THE JOURNAL OF BIOLOGICAL CHEMISTRY

Vol. 268, No. 34, Issue of December 5, pp. 25617-25623, 1993
Printed in U.S.A.

Retinoic Acid Posttranscriptionally Up-regulates Proteolipid Protein Gene Expression in C6 Glioma Cells*

(Received for publication, May 18, 1993, and in revised form, August 2, 1993)

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The proteolipid protein (PLP) gene codes for the major central nervous system myelin protein. We have studied the effects of different agents on the expression of the PLP gene in C6 glioma cells. Retinoic acid (RA), but not dexamethasone, estradiol, insulin, growth hormone, or vitamin D_3, had a drastic effect, increasing 10–20-fold the level of PLP mRNA. Concomitantly, RA also induced the appearance of the corresponding immunoreactive protein. The increase in PLP RNA level showed a slow kinetics and was blocked by cycloheximide, suggesting a posttranscriptional regulation by RA. Nuclear run-on assays confirmed that the rate of PLP gene transcription was unchanged by RA. In contrast, we found that retinoic acid augmented PLP mRNA stability, causing a substantial increase in its half-life. RA action was independent of cell density, serum, or PDGF but was partially inhibited by bFGF. On the other hand, thyroid hormone caused a moderate increase in PLP mRNA levels in C6 cells but only when the low numbers of thyroid receptors in these cells were increased by retrovirally mediated expression of an exogenous c-*erbA*/TRα-1 gene. Our results indicate that RA specifically up-regulates PLP expression in glioma C6 cells at a posttranscriptional level by increasing PLP RNA half-life.

Proteolipid protein (PLP)[1] is the major protein component (50% of total) of central nervous system myelin. PLP is essential for the adequate compaction of the myelin sheath which in turn is critical for normal brain function as shown by the severe consequences of PLP mutations or deficiency in rodents (*jimpy*, *msd*, *md*) (Hogan and Greenfield, 1984; Gardinier *et al.*, 1986; Boison and Stoffel, 1989; Simons and Riordan, 1990; Macklin *et al.*, 1991: Shiota *et al.*, 1991) and humans (Pelizaeus-Merzbacher disease and other demyelinizing disorders; Fahim and Riordan, 1986; Hudson *et al.*, 1989; Gencic *et al.*, 1989). Myelination is impaired by thyroid hormone deficiency during development (for review see Dussault and Ruel, 1987). Accordingly, expression of myelin genes including PLP has been reported to be affected by thyroid hormones (T_3/T_4) both *in vivo*[2] (Muñoz *et al.*, 1991) and in primary cultures of glial cells (for review see Campagnoni and Macklin, 1988). Also, conflicting results have shown dexamethasone-induced up-regulation (Kumar *et al.*, 1989) or down-regulation (Tsuneishi *et al.*, 1991) of PLP and other myelin genes during rat brain development.

To study in detail the regulation of the PLP gene circumventing the restrictions imposed by the low number of glial precursor cells obtained in primary cultures, the rat C6 glioma cell line was chosen because it retains certain characteristics of glial precursors, expresses some astrocyte and oligodendrocyte genes including some myelin genes, and is able at least partially to differentiate *in vitro* under some conditions (McMorris, 1977; Kumar *et al.*, 1984; Milner *et al.*, 1985). Nave and Lemke (1991) have reported recently that high cell density as well as serum deprivation cause an increase in PLP expression in C6 cells. These authors have found positive and negative *cis*-regulatory elements in the PLP promoter. However, no *trans*-activating factors have been described.

