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Oligodendrocytes as Glucocorticoids Target Cells: Functional Analysis of the Glycerol Phosphate Dehydrogenase Gene

Janet D. Cheng and Jean de Vellis*
Mental Retardation Research Center, Departments of Neurobiology and Psychiatry, Neuropsychiatric and Brain Research Institutes, UCLA School of Medicine, Los Angeles, California

Previous research has established that the development and function of oligodendrocytes are influenced by glucocorticoids. The enzyme glycerol phosphate dehydrogenase (E.C.1.1.1.8) has been used as a model to study glucocorticoid regulation of gene expression in oligodendrocytes and the C6 glial cell line. In the rat brain this enzyme is exclusively localized to oligodendrocytes. The sequence of the 5′ flanking region for the rat gene encoding Glycerol Phosphate Dehydrogenase (GPDH; EC 1.1.1.8) was determined. 4 kb of sequence from the 5′ flanking region, exon 1, and part of intron 1 of the rat GPDH gene was compared to the corresponding mouse sequence. Dotplot matrix comparison revealed that the rat sequence is more than 80% similar to the mouse sequence, but differs from the mouse sequence in two regions: the rat sequence is devoid of 200 bp of B1 repeat sequence that is present in the mouse, and the rat sequence has an excess 700 bp of B2 repeat sequence inserted between −0.7 kb and −1.4 kb that is absent in the mouse. To determine the regulatory activity of the rat GPDH 5′ flanking region, various portions of the rat GPDH 5′ flanking region were placed in luciferase reporter constructs and tested for transcriptional activity. Transient transfection of reporter constructs into the C6 glial cell line revealed that the distal end of the 5′ flanking region was glucocorticoid-inducible. A 385 bp Glucocorticoid Response Unit (GRU) was identified whose glucocorticoid induction was enhanced by dibutyryl-cAMP and reduced by phorbol esters. Sequence analysis of the GRU revealed the presence of four consensus GRE sequences and other putative consensus elements. Results here suggest that the 5′ flanking region of the GPDH gene mediates the ligand-inducible regulation of GPDH, and that multiple signaling pathways converge at the 5′ regulatory sequence to modulate GPDH gene expression in oligodendrocytes. J. Neurosci. Res. 59: 436–445, 2000.

Key words: glucocorticoids; glycerol-3-phosphate dehydrogenase; glia; oligodendrocytes; C6 glioma; glucocorticoid response elements; phorbol esters; cAMP

The concept of brain cells as targets for glucocorticoid hormones originated from radioligand and functional studies that correlated the presence of specific glucocorticoid receptors to discrete groups of neurons in the limbic system (for review see McEwen, 1999; McEwen et al., 1986). These localization experiments, however, failed to provide evidence for involvement of neuroglial cells as glucocorticoid target cells. The discovery that glycerol phosphate dehydrogenase (sn-glycerol-3-phosphate dehydrogenase, EC.1.1.1.8; GPDH) is specifically regulated by glucocorticoids in most regions of the developing and adult rat brain (de Vellis and Inglish, 1968, 1973), but not in other organs raised the question whether or not this induction is a differentiated property of one more brain cell types. The exclusive localization of GPDH to oligodendrocytes in the adult (Leveille et al., 1980; Meyer et al., 1982) and also in the developing brain (Gordon et al., 1992) as well as in vitro (Breen and de Vellis, 1974; McCarthy and de Vellis, 1980; Lopez-Cardozo et al., 1989) provided strong evidence for the concept of oligodendrocytes as glucocorticoid target cells. GPDH enzymatic activity (de Vellis and Inglish, 1973; Breen and de Vellis, 1974), protein level (McGinnis and de Vellis, 1978), and mRNA level (Kumar et al., 1984, 1986) increase at least 5–10-fold upon stimulation by glucocorticoids. Much of the regulation of GPDH occur at the transcriptional run-off rate level (Kumar et al., 1985, 1989; for review Kumar et al., 1988) indicating that transcription of the GPDH gene is highly regulated.

Previously, the 5′ regulatory regions of the mouse and the human GPDH-encoding genes (the Gdc-1 locus) have been determined (Ireland et al., 1986; Dobson et al., 1987; Gwynn et al., 1990). Significant regions of homology were found between the mouse and the human 5′ flanking sequences (Gwynn et al., 1990). Several DNase hypersensitive sites also exist in the human 5′ flanking sequence (Gwynn et al., 1990). These data indicate that the 5′ flanking region of the GPDH gene is evolutionarily conserved, and may be important for regulation. Whether

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*Correspondence to: Jean de Vellis, Ph.D., 68-177 NPI, 760 Westwood Plaza, Los Angeles, CA, 90024. E-mail: jdevellis@mednet.ucla.edu

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the 5′ flanking region of GPDH contains cis-elements that influence the transcriptional regulation of GPDH expression remains unclear.

In this study, we examined the role of GPDH 5′ flanking region in mediating the glucocorticoid response of GPDH in rat glial cells. In both rat oligodendrocytes and C6 glioma cells, GPDH transcriptional run-off rate is up-regulated by glucocorticoids (Kumar et al., 1985, 1989). This induction has been determined to be a molecular mechanism in the stress response in the brain (Nichols et al., 1990, 1996; Masters et al., 1994), and has been shown to be dependent on the glucocorticoid receptors (McGinnis and de Vellis, 1981; Bennett et al., 1977; Finch et al., 1990). Most of the characterization for glucocorticoid regulation of GPDH in glial cells, however, has been carried out in rats. Comparatively little is known about the glucocorticoid regulation of GPDH in other species, except for a report of GPDH inducibility in pig oligodendrocyte cultures (Montz et al., 1985). Moreover, the information available seems to indicate that the mouse GPDH in the brain is not as inducible, if at all, as the rat GPDH (Thurston et al., 1978; McGinnis and de Vellis, 1977). Although both the human and the mouse GPDH 5′ flanking sequences are available, the rat GPDH 5′ flanking sequence has not been determined. The lack of information and the discrepancy in regulation prompted us to obtain the rat sequence for characterization. In this report, we report the sequence of the rat GPDH 5′ flanking sequence, and the points of differences between the rat and the mouse sequences. Furthermore, we tested the response of the rat 5′ flanking sequence to glucocorticoids using transient transfection analysis. A 385 bp sequence was identified in the distal portion of the rat GPDH 5′ flanking region that was glucocorticoid-inducible. The induction was enhanced by dibutyryl cyclic AMP, and reduced by phorbol esters. Results here define a functional role for the GPDH 5′ flanking region, and provide a model for the study of interaction among different signal transduction pathways in oligodendrocytes.

