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HUMAN GLUCOCORTICOID RECEPTOR GENE PROMOTOR—HOMOLOGOUS DOWN REGULATION

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Summary—To study the regulation of the human glucocorticoid receptor (hGR), we characterized the promoter region by primer extension, S_1 nuclease mapping and by DNA sequencing. We found that the promoter is extremely G + C rich (72% GC content) and contains a "TAATA" and a "CAT" box, eight "GGGCGG", three "CCGCCC" and two "CACCC" motifs and a motif similar to the glucocorticoid responsive element (GRE) which included two interchanged nucleotides "TCTTGT". In contrast to other steroid receptor genes, exon I or GHGR contains the major part of the 5′ non-coding sequences of hGR mRNA while exon II contains coding sequences for the first 394 amino acid residues of the A/B region of hGR. The major transcriptional start site was found to be 134 bp upstream of the ATG initiation codon. Transfection of HeLa cells with plasmids containing various deletions of GHGR promoter fused to a promoterless CAT vector suggested the region between −470 and −1030, at the 5′ end of the mRNA start site, to contain sequences required for down regulation by hormone.

INTRODUCTION

Steroid receptors belong to a superfamily of ligand dependent transacting nuclear transcription factors that stimulate or suppress transcriptional activation of their target genes following binding by their cognate ligands [1, 2]. In the absence of the ligand, the receptor is primarily cytoplasmic and upon binding, the receptor–hormone complex translocates to the nucleus [3, 4], interacts with specific DNA regulatory sequences termed the glucocorticoid responsive element (GRE) and modulates transcription of nearby genes [5–8]. The cDNAs for many steroid receptors have been cloned. Comparison of the receptors from many species has demonstrated that the domains of DNA and hormone binding are highly conserved [1]. Furthermore, studies on the structure of organization of the genes for the chicken progesterone receptor [9], the human estradiol receptor [10] and the human androgen receptor [11] have shown conserved regions and similarities.

The *in vivo* down regulation of the glucocorticoid receptor (GR) by its ligand has been demonstrated by hormone binding studies [12, 13] and utilization of the cDNA probes in determining the level of GR mRNA, suggested the role of glucocorticoids in the homologous down regulation of its mRNA [14, 15].

To understand the organization of the human GR (hGR) gene promoter and to study the mechanism of the receptor down regulation, we have isolated the 5′ region of the hGR gene. We are interested in studying the transcriptional regulatory elements present in the promoter region and the analysis of the promoter activity. Various fragments of the 5′ region were inserted into CAT reporter plasmids and used to co-transfect AtT-20, HeLa, COS-1 or CV-1 cells in the presence and absence of hGR expression vector in order to study the phenomena of homologous down regulation.

MATERIAL AND METHODS

The hGR cDNA probe and expression vector

A full-length hGR cDNA encoding 777 amino acids was constructed from overlapping cDNA clones isolated from human MCF7-λgt11 and λgt10 cDNA libraries [16]. The deduced amino acid sequence of this hGR was identical to the cDNA sequence reported by Hollenberg *et al.* [17].

Isolation of the hGR gene promoter

Five genomic clones were isolated from a human leucocyte genomic library in λEMBL3 using oligonucleotide probes and purified by

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three rounds of screening and their inserts were mapped by restriction digestion and Southern hybridization [18].

Construction of GHGR–CAT chimeric plasmids

A 1151 bp fragment (between −1030 and +121) was inserted into the promoterless pBL–CAT vector at the HindIII–BglII site (HGR 1.0 CAT). No viral enhancers were present in the final construction such that the promoter and CAP site were contributed by the hGR genomic fragments. GR genomic fragments (−470 +121) were constructed deleting the sequences between the PstI (−470) and SmaI (−1030) restriction sites (HGR 470 CAT).

