binding Sites by DNase I Footprinting. To determine the possible binding sites for nuclear proteins,



Relative Luciferase Activity (fold)

Plasmid	CV1	NIH3T3	HeLa
N19	4.5	3.8	5.1
N9	6.8	4.3	5.7
N21	6.1	6.5	6.8
N11	5.8	4.9	6.2
N20	6.4	4.3	6.9
N12	6.4	5.1	7.3
pT109-Luc	1.0	1.0	1.0

FIGURE 2: Stimulation of the heterologous thymidine kinase promoter by hGR promoter fragments. Plasmid constructs with the luciferase gene under the control of the tk promoter and *Sac*I (−700 to −183 bp), *Not*I−*Sac*I (−374 to −183 bp), and *Sac*I−*Not*I (−700 to −375 bp) fragments were tested for luciferase activity by transfection into HeLa, NIH3T3, and CV1 cells. Arrows indicate the orientation of the fragment relative to transcription via the hGR promoter. Promoter activity was corrected for transfection efficiency using β-galactosidase activity and expressed relative to that of pT109-luc.

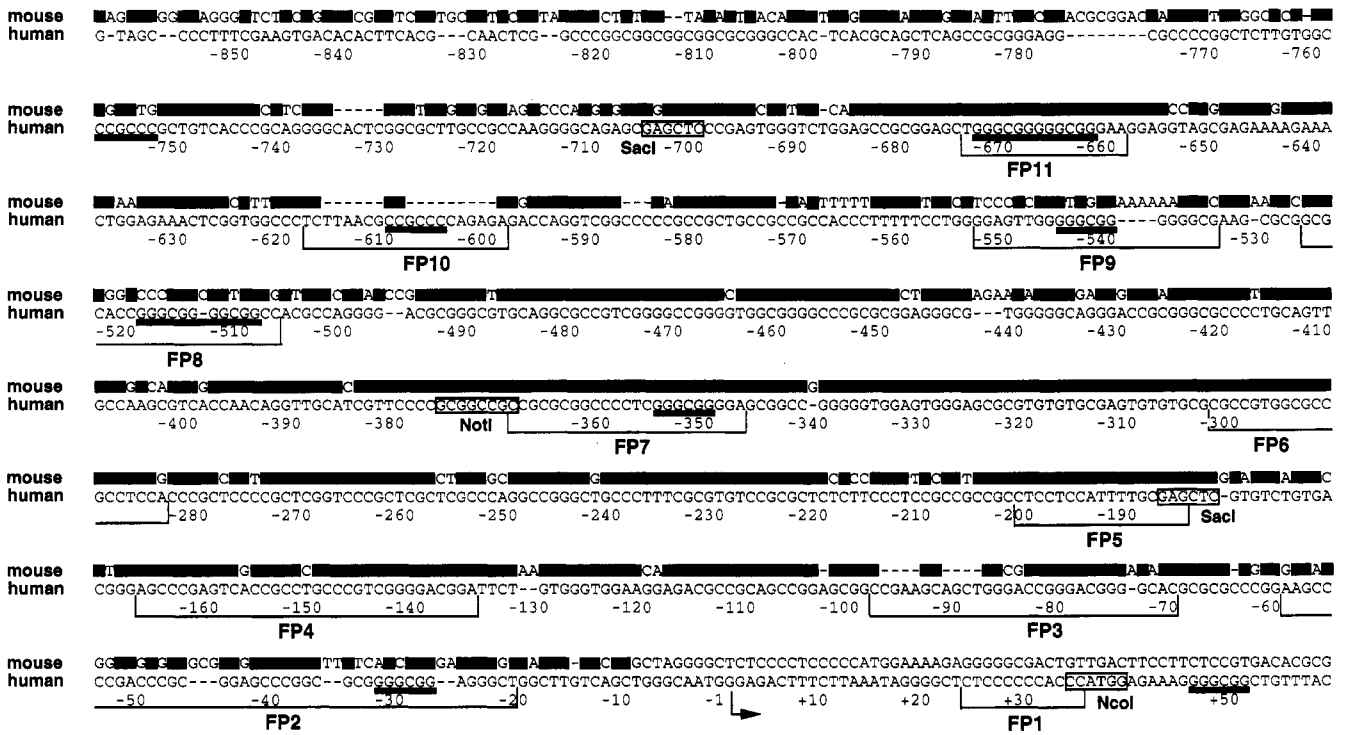


FIGURE 3: Human and mouse GR promoter sequences and DNase I footprinted regions on the human promoter. The mouse 1C (Strähle et al., 1992) and hGR promoters (GenBank Accession Number U10403) are aligned. Identical sequences are represented by thick solid bars. Sequence gaps are denoted by dashes. The transcriptional start site of the IM9 cells is located at nucleotide +1. Discrepancies between the previously reported sequence (Encio & Detera-Wadleigh, 1991) and