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Differential regulation of oligodendrocyte markers by glucocorticoids: Post-transcriptional regulation of both proteolipid protein and myelin basic protein and transcriptional regulation of glycerol phosphate dehydrogenase

(development/primary cultures of glia/immunocytochemistry/RNA blots/run-off transcription)

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ABSTRACT **During neonatal development glucocorticoids potentiate oligodendrocyte differentiation and myelinogenesis by regulating the expression of myelin basic protein, proteolipid protein, and glycerol phosphate dehydrogenase (*sn*-glycerol-3-phosphate: NAD$^+$ 2-oxidoreductase, EC 1.1.1.8). The actual locus at which hydrocortisone exerts its developmental influence on glial physiology is, however, not well understood. Gycerol phosphate dehydrogenase is glucocorticoid-inducible in oligodendrocytes at all stages of development both *in vivo* and *in vitro*. In newborn rat cerebral cultures, between 9 and 15 days *in vitro*, a 2- to 3-fold increase in myelin basic protein and proteolipid protein mRNA levels occurs in oligodendrocytes within 12 hr of hydrocortisone treatment. Immunostaining demonstrates that this increase in mRNAs is followed by a 2- to 3-fold increase in the protein levels within 24 hr. *In vitro* transcription assays performed with oligodendrocyte nuclei show an 11-fold increase in the transcriptional activity of glycerol phosphate dehydrogenase in response to hydrocortisone but no increase in transcription of myelin basic protein or proteolipid protein. These results indicate that during early myelinogenesis, glucocorticoids influence the expression of key oligodendroglial markers by different processes: The expression of glycerol phosphate dehydrogenase is regulated at the transcriptional level, whereas the expression of myelin basic protein and proteolipid protein is modulated via a different, yet uncharacterized, mechanism involving post-transcriptional regulation.**

The expression of glycerol phosphate dehydrogenase (GPDH; *sn*-glycerol-3-phosphate: NAD$^+$ 2-oxidoreductase, EC 1.1.1.8), myelin basic protein (MBP), and proteolipid protein (PLP) marks the differentiation of oligodendrocytes, the myelinating cells in the central nervous system (1, 2). MBP and PLP are components of the myelin sheath, constituting 30% and 50% of the proteins in myelin, respectively. The expression and biosynthesis of MBP and PLP in oligodendrocytes have been shown to be coordinately expressed with the process of myelination (3–6).

Peak expression of GPDH during neonatal development also coincides with the active period of myelination (7, 8). Localized in oligodendrocytes (9), GPDH provides glycerol phosphate for the biosynthesis of phosphatides (10), a precursor for membrane components (11). The developmental regulation of GPDH gene expression and its modulation by glucocorticoids in the rat brain and in primary cultures of oligodendrocytes have been well characterized (12).

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Glucocorticoids and other hormones have been implicated in the stimulation of myelinogenesis (13–16). During glial differentiation, the expression of 2′,3′-cyclic-nucleotide 3′-phosphodiesterase (CNPase) has been shown to be increased by dexamethasone, a potent glucocorticoid agonist, both in reaggregating (13) and dissociated (14) rat brain-cell cultures in a serum-free medium. Other trophic factors have also been shown to influence myelinogenesis by regulating gene expression. For example, a treatment of brain-cell aggregate cultures at days 2 and 5 with epidermal growth factor causes a dose-dependent increase of CNPase and MBP by culture days 12 and 19 (15). In addition, the continuous presence of bovine growth hormone in the culture medium results in an accumulation of MBP by day 19. A direct effect of growth hormone and thyroid hormone in cerebral myelinogenesis has been documented during the critical first 20 days of postnatal life, when both growth hormone and thyroid hormone stimulate the expression of CNPase and MBP (16).