Analysis of promoter activity and S_1 mapping

A 560 nucleotides long single stranded probe was prepared by PstI digestion after synthesis with the primer "338" (Fig. 1) and single stranded template was purified on 6% denaturing gel. This probe was relabeled with (γ^{-32}P)ATP and T4-polynucleotidekinase at the 5′ end to high specific activity before S_1 mapping experiment. The probe was hybridized with 2 μg each of the polyA$^+$ [18] purified from MCF-7 and LNCaP cells, 20 μg of tRNA as control and 50 μg of total RNA purified from CV-1 cells transfected with hGR 1.0 CAT, and hGR 470 CAT.

Transfection

Transfections at AtT-20, HeLa, CV-1 or COS-1 cells were performed with the GHGR–CAT constructs (10 μg of the 2 × CsCl purified plasmids) in the presence or absence of 2.5 μg of hGR expression vector or with 2.5 μg of MMTV-CAT in the presence or absence of 2.5 μg of hGR expression vector by calcium phosphate co-precipitation [18]. As internal standard 5 μg of β-galactosidase expression vector CH110 (Pharmacia, Canada) was included during co-transfection. For the CAT assay, extracts containing 5 units of β-galactosidase (~50 μg of protein determined by Bio-rad protein assay kit) were used.

RESULTS

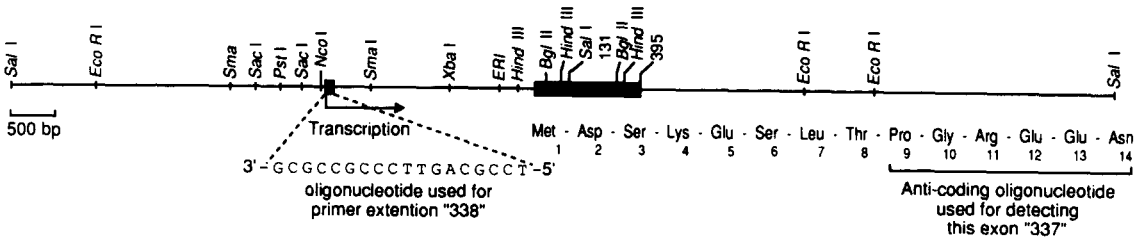
Sequence analysis of the SamI–SmaI subfragment of pGHGR 4.5 hybridizing to the oligonucleotide 338 (from the 5′ non-translated region, Fig. 1) showed to contain the complete 5′ non-coding sequences present in hGR cDNA [18]. The donor of exon I contained AG, whereas the acceptor of exon II is TT in GHGR. The genomic subclone pGHGR 3.5, contained codons for the first 394 amino acids (splice junction between Ser 395 and Pro 396), but did not extend to the DNA binding domain. An additional intron between the carboxyterminal part of A/B region and the amino terminus of the DNA binding region was also found.

Primer extension

To identify the 5′ boundary of the hGR mRNA, 5′ end ^{32}P-labelled oligonucleotide "338" (+73 to +90) corresponding to the antisense strand was prepared and hybridized to 1 and 2.5 μg of LNCaP and MCF-7 polyA$^+$ RNA (Fig. 2, lanes 1, 2, 3 and 4, respectively). The most abundant extension product has a length of 89 bases (Fig. 2 indicated by an arrow). Minor extension products containing 163, 136, 132, 120, 110 and 39 nucleotides were observed, in which the 136 nucleotides product appears to be more predominant in LNCaP than in MCF-7 mRNA. No extended products were visible in the control analysis (Fig. 2, lane 5) with yeast tRNA. This may suggest that the hGR gene transcription may be heterogeneous. These results indicated that the major hGR mRNA population is transcribed from the same transcription start site.