Retinoic acid (all-*trans*; RA) is together with thyroid hormone (T_3) the main known regulator of metabolism, differentiation, and development in vertebrates (for reviews see Sherman, 1986; Brockes, 1989; Eichele, 1989; Lotan, 1991). Both agents, RA and T_3, exert their actions by regulating gene expression in a tissue-, cell- and time-specific fashion (for reviews see Glass *et al.*, 1991; Glass and Holloway, 1990; Brent *et al.*, 1991). Gene regulation by RA and T_3 follows binding to specific nuclear receptors (RARs and TRs/*erbA*, respectively) and formation of heterodimers with members of the RXR proteins (for a review, see Stunnenberg, 1993). RXRs themselves are receptors for 9-*cis*-retinoic acid (Heyman *et al.*, 1992; Levin *et al.*, 1992). Binding of liganded RAR-RXR or TR-RXR heterodimers to short specific nucleotide sequences (retinoic acid response elements, RAREs, and thyroid hormone response elements, T_3REs) leads to the up- or down-regulation of genes harboring these elements. Several RAREs and T_3REs, and consequently several RA- and T_3-transcriptionally regulated genes, are known (for reviews see Glass *et al.*, 1991; Lehmann *et al.*, 1992; Brent *et al.*, 1991; Stunnenberg, 1993). In addition, several genes have been

* This work was supported in part by CICYT Plan Nacional de I+D Grant SAF92-0396 and by DGICYT Grant PM-880006. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ Recipients of Ministerio de Educación y Ciencia fellowships.

‖ Recipient of a European Molecular Biology Organization short term fellowship.

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[1] The abbreviations used are: PLP, proteolipid protein; RA, all-*trans*-retinoic acid; RAR, nuclear all-*trans*-retinoic acid receptor; RXR, retinoid X receptor; T_3, triiodothyronine; T_4, thyroxine; TR, thyroid hormone receptor; RARE, all-*trans*-retinoic acid response element; T_3RE, thyroid hormone response element; PDGF, platelet-derived growth factor; bFGF, basic fibroblast growth factor; kb, kilobase(s).

[2] A. Rodríguez-Peña, N. Ibarrola, A. Muñoz, M. A. Iñiguez, and J. Bernal, unpublished results.

described in the last years as regulated posttranscriptionally by RA (Glick *et al.*, 1989; Antras *et al.*, 1991; Gellersen *et al.*, 1992; for review see Glass *et al.*, 1991) or T_3 (Shambaugh *et al.*, 1969; Davidson *et al.*, 1988; Glass and Holloway, 1990; Brent *et al.*, 1991). For some of these genes, an increase or decrease in mRNA stability has been the regulatory mechanism proposed. Moreover, for some genes dual transcriptional and posttranscriptional regulation by RA or by T_3 has been found (see reviews cited above).

The PLP gene is expressed postnatally in development. Data from different groups indicate that PLP is expressed at a low level in immature oligodendrocytes, suggesting that the protein may be necessary during the early steps of myelination (Macklin *et al.*, 1991). Here we have investigated the regulation of PLP gene expression in glioma C6 cells. We report an increase of steady-state PLP mRNA levels by RA mirrored by an increase in the amount of immunoreactive protein. This effect appears to be the result of a posttranscriptional regulation causing an increased half-life of PLP mRNA. Thyroid hormone also has an analogous but smaller effect that is dependent on the overexpression of active thyroid receptors in the C6 cells. Our results demonstrate the regulation of the PLP gene by retinoic acid, supporting a role of this agent in oligodendrocyte differentiation.

EXPERIMENTAL PROCEDURES

Cells and Culture Conditions—C6 cells were grown in RPMI medium containing 10% horse serum and 5% fetal calf serum (all from GIBCO, Paisley, Scotland). They were maintained at 37 °C in a 5% CO_2 atmosphere. When incubated in serum-free conditions 1 μg/ml bovine serum albumin and 100 μg/ml human transferrin (Sigma) were added into the medium. To generate *erbA*-overexpressing cells, recombinant retroviruses encoding the chicken c-*erbA*/TRα-1 and v-*erbA* genes were used which have been described previously (Muñoz *et al.*, 1990). Both are derived from the Moloney murine leukemia virus and encode the neomycin-resistant gene providing resistance to the antibiotic G418. By this approach and after infection and selection with G418, three different types of C6 cells became available: control uninfected cells, c-*erbA*/TRα-1-overexpressing cells, and v-*erbA*-overexpressing cells.