The ability of glucocorticoids to influence the developmental expression of genes in the brain correlates highly with the ontogeny of glucocorticoid receptor in target cells. A developmental rise in the levels of unoccupied glucocorticoid receptor and nuclear acceptor site (or nuclear binding sites) has been seen in the rat brain during the first week of life, reaching its maximum during the second week of development (17, 18). Primary cultures of rat oligodendrocytes and astrocytes have abundant levels of glucocorticoid receptors (12).

In this study, we show that like GPDH, MBP and PLP are also glucocorticoid-inducible in oligodendrocytes during development. In contrast to GPDH, which is transcriptionally regulated throughout the age of the culture, the glucocorticoid induction of MBP and PLP is developmentally restricted and involves post-transcriptional regulation during the second postnatal week, the period of myelinogenesis in the rat cerebrum.

METHODS

Cell Culture Preparation. Primary glial cell cultures were prepared from cerebral cortices of 1- to 2-day postnatal Wistar rats as described (19). Secondary cultures of astrocytes and oligodendrocytes were prepared by mechanically shaking 7- to 9-day-old confluent primary glial cultures, essentially as described by McCarthy and de Vellis (20),

Abbreviations: MBP, myelin basic protein; PLP, proteolipid protein; GPDH, glycerol phosphate dehydrogenase; CNPase, 2′,3′-cyclic-nucleotide 3′-phosphodiesterase.

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except that the cultures were shaken for 6 hr to remove macrophages before the 18-hr shake, which yields oligodendrocytes.

Immunocytochemistry. The rabbit antibody to GPDH has been described (21); the rabbit anti-MBP was from L Bologa (Zurich); and the rabbit anti-PLP was generated by the method of Vaitukaitis (22) from a purified rat brain PLP preparation. For immunostaining, cultures were fixed and treated with primary antibodies at 1:50 dilutions by a described method (23). A 1:200 dilution of goat anti-rabbit IgG was used as a second antibody, followed by peroxidase–antiperoxidase treatment (24).

Preparation of RNA Blots. For all RNA expression studies, cell cultures were treated with 1 μM hydrocortisone succinate (diluted from 0.5 M stock solution in ethanol) for 12 hr before RNA extraction. Total RNA was rapidly extracted from the primary cultures of mixed glia, astrocytes, or oligodendrocytes by the acid guanidinium thiocyanate/phenol/chloroform method (25). Two micrograms of poly-$(A)^+$ RNA samples were electrophoresed on a 1% formaldehyde denaturing agarose gel and blotted on nitrocellulose (26). Two micrograms of RNA samples were also slot-blotted on nitrocellulose using a Manifold II apparatus (Schleicher & Schuell).

Probes. In these studies, a human PLP cDNA clone, pRI containing a 1.9-kilobase (kb) insert (a gift from J. R. Riordan, Hospital for Sick Children; Toronto) (5), a 600-base-pair (bp) insert of rat glial GPDH cDNA clone (8), and a mouse MBP cDNA clone, pdF191, containing a 380-bp insert (a gift from R. A. Lazzarini, Mount Sinai Medical Center, New York) (27) were used. To examine the overall expression of mRNAs, pCHOB cDNA from a Chinese hamster ovary (CHO) cell line was used as a probe (a gift from H. R. Herschman, Univ. of California at Los Angeles). The *CHOB* gene is expressed constitutively in all mammalian tissues and cell lines examined (28, 29). All the densitometric values obtained from the scanning of MBP, PLP, and GPDH RNA-cDNA hybridized signals were normalized to values obtained for pCHOB to adjust for any variation in the amount of RNA loaded in each slot.

***In Vitro* Transcription Assays.** The run-off transcription of MBP, PLP, and GPDH from cultured cells followed the described method (8, 30).