the above sequence are represented by an insertion (in the current sequence) of a C, GC, CGC, G, and C at positions −148, −304, −366, −555, and −578 bp, respectively. Footprinted sites (FP1 to FP11) are bracketed. The GC boxes (GGGCGG or CCGCCC) are underlined. Restriction enzyme sites are enclosed in boxes.

DNase I footprinting experiments were performed. Eleven protected segments, referred to as FP1 through FP11, mapping within the −700 to +38 bp were detected (Figures 3 and 4). In the proximal region spanning −182 and +38 bp, four footprints, FP1, FP2, FP3, and FP4, were found (Figures 3 and 4A,B). The highly conserved region between −374 and −183 bp was shown to contain three protected sites: FP5, FP6, and FP7 (Figures 3 and 4C). Four footprints, FP8, FP9, FP10, and FP11, were detected in the GC box-rich region between −700 and −375 bp (Figures 3 and 4D). In this study we focused our investigation on the F5 through F11 sites since these footprints were located in regions that might be involved in GR promoter activation.

Nuclear Proteins Binding to FP5, FP6, and FP7 Sites. To characterize the cis elements in the highly conserved −374 to −183 bp region and the nuclear factors that recognize these elements, gel mobility shift assays were performed.

FP5 did not contain a consensus sequence for common transcription factors. Two retarded bands were detected, but