EXPERIMENTAL PROCEDURES

DNA Sequence Determination and Analysis

A lambda Charon 4A clone containing the genomic sequence of the rat GPDH gene was isolated based on hybridization with a 600 bp rat GPDH cDNA and a 42 mer synthetic oligonucleotide corresponding to the mouse Gdc-1 exon 1 (Kumar and de Vellis, unpublished data). This phage clone was restriction mapped and subcloned into smaller fragments in pBluescript. Each subclone was then subjected to exonuclease III digestion (Erase-A-Base; Promega) and sequenced (Sanger et al., 1997; US Biochemical). Sequences were compiled and assembled using the University of Wisconsin Genetic Computing Group (GCG) sequence analysis program. Comparison of sequences was carried out using the Needleman and Wunsch algorithm (Needleman and Wunsch, 1970) in the GAP program of GCG at a gap weight of 5.0 and a length weight of 0.3. Global dot matrix comparisons were carried out using the Compare and Dotplot programs in GCG (Maizel and Lenk, 1981). Also, the database for Transcription Factor Binding Sites (TFSITES) from GenBank was used for search of putative *cis*-elements in the 5′ flanking region.

Construction of Reporter Plasmids

Restriction fragments containing the 5′ flanking region of the GPDH gene were isolated using the enzymes shown in Figures 3A and 4A. Fragments were blunt end cloned into the NheI site of the luciferase reporter vector pGL2-basic (pGLB; Promega). The TATA-like box, transcription initiation site, as well as the 5′ un-translated region were included in each construct. The vectors pGLP-PE and pGLP-SPE were generated by ligating the PstI-EcoRV fragment into the XhoI site of the pGL2-promoter vector (pGLP, Promega), that contains the SV40 promoter sequence in front of the luciferase cDNA. The pGLP-SPE vector contains the PstI-EcoRV fragment downstream of the luciferase gene. Constructs containing 5′ and 3′ deletions of the PstI-EcoRV fragments (Fig. 3A) were derived from Exonuclease III-digestion using the Erase-A-Base kit. The inserts for pFC0.6 and pFCR0.6 were generated from a 0.6 kb PflMI-PflMI restriction fragment. This fragment was blunt-end cloned into the NheI site of p0.9. The plasmid pGRE5CAT was a generous gift of J. White (Mader and White, 1993).

Cell Culture and Transfection

The transient transfection studies used C6 cells (ATCC) from passage 38–44 and Rat-1 fibroblast cells from passage 5–9. Both types of cells were plated and maintained in Dulbecco's Minimal Essential Media and Ham's F12 media in a 1:1 ratio (DMEM/F12) with 5% fetal calf serum (FCS). Three days before transfection, 300×10^3 of C6 cells and 100×10^3 Rat-1 cells were plated onto 35 mm culture wells and incubated at 37°C in humidified air containing 5% CO_2. Cells in logarithmic phase at approximately 80% confluence were used for transfection. Six hours before transfection, the media was replaced with fresh DMEM/F12 and 10% FCS to ensure active cell proliferation.

A pCAT construct (Promega) containing the chloramphenicol acetyltransferase reporter (CAT) gene driven by the SV40 promoter sequence was used in every transfection to normalize the transfection efficiency. The cells were transfected with the standard calcium phosphate precipitation method using the Profection kit provided by Promega. Cells were incubated with DNA precipitates at 37°C for 12 hr, rinsed, and incubated in 5% FCS for an additional 21 hr. For transient transfection, cells were induced with ligands for 24 hr before harvesting. For stable transfection, the pSV2neo expression vector (Okayama and Berg, 1983) instead of pCAT was co-transfected with the reporter construct. Ten μg of the expression vector DNA and one μg of the pSV2neo DNA were transfected into C6 cells grown in 100 mm culture dishes. Twenty-four hours after precipitate removal, transfected cells were trypsinized and replated at a 1:20 dilu-

tion. Replated cells were selected with the antibiotic G418 (400 μg/ml; Gibco) until colonies were visible. All colonies from each transfection were pooled and each pool was denoted according to the name of the plasmid transfected, e.g., the cell line p4.3 was derived from transfection with the plasmid p4.3. Ligands were purchased from Sigma and prepared at a 1000-fold concentrated stock solution. Hydrocortisone (HC) and 12-*O*-tetradecanoyl 13-phorbol acetate (TPA) were dissolved in ethanol. Dibutyryl cyclic AMP (db-cAMP) was prepared in media. The final concentrations of ligands used for stimulation were: 1 uM HC, 100 μM db-cAMP, and 100 ng/ml TPA.

Luciferase and CAT Assays

Transfected cells were rinsed three times with 1× PBS and lysed in lysis buffer (50 mM NaH_2PO_4, pH7.4, 1 mM EDTA, 1 mM 2-mercaptoethanol). Cell lysates were centrifuged at 10,000 × *g* for 10 min, and the supernatant was used for luciferase and CAT analyses. Luciferase assays were carried out in a luminometer (Berthold, San Diego). One hundred microliters of the Luciferase Reaction Buffer (Promega; 530 μM ATP, 470 μM luciferin, 270 mM Coenzyme A, 20 mM Tricine, 2.67 mM $MgSO_4$, 0.1 mM EDTA, 1.07 mM $(MgCO_3)_4Mg(OH)_25H_2O$ and 33.3 mM dithiothreitol) was injected into 30 μl of the lysate supernatant in a tube placed inside the luminometer. Light emission was measured for 10 sec after addition of the reagent.

CAT activity was determined by the liquid scintillation counting method using a kit provided by Promega. Activity was determined from 30 μl of cell lysate at an incubation time of 30 min at 37°C. Counts of butyrylated ^{14}C-chloramphenicol were subtracted from background (no enzyme) and compared to a standard curve. After CAT assay, protein concentration of each sample was determined using the Bradford method. Values of luciferase activity were normalized to values of CAT activity and divided by mg of protein in each well. Expression of luciferase reporter gene from the positive control (SV-40 promoter, pGLP) was made 100% and all other experimental values were expressed as a percent of the SV-40 positive control.