RESULTS

Primary cell cultures prepared from neonatal rat cerebral cortex consist of a bedlayer of astrocytes overlaid by phase-dark process-bearing cells that mature into oligodendrocytes (20). Immunostaining of these cultures demonstrates that some process-bearing cells lying on top of an astrocyte bedlayer express the oligodendrocyte marked GPDH (Fig. 1*A*). The treatment of the cultures with hydrocortisone for 24 or 48 hr considerably increased the expression of GPDH antigen (Fig. 1 *B* and *C*). For example, the level of expression of GPDH in 8-day-old mixed glia treated with hydrocortisone for 48 hr resembles levels found in 2- to 3-week-old cultures. For comparison, the immunostaining of GPDH in an 18-day-old culture not treated with hydrocortisone is shown (Fig. 1*D*). To more closely compare the basal and induced levels of GPDH, the total numbers of process-bearing cells and GPDH-positive cells were counted at various times over a 4-week period. The total number of process-bearing cells in either the control or glucocorticoid-treated cultures was the same at each corresponding age. Fig. 2 shows that the percent of GPDH-positive cells increases several-fold in response to hydrocortisone throughout the culture time.

Immunostaining shows MBP and PLP to be primarily membrane-bound, forming a halo around the cell boundary (Fig. 3), in contrast to GPDH, which is uniformly distributed

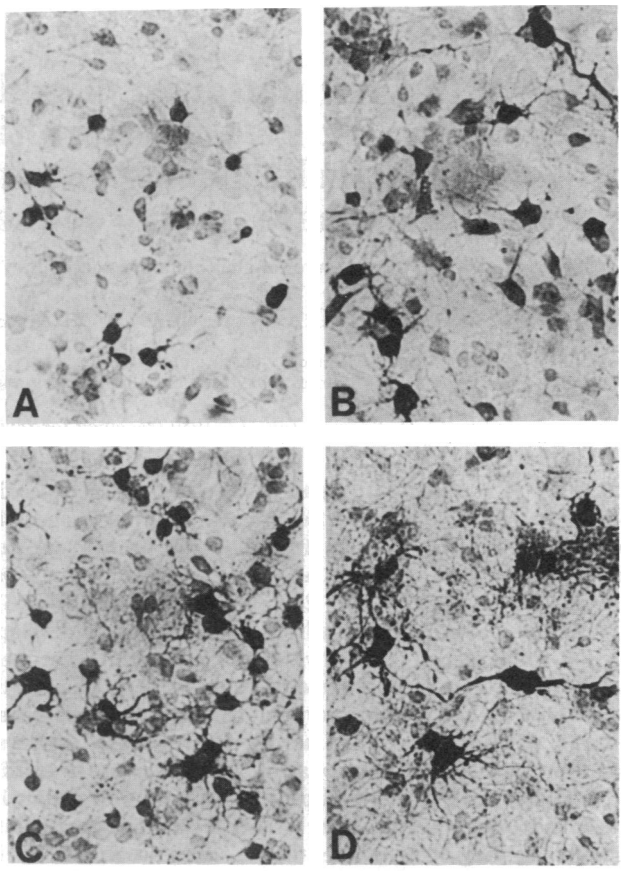

FIG. 1. Immunostaining of primary glial cell cultures with anti-GPDH. (*A*) Day 8, untreated. (*B*) Day 8, treated with 1 μM hydrocortisone during the last 24 hr. (*C*) Day 8, treated with hydrocortisone during the last 48 hr. (*D*) Day 18, untreated. Bright field (×188).

in the cytoplasm (Fig. 1). Because immunostains *in vivo* show that MBP and PLP appear relatively late in development, a striking result has been the precocious expression of both MBP and PLP in *in vitro* hydrocortisone-treated phase-dark cells (Fig. 3 *B*, *C*, *E*, and *F*). This increase in MBP and PLP antigens in oligodendrocytes in response to hydrocortisone is evident only in early cultures, ranging between day 9 and day 15. No significant glucocorticoid-mediated induction of immunodetectable MBP and PLP was evident in the cultures before day 9 (data not shown). In hydrocortisone-treated older cultures, both the MBP- and PLP-positive cells appeared in large clusters, making single-cell evaluation more difficult. Once again, in hydrocortisone-treated cultures, the percent of MBP- or PLP-positive process-bearing cells increased, although not as much as seen for GPDH (Fig. 2).