Analysis of promoter activity and the transcription initiation sites of the chimeric genes containing hGR regulatory elements

Mapping of the transcription initiation sites of the MCF-7 and LNCaP cellular hGR mRNA as well as the transcription initiation site of the chimeric gene containing the hGR promoter elements were performed by S_1 nuclease mapping [18]. A single stranded probe was synthesized using the oligonucleotide "338" and single stranded DNA containing the hGR gene fragment and purified after digestion with PstI. This 560 nucleotide probe hybridized to polyA$^+$ RNA from MCF-7 and LNCaP cells (Fig. 3) as well as the total RNA prepared from CV-1 cells transfected with hGR 1.0 CAT and HGR 470 CAT. An 89-nucleotides-long protected fragment was observed in the autoradiograph in all samples which co-migrated with the primer "338" extension products using polyA$^+$ RNA isolated from MCF-7 and LNCaP cells (Fig. 2) suggesting that the major initiation of transcription *in vivo* as well as *in vitro* after transfection occurs at an identical hGR–CAP site. Neither primer extension product nor S_1 protected



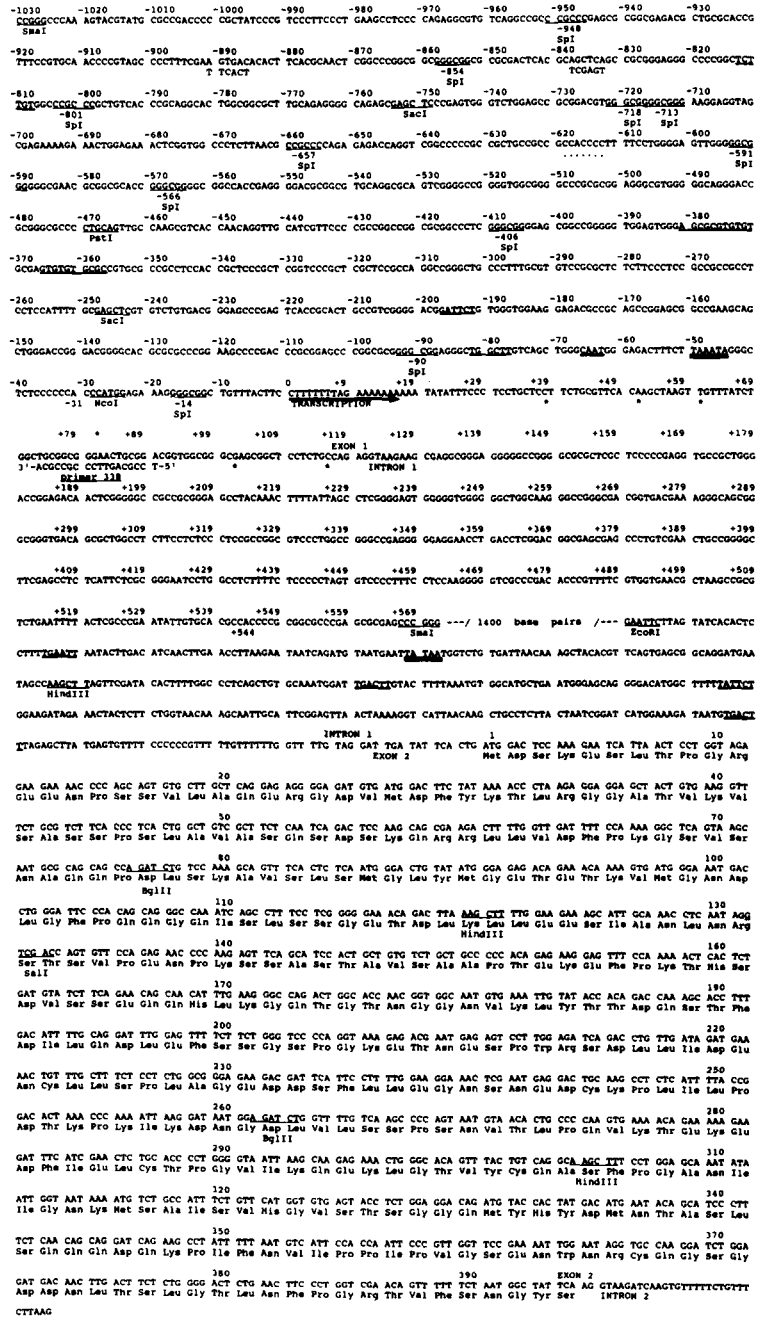


Fig. 1. Characterization and sequence of GHGR promoter. Partial restriction map of λGHGR is shown for reference. The oligonucleotide used for screening and the 5′ end of exon I are dark lined. Oligo "337" used in identifying exon II and oligo "338" used in identifying exon I are indicated. The sequence of GHGR 1.6 kb promoter region between SmaI and SmaI and the sequence part of exon II containing the codons for 394 N-terminal amino acid residues of hGR are shown in the sequence. The "GC" boxes "GGGCGG" or CCGCCC are marked with SpI in boxes. The *in vivo* transcription site is indicated by an arrow at +1 in the direction of transcription.