RESULTS

Determination of Sequences Upstream of the Rat GPDH Exon 1

A rat genomic clone containing the GPDH gene was previously isolated (Kumar and de Vellis, unpublished data), based on its ability to hybridize to a 600 bp rat GPDH cDNA (Kumar et al., 1985) and a synthetic oligonucleotide corresponding to exon 1 of the mouse GPDH-encoding locus (Ireland et al., 1986; Dobson et al., 1987). Restriction mapping and Southern blot hybridization analyses revealed that this clone contained at least 6 kb of sequences upstream of exon 1. The relevant region of the genomic clone was then subcloned into smaller restriction fragments and sequenced. 4596 base pairs of sequence, containing part of intron 1, the entire exon 1, and sequence upstream of exon 1 were determined (Fig. 1). To confirm the identity of this sequence as GPDH, a Gap comparison was carried out using the GCG program. In the mouse genomic sequence, the first nucleotide of the ATG codon in exon 1 was designated +1, and all sequence upstream was negative numbered (Ireland et al., 1986). To facilitate comparison, we adopted the same numbering system for our sequence. The rat exon 1 contains 14 amino acids, and is identical to the mouse exon 1 except in three nucleotide positions. They corre-

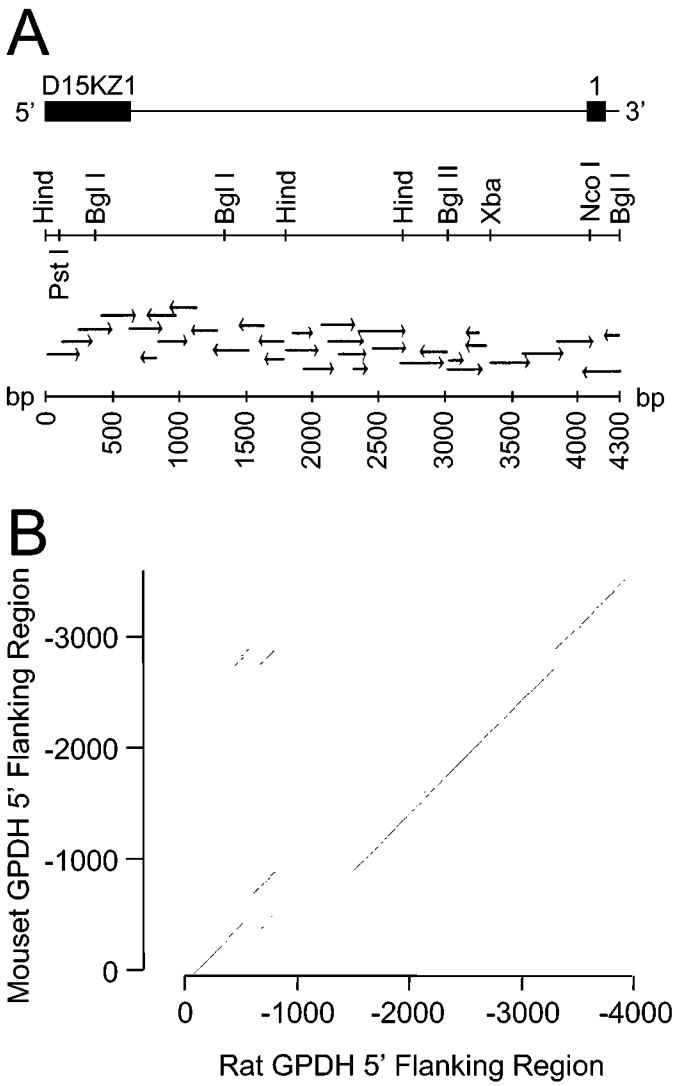

Fig. 1. (**A**) Organization of the 5′ flanking region of the rat GPDH-encoding gene. The black box represents the sequence for D15kz1 or GPDH. The arrows represent the direction of sequencing reaction. (**B**) Dotplot sequence comparison between the rat and the mouse GPDH 5′ flanking sequences. Comparison was carried out at a window of 20 with a stringency of 16 using the Compare and Dotplot program of GCG. The line represents the points of similarity, the discontinuity represents points that do not have any similarity between the two sequences.

spond to a T to C change at position 24, a C to A change at position 27, and a T to C change at position 33. These substitutions all occur at the third position of the codon, and do not lead to changes in amino acid identity. Downstream of exon 1, 226 bp of the rat intron 1 sequence was determined and compared. This region of the rat intron 1 shares an overall homology of 88% with the mouse intron 1 sequence. Furthermore, 3.8 kb upstream of exon 1, another transcription unit previously identified as D15kz1 in the mouse (Johnston et al., 1989) was also present in the rat sequence. Based on the high sequence identity in exon 1, intron 1, and the upstream gene, we concluded that the genomic sequence we obtained corresponded to the 5′ flanking region of the rat gene encoding GPDH.

Comparison Between the Mouse and the Rat GPDH 5′ Flanking Sequences

Upstream of exon 1, at position −50, the sequence TAAATA exists, that is identical to the human and the mouse TATA-like box. The transcription initiation site previously identified in the mouse gene (Dobson et al., 1987) was also conserved in the rat (located at 26 bp downstream of the TATA-like box). Operationally, we have defined the 5′ flanking sequence of the rat GPDH gene from start at position 1 of the first ATG codon in exon 1, upstream to the end of polyadenylation signal of the upstream gene D15kz1.

To identify differences between the rat and the mouse GPDH 5′ flanking sequences, we carried out a dot-plot matrix analysis using these two sequences. The entire GPDH 5′ flanking regions of the mouse and the rat genes were compared using a window of 20 and a stringency of 16 nucleotides. Each dot made on the graph thus represents a 80% sequence similarity (or more) between the two sequences. As shown in Figure 1B, all dots from the analysis formed a linear line, indicating that the two sequences are at least 80% homologous; however, two distinct regions of differences exist between the mouse and the rat: a deletion of 200 bp located at 3 kb upstream of exon 1, and an insertion of 700 bp located between −0.7 kb and −1.4 kb of the rat sequence (Fig. 1B). The deleted sequence corresponds to the B1 repetitive elements in the mouse (Ireland et al., 1986; Gwynn et al., 1990), and partially matches to sequences approximately 0.6 kb upstream of exon 1. On the other hand, the 700 bp insertion does not match to any of the mouse sequence. A search in the GenBank database revealed that sequence within this insertion is similar to the B2 repeat element.