The hormonally regulated developmental expression of MBP and PLP was also analyzed by Northern (RNA) blot (Fig. 4). A Northern blot sequentially probed with ^{32}P-labeled GPDH, MBP, and PLP cDNAs shows a single 2.1-kb band of MBP mRNA and two PLP mRNA bands of 3.3 and 1.4 kb. These values are the same as reported values for MBP and PLP mRNAs (5) and are not influenced by hormone treatment. A further quantification of these oligodendrocyte marker mRNAs was done with slot blots prepared from poly$(A)^+$ RNA isolated from untreated and hydrocortisone-treated primary cultures of mixed glia, astrocytes, and oligodendrocytes. The 31-day-old rat brain mRNA was also analyzed as an index of *in vivo* developmental levels of these genes. The densitometric scanning after normalization to pCHOB shows a 2- to 3-fold increase in MBP and PLP mRNA in 2-week-old primary cultures of mixed glia and secondary cultures of oligodendrocytes treated with hydrocortisone for

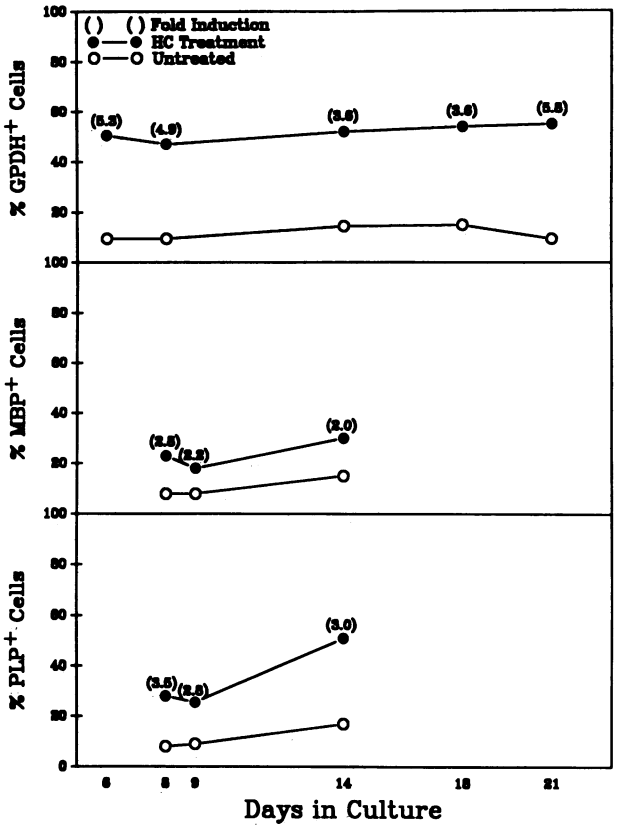

FIG. 2. Percent of the total population of phase-dark process-bearing cells expressing GPDH, MBP, and PLP as determined by immunostaining. Total process-bearing cells and process-bearing cells positive for GPDH, MBP, or PLP in primary glial cultures were counted in various fields similar to those in Figs. 1 and 3. The fold-increase is the ratio of the hydrocortisone (HC)-treated to the untreated values.

12 hr (Fig. 5). No detectable levels of MBP, PLP, and GPDH were seen in astrocytes. The glucocorticoid-mediated increases of MBP and PLP mRNAs were much less than the

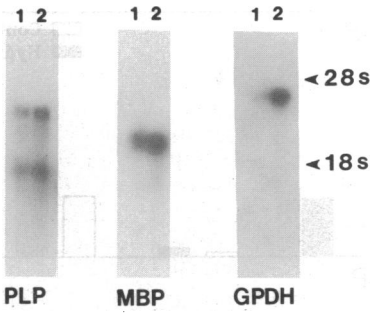

FIG. 4. Northern blot of RNA from 2-week-old oligodendrocyte cultures, sequentially probed with GPDH, MBP, and PLP cDNAs. Lanes: 1, untreated; 2, hydrocortisone-treated. Arrowheads represent 18S and 28S ribosomal RNA bands.