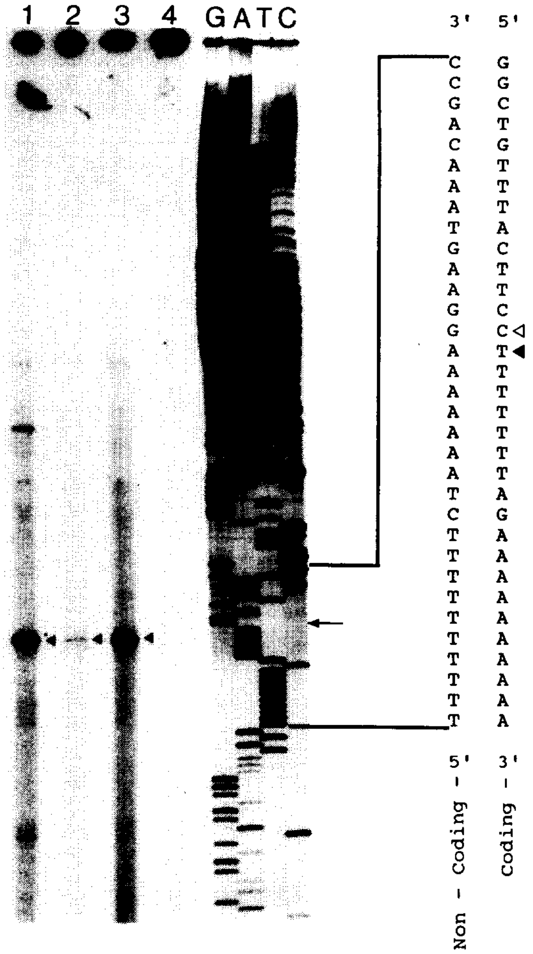


Fig. 2. Primer extension. The primer extension product of the ^{32}P-end labelled primer 5'TCCGCAGTTCCCGCCGCA3' (Primer 338) with 1 and 2.5 μg of LNCaP polyA+ RNA (lanes 1 and 2, respectively) and 1 and 2.5 μg of MCF-7 polyA+ RNA (lanes 3 and 4). The 10 μg of tRNA control primer extended product is shown in lane 5. The DNA sequencing lanes GATC show the dideoxy chain termination reaction products with the same primer using the 1600 bp SmaI–SmaI single stranded DNA on template. The sequence of the 5' end mRNA extension site is marked with an arrow.