The Distal 5′ Flanking Sequence of the GPDH Gene is Glucocorticoid-Inducible in C6 Glioma Cells

Glucocorticoid induction of GPDH in oligodendrocytes is an in vivo event that can be reproducibly observed in culture, including primary mixed cultures of the cerebrum (Breen and de Vellis, 1974; Breen et al., 1978), enriched oligodendroglial cultures (McCarthy and de Vellis, 1980; Warringa et al., 1987), or C6 glioma cell line (McGinnis and de Vellis, 1978; for review see Kumar and de Vellis, 1988). To determine if the 5′ flanking region of the rat GPDH gene was glucocorticoid-inducible in glial cells, we carried out transient transfection analyses using C6 glioma cells. Restriction fragments containing GPDH 5′ flanking region were placed upstream of the luciferase reporter gene. A GPDH-negative cell line, Rat-1 fibroblast cells, was used as a negative control. C6 and Rat-1 cells were transfected with various reporter constructs, and subsequently induced with ethanol, or 1 μM hydrocortisone (HC) for 24 hr before luciferase activity of the reporter gene was assayed.

Luciferase activity was detectable in both C6 and Rat-1 cells transfected with the GPDH-luciferase reporter constructs (Fig. 2). The values of luciferase activity from each construct were as high as the SV40-driven luciferase reporter construct (pGLP), indicating that the promoter of GPDH was very active in both cell types. Only the p4.3 construct exhibited a 4-fold HC-induced increase in luciferase activity. All other constructs, including the SV-40 driven pGLP construct, did not exhibit any HC-induced increase in luciferase activity. The response of the p4.3 construct to HC was specific to C6 glioma cells, because the same induction was not observed in fibroblasts. Simultaneous to the luciferase assays, the endogenous GPDH activities were also measured. Accordingly, the C6 glioma cells had an average basal GPDH activity of approximately 40 units/mg protein, and an average induced activity of 220 units/mg protein. On the other hand, the Rat-1 fibroblasts had a basal activity of 5 units/mg protein, and HC did not cause any significant increase in GPDH enzymatic activity.

The Distal Portion of the 5′ Flanking Region Contains Glucocorticoid Enhancer Activity

The transfection data from the deletion experiment above indicated that the distal deleted portion of p4.3 was important for glucocorticoid inducibility in C6 cells. To test if the distal portion alone could function independently of downstream sequences, a PstI-EcoRV restriction fragment encompassing this region (from −4232–−2734) was isolated. Reporter constructs were assembled in a variety of configurations to test the distance- and orientation-dependence of this fragment (Fig. 3). To test the position-dependence of this fragment, the Pst-EcoRV fragment was placed 0.9 kb upstream of GPDH's transcription initiation in construct p0.9. This shortens the distance between the PstI-EcoRV fragment and transcription initiation by 1.8 kb. To test if the PstI-EcoRV fragment was capable of enhancing transcription from a heterologous promoter, this fragment was cloned either upstream of the SV40 promoter (pGLP-PE), or downstream of the luciferase reporter gene (pGL-SPE). Transfection of these constructs into C6 cells revealed that those constructs containing the PstI-EcoRV fragment (i.e., p0.9PE, pGLP-PE, and pGLP-SPE) were glucocorticoid-inducible, whereas those that did not contain the fragment (p0.9 and pGLP) were not inducible (Fig. 3). Additionally, the basal, un-induced levels of PstI-EcoRV-containing constructs seem to be lower than those constructs without

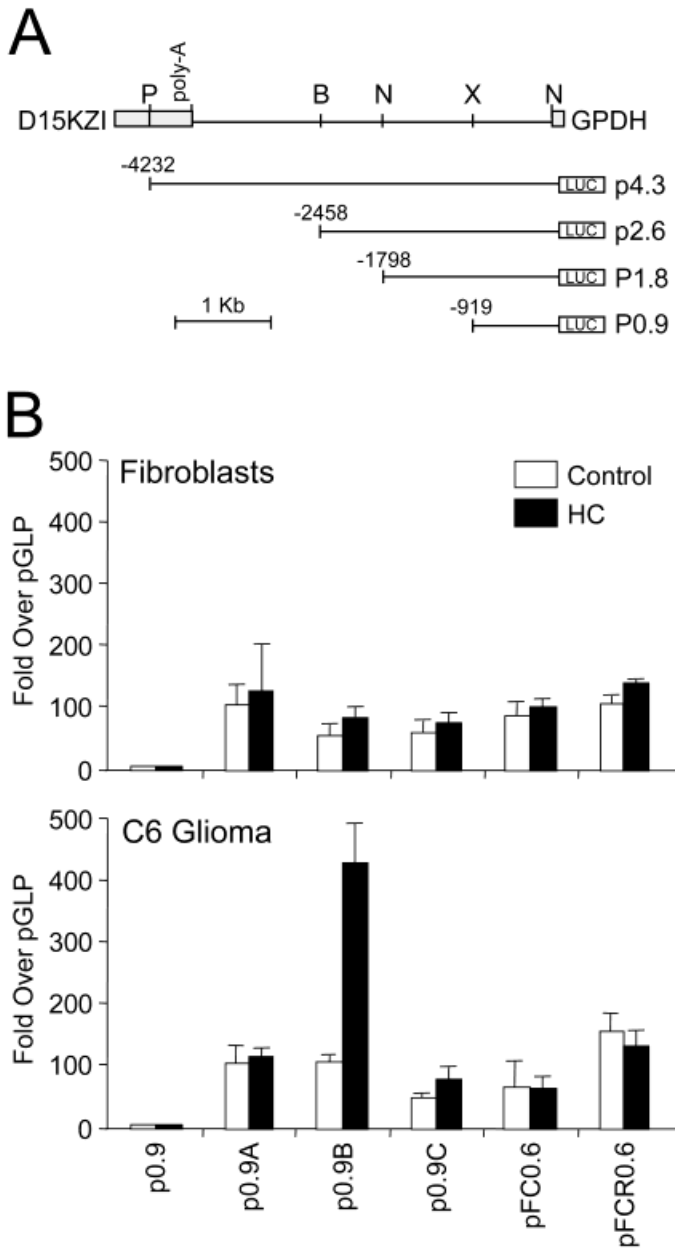

Fig. 2. Transcriptional activity of the rat GPDH 5′ flanking region assayed by luciferase reporter activity in transfected C6 glioma and Rat-1 fibroblast cells. (**A**) Schematic restriction map of GPDH 5′ flanking region in luciferase reporter constructs. (**B**) Results of luciferase assay. Different portions of the GPDH 5′ flanking region were cloned into the pGLB luciferase reporter construct using the convenient restriction sites listed. Each construct was introduced into C6 glioma and fibroblast cells by the standard calcium phosphate-mediated DNA transfection. 24 hr after transfection, cells were treated with ethanol (control) or 1 uM hydrocortisone. Cellular extracts were harvested after 24 hr of induction. Values of luciferase activity from each construct were normalized to the CAT activity per ug protein, and expressed as percent expression of the SV40-driven pGLP reporter activity in untreated cells. The result is an average of five independent experiments with triplicate samples from each experiment. P, PstI; B, BstXI; N, NcoI; X, XbaI.