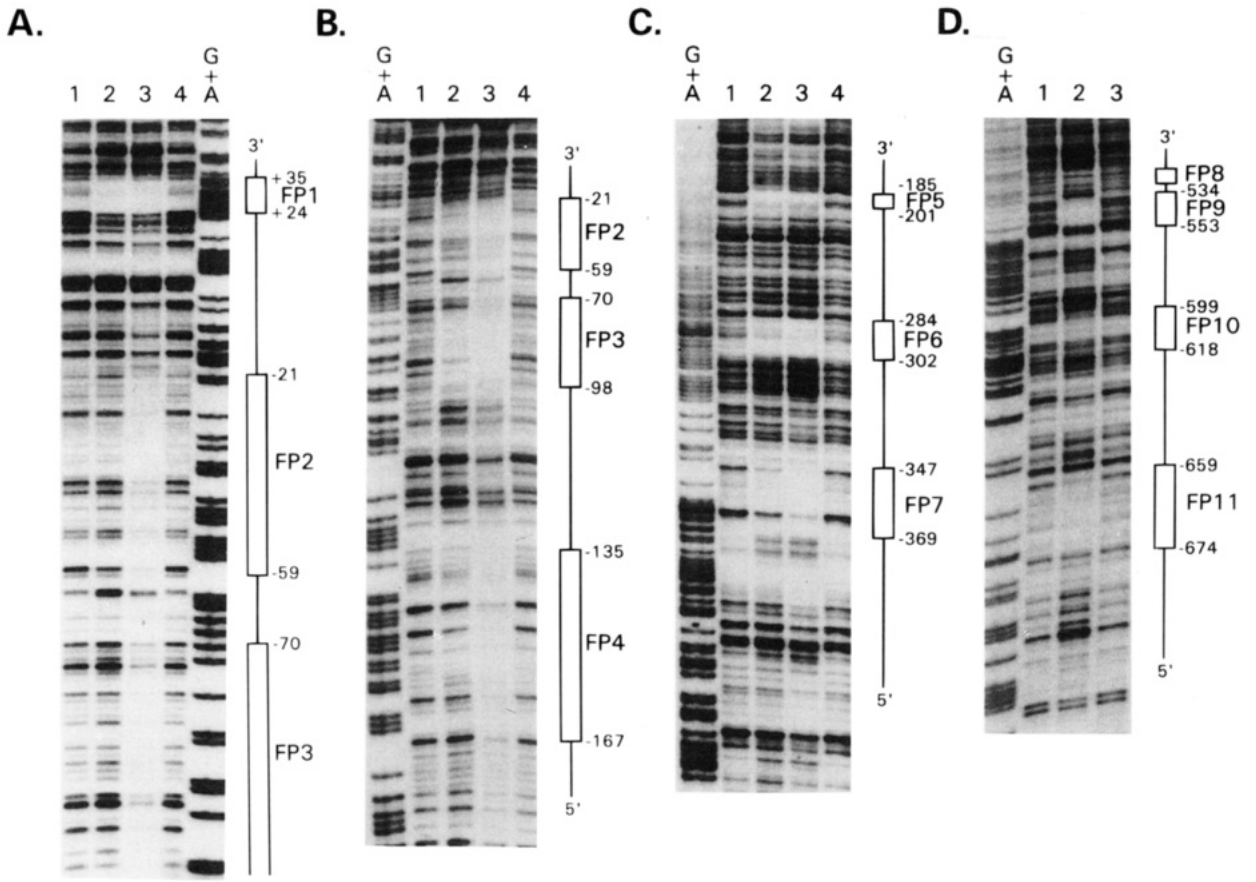


FIGURE 4: DNase I footprinting analysis of HeLa nuclear protein-binding sites in the hGR 5′-flanking region. Locations of FP1 to FP11 footprints are indicated in base pairs and by open boxes. Panels A and B: FP1, FP2, FP3, and FP4 footprints were detected in the proximal region from −182 to +38 bp. A 260 bp *Bam*HI−*Xba*I fragment from N24 was used as probe. DNase I cleavage patterns are shown without HeLa nuclear extract (NE) (lanes 1 and 4), with 50 μg of NE (lane 2), and with 100 μg of NE (lane 3). G+A refers to the guanine and adenine marker obtained by Maxam−Gilbert sequence reaction. Panel C: FP5, FP6, and FP7 footprints in the −374 to −183 bp region were detected by a 500 bp labeled *Bam*HI−*Xba*I fragment from N5. Cleavage patterns are shown without NE (lanes 1 and 4) and with 50 μg (lane 2) or 100 μg (lane 3) of NE. Panel D: FP8, FP9, FP10, and FP11 footprints within the −700 to −375 bp region were detected by a 400 bp *Bam*HI−*Not*I probe from N1. Cleavage patterns are shown without NE (lanes 1 and 3) or with 100 μg of NE (lane 2).