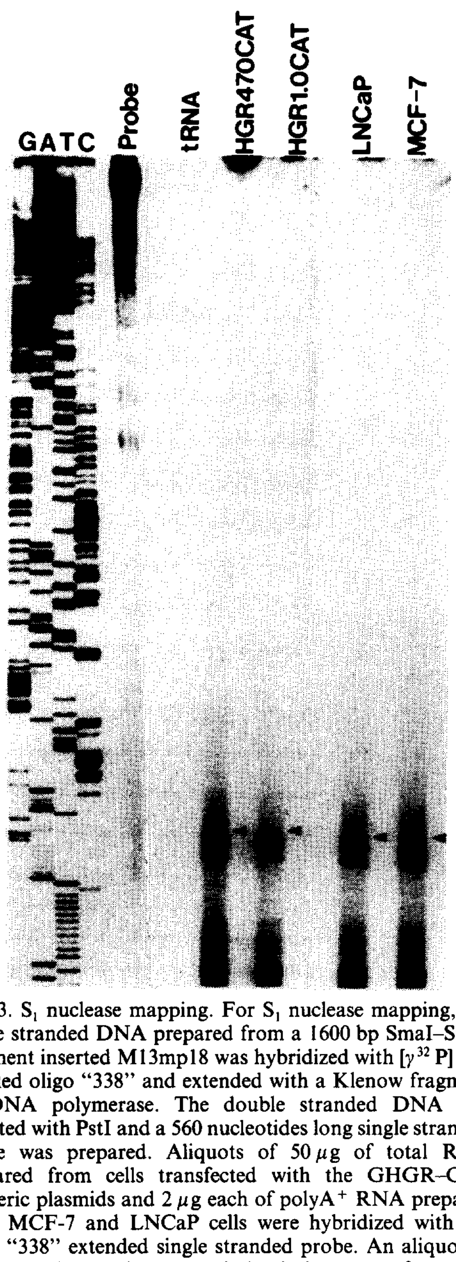


Fig. 3. S_1 nuclease mapping. For S_1 nuclease mapping, the single stranded DNA prepared from a 1600 bp SmaI–SmaI fragment inserted M13mp18 was hybridized with [γ^{32}P] end labelled oligo "338" and extended with a Klenow fragment of DNA polymerase. The double stranded DNA was digested with PstI and a 560 nucleotides long single stranded probe was prepared. Aliquots of 50 μg of total RNA prepared from cells transfected with the GHGR–CAT chimeric plasmids and 2 μg each of polyA+ RNA prepared from MCF-7 and LNCaP cells were hybridized with the oligo "338" extended single stranded probe. An aliquot of 20 μg tRNA served as control. Analysis were performed in 8% denaturating polyacrylamide gels. The largest protected fragment is indicated by an arrow. The DNA sequencing shown in lanes GATC are the products of dideoxy chain termination reactions as described in Fig. 4.

fragments were visible in the tRNA control assays. These experiments show that the hGR transcription site is functionally active.

Analysis of promoter function by transfection of chimeric GHGR–CAT plasmids into AtT-20, HeLa, CV-1 or COS-1 cells

To study the homologous down regulation of hGR, we introduced the promoter containing chimeric plasmids into AtT-20 cells. For transfection, we used HGR 1.0 CAT in both orientations. The cells were treated in the absence or presence of 10^{-8} M dexamethasone or 10^{-5} M forskolin alone or in combination for 12 h (Fig. 4, Panel B). Addition of 10^{-8} M dexamethasone suppressed CAT activity by 60%. The addition of 10^{-5} M forskolin into the medium had a stimulatory effect, increasing the CAT activity 1.4 fold. Treatment of the cells with both 10^{-8} M dexamethasone and 10^{-5} M forskolin did not affect the high level of CAT activity observed with forskolin alone. No CAT activity was observed with HGR 1.0 CAT in the reverse orientation.