Expression of the corresponding genes was assessed by Northern analysis and T_3 binding assays (not shown). These analyses showed that the concentration of thyroid hormone receptors increased around 10-fold in c-*erbA*/TRα-1 cells.

RNA Isolation and Analysis—Poly(A)$^+$ RNA from cells plated in 150-mm dishes (Nunc, Denmark) was purified using oligo(dT)-cellulose as described (Vennström and Bishop, 1982). The RNA was electrophoresed on a 1.2% agarose gel containing 2.2 M formaldehyde and blotted onto GeneScreen membranes (DuPont NEN) according to standard protocols (Sambrook *et al.*, 1989). All probes were labeled by the random priming method (Feinberg and Vogelstein, 1983) using [α-^{32}P]dCTP. The source of probes were as follows. Rat PLP and cyclophilin were from Dr. J. G. Sutcliffe, mouse vimentin from Dr. E. Reichmann, rat 2′,3′-cyclic nucleotide 3′-phosphodiesterase (hereafter referred to as cyclic nucleotide phosphodiesterase) was from Dr. L. Bernier/Dr. D. R. Colman, human RAR-α and RAR-γ from Dr. P. Chambon, mouse RAR-β from Dr. A. Dejean, human RXR-α from Dr. R. M. Evans, RXR-β from Dr. K. Ozato, and chicken β-actin from Dr. M. Zenke. Hybridizations were carried out overnight at 65 °C in 7% SDS, 500 mM sodium phosphate buffer, pH 7.2, and 1 mM EDTA according to Church and Gilbert (1984). Filters were washed twice for 30 min each in 1% SDS and 40 mM sodium phosphate buffer, pH 7.2, at 65 °C. Before rehybridizing the nylon membranes with probes for other genes, the radioactive probe was stripped off the membrane by placing it in a 75 °C water bath for 5 min. Sizes of respective mRNAs were calculated using RNA ladder as markers (Life Technologies, Inc.). Membranes were exposed to Kodak X-Omat AR films.

In Vitro Transcription in Isolated Nuclei—Nuclear run-on reactions were performed basically according to Linial *et al.* (1985) using [α-^{32}P]UTP and 2×10^7 nuclei. The RNA polymerase II inhibitor α-amanitin was included in one control reaction. As expected (Linial *et al.*, 1985), α-amanitin (2 μg/ml) reduced isotope incorporation by 60%. Labeled RNA was purified by phenol:chloroform extraction after the addition of 3 volumes of guanidine isothiocyanate solution (Chomczynski and Sacchi, 1987) and isopropyl alcohol precipitation. Unincorporated label was removed by centrifugation through Sephadex G-50 spin columns.

Recovery as high molecular weight material from the columns was 7% of the total radioactivity present in the run-on reactions. The probes were 5 μg of linearized PLP or β-actin plasmid DNA immobilized on nylon filters after denaturation in NaOH. Linearized pTZ was also present on the filters to control for background hybridization. Hybridizations were performed in 0.2 M sodium phosphate buffer, pH 7.2, 1 mM EDTA, 7% SDS, and 45% formamide containing 250 μg/ml *Escherichia coli* tRNA as a carrier, at 42 °C for 3 days. Following hybridization the filters were washed in 40 mM sodium phosphate buffer, pH 7.2, containing 1% SDS at 37 °C.

Immunofluorescence—Cells seeded on poly-D-lysine-coated coverslips were treated with or without 1 μM RA for 24, 48, or 72 h. Fixation was done in 4% paraformaldehyde/phosphate-buffered saline for 20 min. Cells were then rinsed several times with phosphate-buffered saline and treated with blocking solution (50% goat serum, 20 mM Hepes, 10 mM lysine/Hanks' balanced salt solution for 30 min. Blocking solution was drained off and cells incubated with the first antibody (rabbit polyclonal anti-PLP antiserum, donated by Drs. A. Espinosa/J. de Vellis, diluted 1:200 in solution A: 20 mM Hepes, 10% newborn calf serum/Hanks' balanced salt solution) for 30 min. After repeated washings with solution A, the cells were incubated with the secondary rhodamine-conjugated goat anti-rabbit antibody (Southern Biotechnology, U. K., diluted 1:100 in solution A). Nuclei were stained with bisbenzimide (Hoechst 33258, Sigma) after permeabilizing the cells with 100% methanol at −20 °C for 10 min. Coverslips were mounted in 2.5% DABCO/glycerol (Sigma) and viewed by epifluorescence on a Zeiss Axiophot microscope.