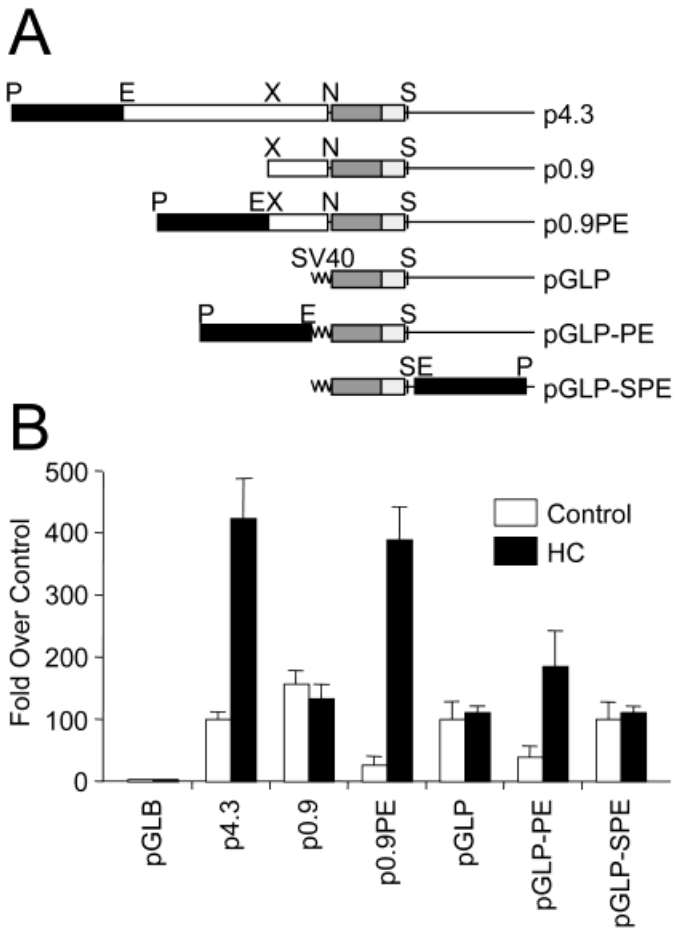

Fig. 3. Glucocorticoid-inducibility of the PstI-EcoRV fragment cloned in different positions and orientations relative to transcription initiation. (**A**) Configuration of each reporter construct. Shaded box represents the luciferase reporter gene. (**B**) Results of luciferase assay from transient transfection. Each construct was introduced into C6 cells and analyzed as described in the legend to Figure 2. The result is an average of six independent experiments with triplicate samples for each experiment. Fold inductions were statistically significant based on Student's *t*-test for plasmids p4.3, p0.9PE, pGLP-PE, and pGLP-SPE. P, PstI; E, EcoRV; X, XbaI; N, NcoI; S, SalI.

this fragment, indicating that a basal repressor activity is also associated with the PstI-EcoRV fragment.

The Glucocorticoid Enhancer Activity Is Located Between −3631 and −3246

Using the PstI-EcoRV fragment, we carried out further deletion to determine the location of enhancer activity. Two 5′ deleted fragments and one 3′ deleted fragment were generated using Exonuclease III. These fragments were blunt-end ligated into p0.9, generating constructs p0.9A, p0.9B, and p0.9C (Fig. 4A). In addition, a fragment deleted at both the 5′ end (600 bp) and the 3′ end (300 bp) was generated. All of these deletion fragments were cloned into the p0.9 construct, that was the non-inducible construct with the highest basal activity.