glucocorticoid-mediated increase in GPDH mRNA. Previously, we demonstrated that the glucocorticoid-mediated induction of GPDH mRNA in these cells is transcriptionally regulated by using *in vitro* run-off transcription assays in nuclei isolated from C6 cells (8). To examine the mechanism of action of hydrocortisone on oligodendroglial markers in process-bearing cells, we performed *in vitro* transcription assays with nuclei isolated from cultures of oligodendrocytes either untreated or treated with hydrocortisone for 12 hr. The ^{32}P-labeled transcribed heterogeneous nuclear RNAs were hybridized to nitrocellulose discs containing GPDH, MBP, or PLP clone cDNAs. As is clear from Fig. 6, glucocorticoids do not increase the transcription of MBP and PLP mRNAs over their untreated levels, whereas an 11-fold increase in the GPDH gene activity was seen in response to hydrocortisone.

DISCUSSION

We have begun to characterize a glial culture system for the study of hormonal regulation of developmental expression of oligodendroglial-specific marker genes. Prepared from the cerebral cortices of 1- to 2-day postnatal rat pups, mixed glial cultures can be maintained for weeks and examined for developmental expression of different markers, both at basal

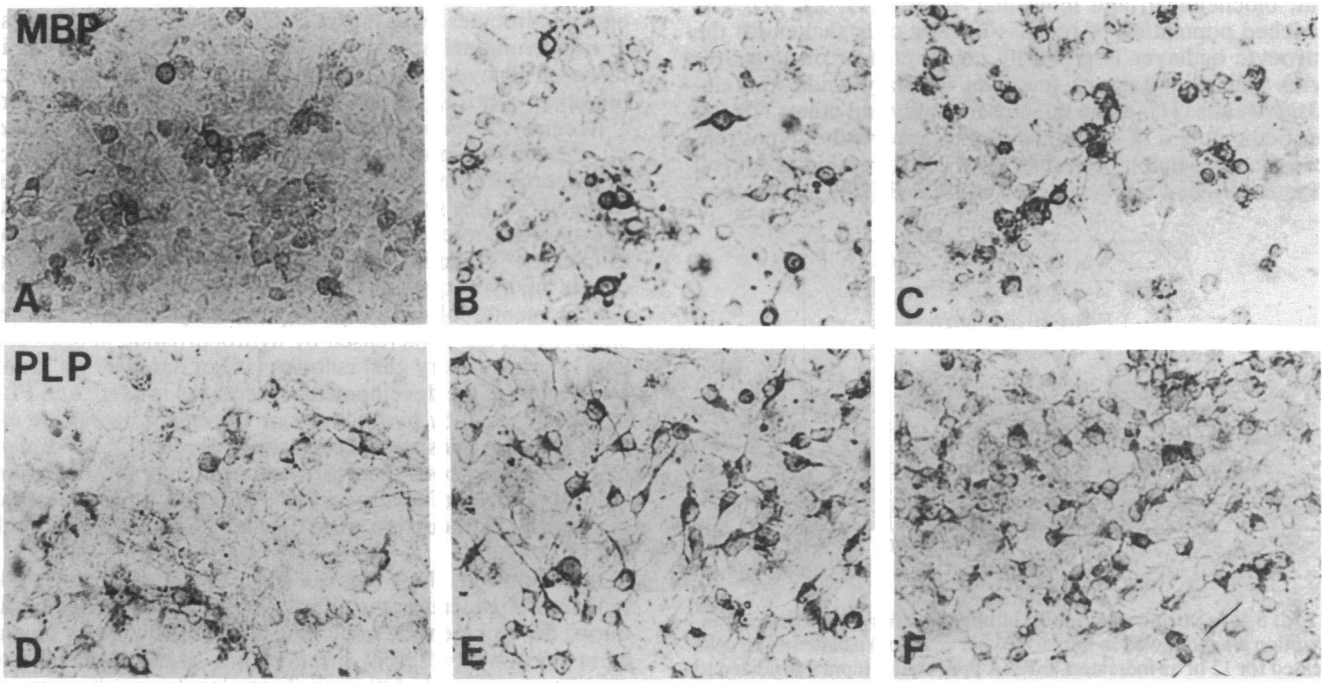

FIG. 3. Immunostaining of the primary glial cultures with anti-MBP (*A–C*) and anti-PLP (*D–F*). (*A* and *D*) Day-9 untreated cells. (*B* and *E*) Day-9 cells treated with hydrocortisone during the last 24 hr. (*C* and *F*) Day-9 cells treated with hydrocortisone during the last 48 hr. Bright field (×188).