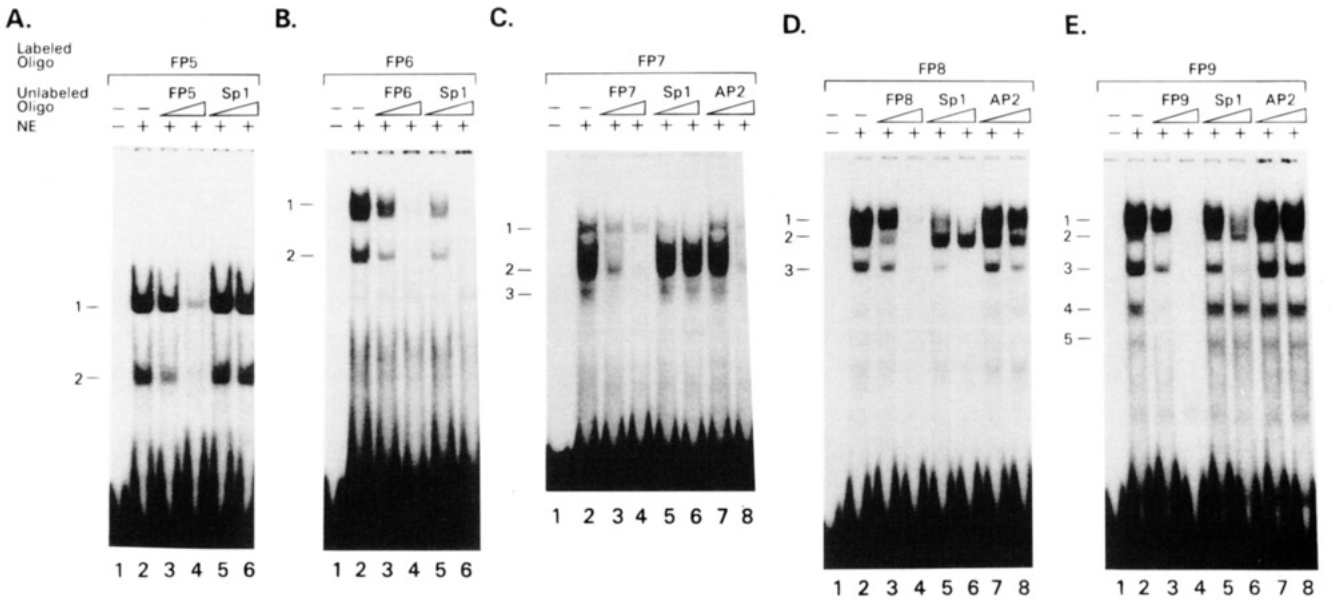


FIGURE 5: Gel mobility shift analysis of the hGR promoter footprinted regions. Panels A−E: gel mobility shift analysis of FP5 to FP9 sites, respectively. DNA binding and competition assays were done using double-stranded synthetic oligonucleotides (Table 1) derived from the protected sites FP5 to FP9 as described in Materials and Methods. Protein−DNA complexes are indicated. Competition was done with either 0.1 or 1 pmol of homologous oligomer or the indicated oligomer.

the identity of the binding protein is not presently known (Figure 5A).

A putative Sp1 binding site (Briggs et al., 1986) was noted in the FP6 sequence (Figure 3). The two visible bands were

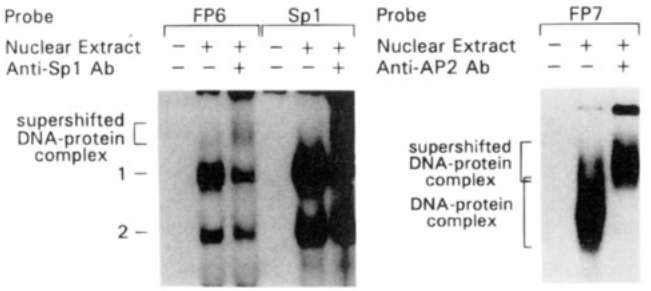


FIGURE 6: Immunologic identification of AP2 and Sp1: FP6, labeled FP6 and consensus Sp1 oligo incubated with 5 μg of NE in either the absence or presence of 0.5 μg of anti-Sp1 antibody; FP7, labeled FP7 oligo (lane 1) incubated with 10 μg of NE in either the absence (lane 2) or presence of 0.5 μg of anti-AP2 antibody (lane 3).

found to be inhibited by an Sp1 consensus oligonucleotide (Figure 5B). An anti-Sp1 antibody caused a supershift in the migration of the DNA−protein complex, providing evidence that Sp1 binds the FP6 site (Figure 6).

FP7 was found to map within the −374 and −347 bp region which when deleted triggered a considerable drop in promoter activity in HeLa and NIH3T3 cells. The FP7 sequence revealed a putative Sp1 site and two putative AP2 elements (Figure 7B). Gel mobility shift assays showed three DNA−protein complexes. Band 1 which was faint appeared to be competed out by an Sp1 consensus oligonucleotide. The formation of bands 2 and 3 was blocked by an AP2 consensus oligonucleotide (Figure 5C). Supershift experiments with an antibody to AP2 elicited the appearance of a well-defined supershifted band (Figure 6). Furthermore, DNase I footprinting using purified AP2 mimicked the footprint pattern shown by the HeLa nuclear extract (Figure 7). These data confirmed that AP2 binds the FP7 site. Purified Sp1 was also found to bind to this site, but its binding was very weak (data not shown).