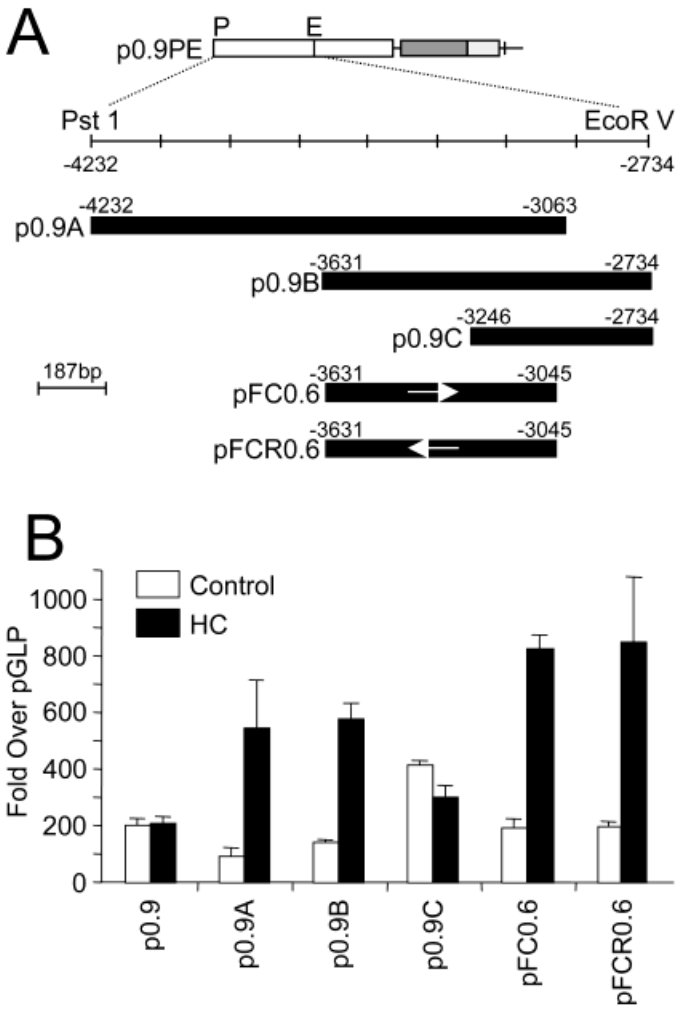

Fig. 4. Delineation of the glucocorticoid-inducible region in the PstI-EcoRV fragment. (**A**) Schematic of deletion constructs containing different portions of the PstI-EcoRV fragment. (**B**) Results of luciferase assay from transfection. The constructs p0.9A, p0.9B, and p0.9C were generated from Exonuclease III deletions of the PstI-EcoRV in p0.9PE. Inserts for the constructs pFC0.6 and pFCR0.6 were generated from a PflMI digestion of the PstI-EcoRV fragment. The arrows within pFC0.6 and pFCR0.6 represent the direction of the insert. The deletion constructs were transfected into C6 cells and analyzed as described in the legend to Figure 2. Results represent the average of three independent experiments with triplicate samples in each experiment. Significant differences were observed in between control and HC-treated cells transfected with p0.9A, p0.9B, pFC0.6, and pFC0.6R. Statistically significant differences in the negative direction were also observed in cells transfected with p0.9C.

When transfected into C6 cells, both p0.9A and p0.9B were glucocorticoid-inducible, but p0.9C was not (Fig. 4B). Comparison of insert size between p0.9B and p0.9C revealed that p0.9C was deleted for an additional 385 bp of sequences at the distal end. This region, from −3631−−3246, was thus termed the glucocorticoid responsive unit (GRU). The GRU sequence was also

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-3630  GCCGGTCGTG CCAGGCTCTG GAGGTCTGGG AAATGAGTCT TTTTCCCCTG
-3580  GGGACTCTGG GTCTTATTAG AGGCTTGTGA CCTCAGCAAT GCCCTTGGTT
-3530  GGTTGCTCCA ACTCCCAGCC ATCAAGAACA GACAAGGCAG TGCGCTACTG
-3480  GTGTCTCCCT CGTCCTTGAA ATCTCCATGT CATGTACTGG CAGGAACAAG
-3430  CTGTCCTTGG CATCTGTGGC CCCTTGATTT TGATGAAACT GGGCTTTGTT
-3380  CTCCCAAGAG TACATGTCCA TCCAGCCGCT GCCAAGGGCC CCAGTGACAG
-3330  GTTTTCCATA CAGACCCACC ACATGCACTG ATCTCATGCT TTCTGAGGTT
-3280  CTGTGAATGG CCCCAAGACA CCGGGGTTTT CTTTGT
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Fig. 5. Nucleotide sequence of the Glucocorticoid Response Unit (GRU) at the distal portion of the GPDH 5′ flanking region. This region was defined based on transfection results in Figure 4. Nucleotide sequences are numbered in relation to the ATG start codon of exon 1. Consensus GRE sequences identified from GenBank search are double-underlined.

present in constructs pFC0.6 and pFCR0.6, both of which exhibited a similar glucocorticoid induction, indicating that GRU can function in both forward and reverse orientations.

A search in the database for consensus transcription factor binding sites (database TFSITES.DAT) within the GRU revealed the presence of four Glucocorticoid Response Elements (GREs; Fig. 5). Two of them have the sequence (A/T)CTG(A/T)TCT in forward or reverse direction, and one has the sequence TGTTCT. Both types of these sequences correspond to the consensus GRE half sites. In addition, a pseudo-palindromic full site was found, that has the sequence GNNACAANNTGTYCT. The search also identified a number of other consensus binding sites for transcription factors, including an AP-2 site, a RXRβ half site overlapped with a NF-IL6 binding site, an E2A binding site for the transcription factors E12/E47, and a NFκB binding site. Comparison of the GRU sequences to the equivalent region in the mouse and human GPDH 5′ flanking region revealed that the GRU was 80% homologous to the mouse, but only 42% to the human sequence. Three of the four consensus GREs in the rat are conserved in the mouse, including the full GRE site; however, none of the rat GREs were conserved in the corresponding region in the human 5′ flanking region.

GRU Activity is Regulated by Phorbol Esters and Cyclic AMP

In C6 cells, glucocorticoid induction of GPDH enzymatic activity is modulated by intracellular second messengers. An increase in intracellular cAMP level causes an increase in the glucocorticoid induction (Breen et al., 1978; Montiel et al., 1986), whereas an increase in the intracellular diacylglycerol (DAG) level abolishes this induction (Bressler, 1987). Because GPDH 5′ flanking region was glucocorticoid-inducible, we examined if the glucocorticoid inducibility of GRU can also be modulated by second messengers.

Intracellular levels of cAMP or DAG were increased by application of 100 μM dibutyryl cyclic AMP (db-cAMP) or 100 ng/ml Tetradecanoyl Phorbol Acetate (TPA). These ligands have been previously tested in C6 cells to increase cAMP or DAG levels, respectively

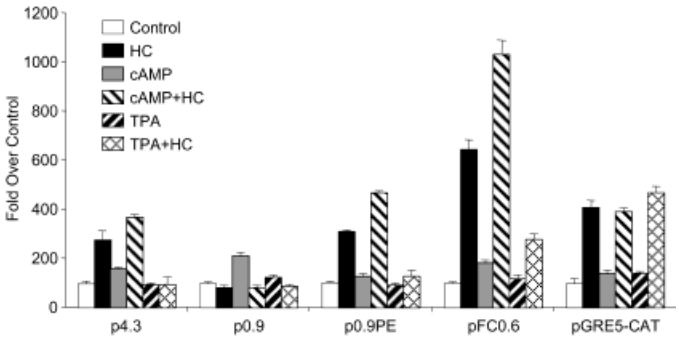

Fig. 6. Modulation of glucocorticoid-induced luciferase expression by co-treatment with db-cAMP or TPA. Stably transfected C6 cells (for constructs p4.3, p0.9, p0.9PE, pFC0.6, and pGRE5CAT) were treated with indicated ligands for 6 hr before luciferase activity was determined from each line. The level of reporter expression was expressed as fold over control (untreated cells). Values represent the average of three independent experiments with triplicate samples within each experiment.