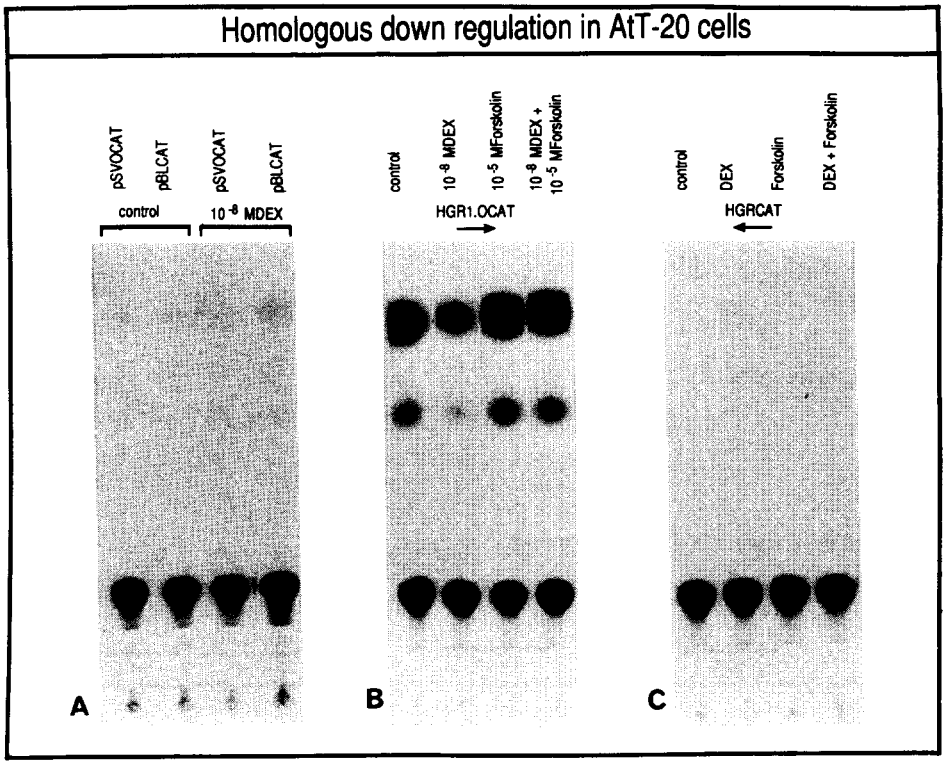


Fig. 4. Homologous down regulation in AtT-20 cells with hGR 1.0 CAT after co-transfection. To study the homologous down regulation of hGR with chimeric plasmids in cells containing intact glucocorticoid receptor system, we used AtT-20 cells. Control experiments with vectors used for construction in the presence and absence of hormone is shown in panel A. Treatment with AtT-20 cells transfected with HGR 1.0 CAT in the right orientation in the presence and absence of 10^{-8} M dexamethasone, 10^{-5} M forskolin or a combination of both is shown in panel B. Identical experiments performed with HGR 1.0 CAT in the reverse orientation is shown in panel C.

The GHGR–CAT constructs were co-transfected into HeLa cells with CH110 or co-transfected in addition with hGR expression vector. As shown in Fig 5, the CAT activity was unchanged when the cells were transfected with HGR 1.0 CAT in the absence or presence of 10^{-8} M dexamethasone. But the hGR expression vector co-transfected cells showed after hormone treatment a considerable decrease (40% compared to the untreated control) in measurable CAT activity (Fig. 5). The CAT activity was unchanged in hGR co-transfected cells without hormone treatment. Transfection of HeLa cells with HGR 1.0 CAT in reverse orientation showed no CAT activity in the cellular extracts.

Transfection analysis were performed with HGR 470 CAT in both orientations. As shown in Fig. 5, the homologous down regulatory signals are observed only with HGR 1.0 CAT. The regulation is orientation-dependent and deletion of sequence between −1030 and −470 results in the loss of homologous down regulation.

Sequence of the hGR gene promoter

The promoter contains 11 putative Sp1 binding sites [19] between −20 and −1030. Three of the motifs "CCGCCC" at −657, −801 and −948 are on the sense strand and 8 of the complementary motifs "GGGCGG" at −14, −90, −566, −591, −713, −718 and −853 are on the anti-sense strand of GHGR. There are two "CACCC" motifs at the promoter region at −31 and at −616. A perfect "TATAA" box is present downstream in intron I. There are motifs such as TCTTCT (+173), TATTCT (+151) and TGACTT (+97 and +261) present in the 3′ region of the "TATTA" motifs in the intron I. The motif "TCTTGT" (−813) is similar to the positive GRE consensus sequence "TGTTCT".