Nuclear Proteins Binding to FP8, FP9, FP10, and FP11 Sites. Gel mobility shift and competition assays were also performed to identify nuclear proteins that bind to FP8 through FP11, within the −700 to −375 bp region.

The gel shift and competition patterns exhibited by FP10 and FP11 were similar to that of FP6 (Figure 5B), indicating that Sp1 binds to these sites (data not shown).

Gel shift assays with FP8 and FP9 revealed three and five protein−DNA complexes, respectively. Competition with consensus Sp1 oligo inhibited bands 1 and 3 (Figure 5D,E). The nuclear binding factor in band 2 of FP8 or in bands 2, 4, and 5 of FP9 is not presently known.

To confirm the finding that Sp1 interacts with sequences on FP8, FP9, FP10, or FP11, we performed gel mobility shift assays with anti-Sp1 antibody. In all cases a supershifted band was visible as with FP6 (Figure 6) which migrated to the same position as in the control experiment (data not shown).

AP2 Can Transactivate hGR Promoter Activity. As described above, FP7 mapped within the −374 to −347 bp region, the deletion of which caused a considerable drop in promoter activity, particularly in HeLa cells. We have shown that AP2 can bind the FP7 site. To further elucidate the role of the FP7 site and AP2 in hGR promoter activity, we first investigated the AP2 binding activity in HeLa, NIH3T3,

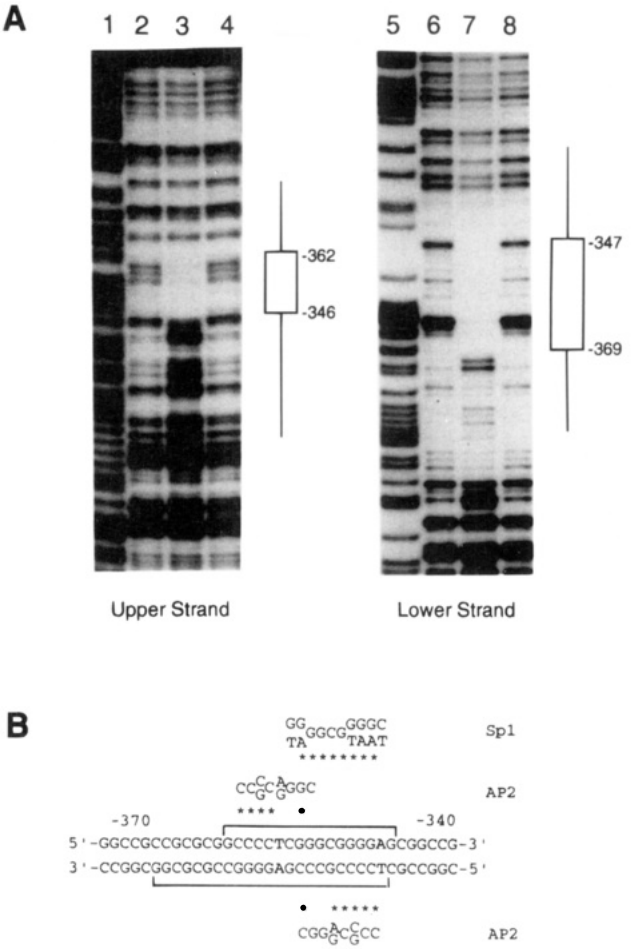


FIGURE 7: DNase I footprinting analysis of AP2 binding sites. (A) DNase I footprinting by purified AP2. A 500 bp *Bam*HI−*Xba*I fragment from N5 and a 320 bp *Bam*HI−*Xba*I fragment from N11 were used as probes. The assay conditions are the same as described in Materials and Methods. The location of DNase I protected sites is indicated on the right of each panel. Lanes: 1 and 5, G+A sequencing reaction; 2, 4, 6, and 8, control without nuclear extract; 3 and 7, with 5 ng of purified AP2. (B) Sequence, AP2 footprint site, and consensus sequence around the FP7 site. Numbers indicate the position relative to the major transcriptional start site. The sequences of both strands are shown, and footprinted sites are bracketed. The asterisk (*) indicates matched base pairs.