(McGinnis and de Vellis, 1978; Bressler, 1987). They were applied to transfected cells alone or in combination with HC; and then luciferase and endogenous GPDH activities were determined in these conditions six hours after stimulation.

Four reporter constructs containing the GPDH 5′ flanking region were tested under the conditions described: p4.3, p0.9, p0.9PE, and pFC0.6. Except the p0.9 construct, all the reporter constructs contained the GRU sequences at various distances from transcription initiation (see Figs. 2A, 3A, and 4A). In addition, a vector containing only the GRE consensus sequences (pGRE5CAT) was tested. This vector contains five consensus GRE sequences from the tyrosine aminotransferase enhancer fused in tandem upstream of the adenovirus major late promoter driving the CAT reporter gene (Mader and White, 1993). Because only the consensus GRE sequences were present in this construct, this construct served as a control to determine if GRE sequences alone were sufficient to mediate the modulatory effects of second messengers.

The large number of ligand treatments and constructs to be tested in this experiment prompted us to use stable transfection instead of transient transfection. Stable transfectants of C6 cells were selected based on G418 antibiotic resistance and pooled. Each pooled cell line was denoted by the name of the plasmid transfected. When stimulated with 1 μM HC, all except the p0.9 cell line showed an increase in luciferase activity (Fig. 6). One hundred μM db-cAMP and 100 ng/ml TPA each alone had no effect on luciferase activities; however, db-cAMP enhanced the HC-induced increase in luciferase activity, and TPA abolished the increase. The modulatory effects were observed in p4.3, p0.9PE, and pFC0.6 cell lines, but not in p0.9 and pGRE5CAT cell lines, correlating well the presence of GRU in the constructs. In p0.9 cells, ligand treatments did not elicit any change in luciferase expression, except cAMP alone caused a 2 fold increase in luciferase activity. In pGRE5CAT cells, a prominent HC-induced increase in luciferase activity was observed, but co-treatment of db-cAMP or TPA with HC did not alter this induction. This indicates that the concatemerized consensus GREs alone were not sufficient to mediate the modulatory effects of second messengers on the HC induction. Sequences outside of the consensus GREs are most likely required for these effects.

DISCUSSION

The 5′ Flanking Region of GPDH is Glucocorticoid-Inducible

In an effort to understand the mechanism of GPDH regulation in oligodendrocytes, we sequenced the rat GPDH 5′ flanking region, compared it to the mouse homolog, and tested its responsiveness to ligand stimulation. Transient transfection of reporter constructs containing the GPDH 5′ flanking region into C6 glioma cells revealed that glucocorticoid inducibility was dependent on the distal portion of the 5′ flanking region. When this portion alone was tested in luciferase reporter constructs, it was found to be glucocorticoid-inducible in a position- and orientation-independent manner relative to transcription initiation. This fragment was also capable of enhancing SV40 promoter activity in the presence of hydrocortisone, indicating that the distal 5′ flanking region of GPDH contains glucocorticoid enhancer activity. Further deletion analysis identified a 385 bp sequence 3 kb upstream of GPDH transcription initiation. This region contains four putative Glucocorticoid Response Elements (GREs) and was termed the "Glucocorticoid Response Unit" (GRU) in this study.

The presence of four putative GRE sequences correlates well with the inducibility of GRU, and suggests that this region may be recognized and activated by glucocorticoid receptors. As a member of the steroid/thyroid receptor superfamily, the glucocorticoid receptors are known to translocate into the nucleus upon ligand binding, and activate transcription via the Glucocorticoid Responsive Elements (GREs; for review see O'Malley, 1990). Several other data exist that also support the involvement of glucocorticoid receptors in the activation of GPDH. First, the GPDH gene is transcriptionally regulated by HC (Kumar et al., 1986, 1989), suggesting a direct mode of action from the glucocorticoid receptors. Second, inhibition of hormone binding to the receptor by cytochalasin B results in a loss of the glucocorticoid-induced increase in GPDH activity (Bennett et al., 1977), indicating hormone binding to the receptors is important for induction of GPDH. Third, down-regulation of the glucocorticoid receptor concentration by concanavalin A treatment in C6 cells causes a loss of GPDH inducibility by glucocorticoids (McGinnis and de Vellis, 1981), indicating that induction is dependent on the glucocorticoid receptors. The transfection results obtained here, and the presence of GRE consensus sequences within the inducible region provide further evidence that the glucocorticoid

receptors are directly involved in mediating GPDH induction.

The transfection result is also the first report that demonstrates a regulatory function for the GPDH 5′ flanking region, and is in line with the finding that numerous DNase hypersensitive sites exist in the 5′ flanking region of the human GPDH 5′ flanking sequence (Gwynn et al., 1990). Previously, Kozak et al. (1990) tested the transcriptional activity of the mouse GPDH 5′ flanking region using a similar type of transient transfection analysis. The mouse GPDH 5′ flanking region was found to be active in both GPDH-positive HepG2 cells, and GPDH-negative Hepa-1 and L cells. Therefore, no tissue-specific regulatory elements could be identified from the mouse GPDH 5′ flanking region. Consistent with their finding, we also observed that the rat 5′ flanking region was transcriptionally active in both GPDH-positive C6 glioma cells, as well as GPDH-negative Rat-1 fibroblast cells. Analysis of the 5′ flanking sequence of the rat GPDH gene in transgenic mice, however, suggests that the rat GPDH 5′ flanking region directs glial-specific expression of GPDH in the brain (Cheng et al., 1997). Experiments here indicate that the 5′ flanking region is inducible by glucocorticoids. Moreover, the induction mimics regulation of the endogenous GPDH enzymatic activity, in that it is enhanced by cAMP, and reduced by TPA. Because the induction was only observed in C6 glioma cells, not in Rat-1 fibroblasts, it is possible that the induction also has certain cell-specificity. Possibly, the GRU identified here contains a glial-specific element, that restricts the glucocorticoid induction of GPDH to glial cells.

Conservation of Glucocorticoid Induction and GRU Sequence Among Species

Despite the large amount of data available on glucocorticoid induction of GPDH in rats, limited data exist on glucocorticoid regulation of GPDH in the mouse. Moreover, those limited data also indicate that regulation of GPDH in the mouse brain is different from that in the rat brain. Specifically, McGinnis and de Vellis (1977) indicated that GPDH in the mouse brain was not HC-inducible, and Fisher et al. (1981) demonstrated that the relative distribution of GPDH immunoreactivity in oligodendroglial and Bergmann glial cells in the mouse brain differs from that in the rat brain. No published reports are available regarding glucocorticoid regulation of GPDH in human glial cells. Therefore, whether or not the glucocorticoid regulation of GPDH is an evolutionarily conserved event between rats and mice, and between rodents and humans remains unclear.