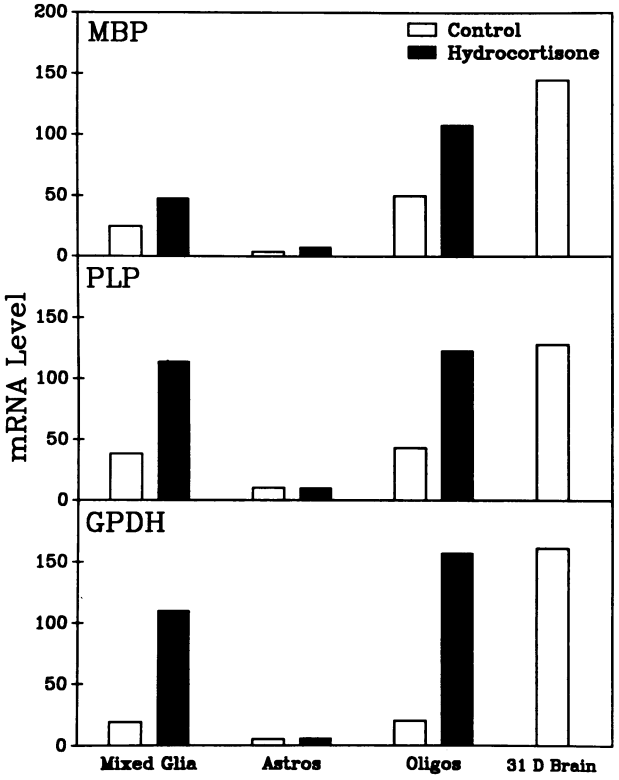

FIG. 5. Slot-blot analysis of RNA from 2-week-old cultures of brain-cell types. The blot was probed with GPDH, MBP, PLP, and pCHOB cDNAs, and the autoradiograms were scanned on a densitometer. The levels of mRNAs expressed here are in relative units obtained by normalizing to pCHOB. 31 D represents 31-day-old whole rat brain.

levels and in response to hormones. In a 7- to 9-day-old culture, the oligodendrocytes appear as phase-dark process-bearing cells overlaying a confluent bedlayer of phase-light astrocytes (20). These process-bearing cells have been previously characterized by ultrastructural, immunocytochemical, biochemical, and molecular criteria (19, 23, 31). The enriched population of process-bearing cells shaken off the astrocyte bedlayer is primarily constituted of oligodendrocytes and exhibits very few glial fibrillary acidic protein-positive cells (31). From the present study and others (19, 23) purified cultures of oligodendrocytes apparently do not represent homogeneous populations. The expression of several oligodendroglial markers, such as GPDH (20), galactocerebroside, CNPase (31), transferrin (23), and MBP (19, 32), has been reported in cultures of phase-dark process-bearing cells; however, these markers are not always co-localized within the same cell (19, 23).