CV1, and HepG2 cells (Figure 8). Gel shift experiments clearly showed the abundance of AP2 and its intense binding to the FP7 oligo in HeLa cells. AP2 binding activity was found to be lower in NIH3T3 and CV1 cells, and no binding was apparent in HepG2 cells. Other DNA−protein complexes which showed different migration patterns from the AP2−DNA complexes were seen in CV1 cells, but the identity of these binding proteins is not presently known. The AP2 binding activity seems to correlate well with the difference in hGR promoter activity in these cell lines (Figure 8). We cannot, however, exclude the possibility that other mechanisms might be involved. Furthermore, we performed cotransfection experiments in HepG2 cells using hGR promoter−luciferase chimeras and AP2 expression plasmid. As shown in Figure 9, AP2 induced the transactivation of the hGR promoter constructs (N5 and N13) that contained the FP7 site by about 2-fold. By contrast, AP2 failed to transactivate the promoter chimeras (N30 and N25) that were devoid of the FP7 site.

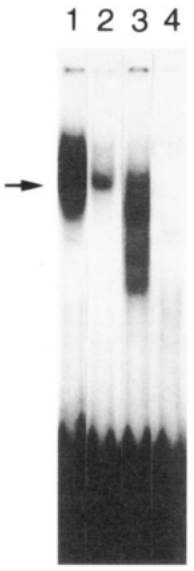


FIGURE 8: Cell line specificity of AP2 binding activity. Gel shift experiments were performed by combining radiolabeled FP7 oligonucleotides with equal amounts (5 μg) of nuclear extracts from HeLa (lane 1), NIH3T3 (lane 2), CV1 (lane 3), and HepG2 (lane 4) cell lines. The binding reaction was conducted by incubating the end-labeled oligonucleotide probe [$(1.0-2.0) \times 10^4$ cpm] with 5 μg of nuclear extract and 1 μg of poly(dI-dC) in a binding reaction buffer (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.05% NP-40, 1 mM EDTA, 1 mM DTT, and 10% glycerol) in a final volume of 20 μL for 30 min at room temperature.

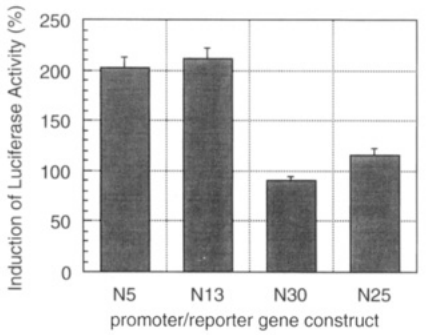


FIGURE 9: Activation of the hGR promoter activity by AP2. hGR promoter−luciferase chimera (N5, N13, N30, and N25) were transfected into HepG2 cells with or without 2 μg of AP2 expression construct. Transfection conditions were described under Materials and Methods. The final concentration of DNA in each transfection was kept constant at 6 μg by adding pUC18. The promoter activity was corrected for variations in transfection efficiency using β-galactosidase. The bars represent ratios of activity in the presence of transfected AP2 versus activity without transfected AP2.

DISCUSSION

To investigate the mechanisms that underlie the regulation of hGR expression, we have conducted a systematic study of the proximal 700 bp region of the hGR promoter with the aim of identifying important functional elements and nuclear factors that bind to them.

Transfections of a series of hGR promoter−luciferase gene chimeras containing various deletions of the hGR promoter suggest that (1) the −374 to +38 bp region is sufficient to direct efficient hGR transcription, (2) the region spanning −374 to −183 bp is crucial for promoter activity, (3) the −374 to −347 bp region is important for differential hGR promoter activity among different cell lines, and (4) additional positive regulatory elements are present within the −182 to +38 proximal region and within the −700 and −375 bp upstream region.

The −374 to −183 bp region which we found to be important for hGR promoter activity contains three footprinted sites, FP5, FP6, and FP7. The sequences of these sites are highly conserved between human and mouse with a 100% [17 nucleotides (nt)/17 nt] identity in FP5, 95% (18 nt/19 nt) in FP6, and 100% (23 nt/23 nt) in FP7 (Figure 3). This high degree of homology further supports the hypothesis that this region exerts an important role in hGR expression.