In our sequence comparison, we found that the glucocorticoid-inducible GRU in the rat was 80% conserved to the mouse sequence, but only 42% conserved between the rat and the human. In fact, the segment where the GRU resides corresponds to a region of low homology between rodents and humans. Additionally, in the mouse, the GRU is located immediately upstream of the extra B1 repeat element that is absent in the rat, indicating that the GRU is located in an evolutionarily unstable part of the 5′ flanking region. It is possible that the GRU is a species-specific enhancer that functions in a few species. The establishment of glucocorticoid inducibility in the rat sequence now allows us to investigate the inducibility of human and mouse GPDH sequences in glial cells.

Basal Repressor Activity is Associated With GRU

In all of the reporter constructs containing the GRU, a repression of basal level luciferase activity seems to be associated with the HC induction. This is evident in constructs p0.9PE, p0.9A and p0.9B, that all exhibit a lower basal level activity than the original p0.9 construct (Fig. 2 and 3). Conversely, in constructs lacking the GRU sequences (as in p0.9C), the basal level was restored to a higher level. The same decrease in basal level was observed in SV40-driven constructs, as the basal levels of pGLP-PE and pGLP-SPE constructs seemed lower than their derivative pGLP (Fig. 3B). This suggests that the basal repressor activity associated with the GRU is not promoter-specific. It is possible that a general cis-acting repressor element exists within the GRU sequence, and the element functions independently of the glucocorticoid enhancer element. Alternatively, the repressor activity could be a property of the glucocorticoid enhancer. It is known that un-liganded hormone receptors, particularly the thyroid hormone receptor, have a constitutive negative effect on basal level transcription for hormone responsive genes (Damm et al., 1989). Because a small portion of the un-liganded glucocorticoid receptors are located in the nucleus (Vielkind et al., 1990), it is possible that the glucocorticoid receptor can also repress the basal level of GPDH transcription before ligand stimulation.

GRU Also Contains Sequences for Second Messenger Modulation of the Glucocorticoid Response

Similar to the endogenous gene, GRU-containing reporter constructs had a glucocorticoid response that was modulated by cAMP and TPA. Because these second messengers by themselves have no effect on reporter gene expression (Fig. 6), they are likely to exert their effects on the glucocorticoid signaling pathway. Both cAMP and TPA are known to trigger a cascade of phosphorylation events via protein kinases, some of which may be able to alter the *trans*-activating ability of the glucocorticoid receptors (DeFranco et al., 1991; Denner et al., 1990). If this were the case, however, one would expect that the construct containing GRE sequences alone (pGRE5CAT) be modulated similarly to those containing the GRU. Interestingly, glucocorticoid induction of pGRE5CAT was not affected by cAMP or TPA, indicating that the *trans*-activating ability of the glucocorticoid receptors per se was not modified by kinase activity. Most likely, additional sequences outside of the GREs are required for this modulation. It is possible that cAMP and TPA each activates a set of transcription factors. Interaction of these factors with glucocorticoid receptors at the DNA level results in stimulation or interference with the glucocorticoid receptor

transactivation. Because the GRU spans 385 bp of sequences, there are many potential binding sites for such an interaction.

A number of transcription factors are known to interact with glucocorticoid receptors and interfere with the *trans*-activating ability of the glucocorticoid receptors. The most prominent example is the interaction between the AP-1 transcription complex and the glucocorticoid receptors. Mutual interference has been observed when the two factors interact, and is thought to mediate some of the antagonistic effects of glucocorticoids on tumor promoters (Yang-Yen et al., 1990; Diamond et al., 1990; Schule and Evans, 1991). An antagonistic effect between the NFκB transcription factor and the glucocorticoid receptors was reported (Ray and Prefontaine, 1994; Scheinman et al., 1995a,b; Auphan et al., 1995), that accounts for some of the anti-inflammatory action of the glucocorticoids. Because both the AP-1 and NFκB transcription factors are activated by TPA, it is conceivable that a similar antagonistic action between these factors and the glucocorticoid receptors exist at the regulatory sequence of the GPDH gene. Further dissection of the GRU in the future might shed light on the mechanism of this interaction.

Glucocorticoids have been used as anti-inflammatory agents for decades. Despite the widely known effect of glucocorticoids as anti-inflammatory, little is known about the intracellular action and specificity of glucocorticoids in oligodendrocytes. In addition, glucocorticoids, along with polypeptide growth factors like the platelet-derived growth factor and fibroblast growth factor, were shown to be an integral part of the "timing mechanism" controlling oligodendrocyte progenitor proliferation and differentiation (Barres et al., 1994). The mechanism by which glucocorticoids interact with these signals remains elusive. Therefore, investigation of the interactive nature of these pathways remains a relevant task in glial cell biology. GPDH regulation provides a simple model to study this interaction, because the glucocorticoid regulation of GPDH occurs in the oligodendroglial population of the rat, and because all of the other modulations occur at the transcriptional level. Furthermore, the regulation can be studied in C6 glioma cell line, that has oligodendroglial/astroglial properties and can be used conveniently for biochemical and molecular analyses. The 385 bp sequence identified in this study further provides a useful tool to dissect the individual components of this interaction. Elucidation of GPDH regulation thus may provide insights to the mechanism by which glucocorticoids interfere or enhance the effects of other extracellular signals in oligodendroglial cells.

The significance of the glucocorticoid regulation of GPDH to oligodendrocyte function needs to be further investigated because glucocorticoids enhance oligodendrocyte differentiation and myelination in vivo (Preston and McMorris, 1984) and in vitro (Kumar et al., 1989; Warringa et al., 1987; Lopez-Cardozo et al., 1989; Byravan and Campagnoni 1994; Chan et al., 1998). For instance, glucocorticoids increase proteolipid protein and myelin basic protein mRNAs in oligodendrocytes by a posttranscriptional mechanism (Kumar et al., 1989). Furthermore, glucocorticoids enhance the translational efficiency of myelin proteins mRNAs (Verdi et al., 1989).

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