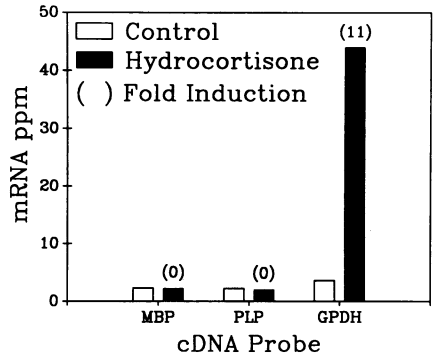

FIG. 6. *In vitro* transcription of oligodendrocyte markers. Transcription was followed in nuclei (10^7 per reaction) isolated from cells treated for 12 hr as indicated. mRNA ppm equals (cpm hybridized to MBP or PLP minus cpm hybridized to pBR322) divided by total input cpm. The mRNA ppm values are the average of repeated experiments and probe discs hybridized in triplicates.

In this study, we have examined the glucocorticoid modulation of the development gene expression of three oligodendroglial-specific markers, GPDH, MBP, and PLP, in rat cerebral primary cultures by measuring protein, mRNA, and transcription rate. In these cultures, basal-level expression of GPDH protein can be detected in the phase-dark process-bearing cells at all times, whereas the expression of MBP or PLP begins later. The basal-level GPDH expression is hydrocortisone-inducible during the entire 3- to 4-week period of culture. Interestingly, we also observed an increase in MBP- and PLP-positive phase-dark cells in 9- to 15-day-old mixed glial cultures treated with 1 μM hydrocortisone for 24–48 hr. However, no significant hydrocortisone-mediated increase in MBP and PLP levels was seen in cultures before day 9. Measurement of hydrocortisone-mediated changes in MBP and PLP protein levels in cultures older than 2 weeks is complicated by the increased basal-level expression and a cell-clustering phenomenon.

The number of phase-dark cells in mixed glial cultures continues to increase with age, but no additional increase in phase-dark cells was seen in cultures treated with hydrocortisone for 24–48 hr. The Northern and slot-blot analyses showed a 2- to 3-fold hydrocortisone induction of MBP and PLP messages over basal levels within 12 hr of treatment at 2 weeks of age; this finding has recently been confirmed by analysis of *in situ* hybridization experiments with MBP riboprobe at 16 days in culture (E. Holmes and J.d.V., unpublished work). Interestingly, a week later, hydrocortisone had no effect on MBP mRNA accumulation in cells. Therefore, this hormonal effect on MBP expression is a developmentally restricted phenomenon and differs from GPDH induction, which is inducible throughout the life-span of the animal and cell culture (12).

We have shown earlier that GPDH is transcriptionally regulated by glucocorticoids in C6, a transformed glial cell line (8). The *in vitro* transcription assays performed here show that glucocorticoids also regulate the expression of GPDH at the transcriptional level in the purified cultures of oligodendrocytes. Within the same experiment, no increase in transcriptional activity of MBP or PLP was evident in response to hydrocortisone, indicating a different mode of hormonal control, possibly a post-transcriptional regulation.

Recently, in an *in vitro* translation assay system, Verdi *et al.* (33) have reported an immediate and direct effect of hydrocortisone on the translation of MBP and PLP messages. Thus, within this system glucocorticoid mediates an increase in translation rate of MBP and PLP, whereas the translation of CNPase is significantly reduced. The direct translational effects of hydrocortisone differ, at least in part, from the developmental observations reported by others. For instance, CNPase is increased by hydrocortisone in aggregating (13) and primary glial cultures (14) of neonatal rat brain. Furthermore, the developmental increases in MBP and PLP mRNAs caused by hydrocortisone treatment cannot be explained by the translational regulation reported by Verdi *et al.* (33). However, both the post-transcriptional and the translational effects of hydrocortisone may contribute to the mechanism of induction of MBP and PLP in glial cell cultures.

In conclusion, these studies suggest that glucocorticoids modulate oligodendrocyte differentiation and myelinogenesis during development by regulating gene expression by means of at least two different mechanisms—transcriptional and post-transcriptional. Although the molecular mechanisms of these inductions remain unclear, the primary culture of oligodendrocytes provides a promising model for the appli-

cation of current molecular techniques and the future investigation of hormonal control of glial development.

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