DISCUSSION

We have shown by DNA sequence analysis that the exon containing 394 amino acids of the A/B region of the hGR is separated from the

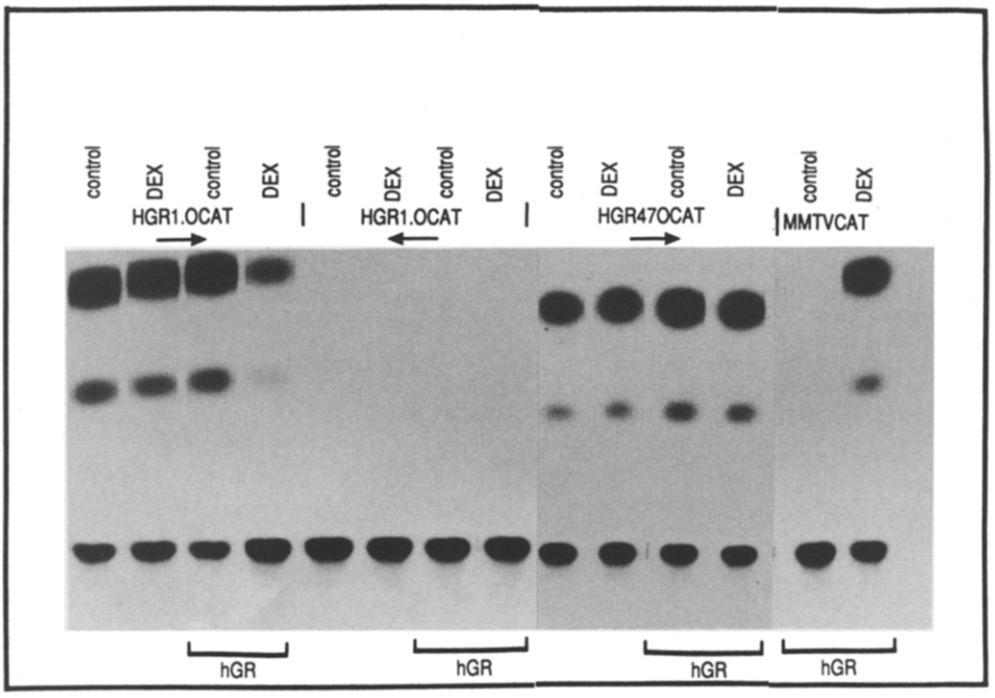


Fig. 5. Homologous down regulation in HeLa cells. Transfection of HeLa cells with HGR CAT chimeric plasmids in the presence and absence of co-transfected hGR expression vector or transfection of MMTV-tk-CAT in the presence and absence of co-transfected hGR expression vector are shown in Fig. 5. Treatment of the hormone is indicated directly on respective assays. Controls contained the non-hormone treated HGR-CAT transfected cells in the absence and presence of 10^{-8} M dexamethasone. The receptor co-transfections are shown in brackets.

exon containing the 5′ non-translated region. There is an intron of 2 kb between exon I and exon II. The structural analysis of the chick progesterone receptor gene [9], the human estradiol receptor gene [10] and the human androgen receptor gene [11] shows that the A/B region and the 5′ non-coding region are in exon I.

Primer extension and S_1 nuclease mapping

Both primer extension and S_1 nuclease mapping showed that irrespective of the cells employed, the CAP site for major hGR mRNA is identical. The S_1 mapping further demonstrated that the GHGR-transcription unit is present in the isolated gene fragment and is active in the fused gene constructs. Thus primer extension and S_1 nuclease mapping defined the hGR mRNA CAP site.

Studies on the homologous down regulation

We tested by co-transfection of AtT-20 cells with HGR 1.0 CAT in both orientations in the presence and absence of forskolin which increases intracellular cAMP levels. The fusion constructs HGR 1.0 CAT in the right orientation produced a 1.4 fold forskolin induction. Akerblom *et al.* [20] have demonstrated the negative regulation of α-subunit gene by glucocorticoids through interference with cAMP responsive enhancer. We found that the treatment of the cells following co-transfection of HGR 1.0 CAT with both forskolin and dexamethasone did not suppress transcription as was observed with 10^{-8} M dexamethasone alone. This down regulation of hGR gene in AtT-20 cells is orientation-dependent. Further characterization of the promoter elements involved in homologous down regulation were studied by co-transfection of HGR 470 CAT demonstrating that homologous down regulatory signals are located between −1030 and −470 upstream of the transcription start site.

Studies are in progress by deletion mutation and analysis of down regulation by transfection into a variety of cells to understand the mechanism of homologous down regulation of the hGR.

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