In particular, the 28 bp sequence between −374 and −347 bp contains a transactivation site that is responsible for the difference in hGR promoter activity among various cells since deletion of this region caused a dramatic decline in promoter activity in HeLa and NIH3T3 cells but not in HepG2. To identify the potential cis element and transacting factor, we performed competition and antibody supershift experiments on gel shift assays. The data reveal binding of AP2 to FP7. DNase I footprinting with purified AP2 supported this finding. Sp1 binding was very weak (data not shown). We have shown that AP2 binding activity correlates well with hGR promoter activity. We also found that AP2 can induce transactivation of the hGR promoter. Taken together, these data suggest that the AP2 site in FP7 and AP2 are important for the activation of GR transcription and that the relative levels of AP2 might account for the difference in hGR promoter activity in various cells.

AP2 was first identified in and purified from HeLa cells (Haslinger & Karin, 1985; Mitchell et al., 1987; Imagawa et al., 1987). This transcription factor is expressed in a cell-type-specific manner (Williams et al., 1988) and is regulated temporally (Mitchell et al., 1991). Phorbol esters, cAMP, and retinoic acid have been shown to induce AP2 activity, which is believed to play an important role in regulating the expression of genes involved in cellular differentiation (Mitchell et al., 1987; Imagawa et al., 1987; Luscher et al., 1989; Snape et al., 1991). Because of the positive regulatory effect of AP2 on GR transcription, it is conceivable that the expression pattern of AP2 could determine, in part, the levels of GR expression in different tissues and at different developmental stages.

Like other TATA-less promoters the hGR promoter is rich in putative Sp1 binding sites (Zong et al., 1990; Encio & Detera-Wadleigh, 1991; Leclerc et al., 1991). We found that Sp1 binds to the FP6 site which is located within −374 to −183 bp, a region shown to be important for maximal hGR promoter activity. Furthermore, we have shown binding of Sp1 to FP8, FP9, FP10, and FP11 sites which are located in the −700 to −375 bp GC box-rich region. This region is also well conserved between human and mouse (73%). In a previous work, Leclerc and co-workers showed that deletion of the region between −750 and −470 bp of hGR resulted in about a 3-fold decrease in promoter activity in CV1 cells (Leclerc et al., 1991). In this study, deletion of the −700 to −375 bp distal region does not significantly change promoter activity. At present the reason for the difference between these studies is not clear. However, we have demonstrated that this same region can induce activation of a heterologous promoter, in an orientation independent manner, in HeLa, NIH3T3, and CV1 cells. This suggests that these Sp1 elements might exert a positive regulatory effect on GR expression and also may be important for the expression of the endogenous GR gene.

Transcriptional regulation through GC-rich sequences is complicated by the fact that several factors other than Sp1 can recognize this sequence (Berg, 1992). Some Sp1-like proteins (Kingsley & Winoto, 1992; Imataka et al., 1992; Hagen al., 1992) and other transcription factors have been shown to have affinity to this site (Kim et al., 1987; Joseph et al., 1988; Chavrier et al., 1989; Christy & Nathans, 1989; Kageyama et al., 1989; Kageyama & Pastan, 1989; Cao et al., 1990; Lemaire et al., 1990; Crosby et al., 1991; Madden et al., 1991; Sogawa et al., 1993). We cannot exclude the possibility that these other proteins are not binding to the putative Sp1 sites on the GR promoter.

Additional nuclear proteins which are presently unidentified could also contribute to the control of GR transcription. In FP5, a computer search revealed that five nucleotides, 5′-CATTT-3′, matched the cis element, CLE0, in the IL-4, IL-5, and GMCSF promoters (Miyatake et al., 1991; Masuda et al., 1993). No significant homology with other known cis-acting elements was found in FP5 so far. FP8 and FP9 also bind unidentified factors. The role of these unknown factors in GR expression remains to be established.

In summary, we have identified regions in the hGR promoter that are important for the expression of this gene and characterized some of the transacting factors that interact with these elements. We have shown that several transcription factors are involved in hGR expression and that AP2 could be an important positive regulator of hGR promoter activity. We believe that these studies provide a foundation for further analyses of the molecular basis of hGR gene expression.

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