Western Blotting—Confluent C6 cell monolayers incubated in serum-free medium were treated or not with 1 μM retinoic acid for 48 h. Cell lysates were obtained, and proteins were separated by SDS-polyacrylamide gel electrophoresis on 14% gels and transferred to a nitrocellulose filter using a Novablot Electrophoretic Transfer apparatus (LKB, Sweden). The blot was incubated with blocking solution (50% fetal calf serum in water) for 1 h at room temperature, rinsed three times with Tris-buffered saline (50 mM Tris-base, 0.9% NaCl, pH 7.5), and subsequently incubated with a 1:10 dilution of a rat monoclonal IgG (hybridoma supernatant) against residues 264–276 of the PLP (AB3, donated by Drs. M. B. Lees and J. Isaac, Boston (Yamamura *et al.*, 1991)) for 1 h at room temperature on a rocking table. The blot was washed three times with Tris-buffered saline and further incubated in a solution containing a 1:1,000 dilution of a peroxidase-conjugated rabbit anti-rat total Ig (Nordic) for 1 h at room temperature. After three new washes with Tris-buffered saline, the blot was incubated with peroxidase-anti-peroxidase soluble complex (Sigma). Antibody binding was detected by incubation with 0.5 mg/ml diaminobenzidine-HCl (Sigma) in 0.5% hydrogen peroxide.

RESULTS

Retinoic Acid Increases PLP mRNA Levels in Glioma C6 Cells—To search for physiological regulators of PLP gene expression and to test whether thyroid hormone could also modulate PLP gene expression in cultured cells as it does in the developing rat brain (Muñoz *et al.*, 1991), rat glioma C6 cells were treated with these or other hormones and agents for 48 h (Fig. 1). No increase in the steady-state PLP mRNA level was observed in T_3-treated cells. In contrast, a major effect of RA was found. RA induced a 10–20-fold increase in the level of PLP mRNA after 48 h which was not detected in cells exposed to dexamethasone, growth hormone, estradiol, or vitamin D_3 (Fig. 1). Insulin also failed to increase PLP mRNA expression, and forskolin, a cAMP-inducing agent, had only a minor effect (not shown).

The time course and dose dependence of the RA-induced up-regulation in PLP mRNA are shown in Fig. 2. The kinetics of appearance revealed a lag period of 2–4 h followed by a slow but consistent increase in the level of PLP mRNA. The maximum effect is reached 24–48 h after RA exposure and remains constant in the continuous presence of RA for another 24 h. Treatment of cells with cycloheximide (8 μg/ml,

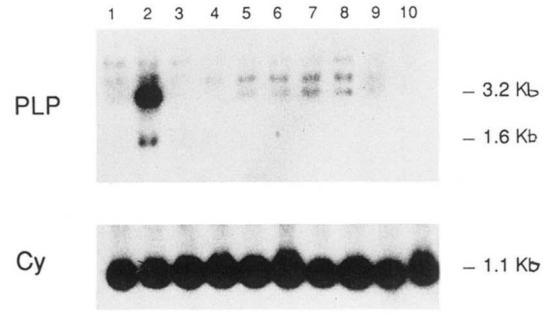

FIG. 1. **Effect of different hormones on PLP mRNA expression in C6 glioma cells.** Poly(A)$^+$ RNA (10 μg) was isolated from confluent C6 cells at 48 h after exposure to no hormones (*lane 1*), 10^{-6} M RA (*lane 2*), 10^{-7} M 1α,25-(OH)$_2$-vitamin D$_3$ (*lane 3*), 10^{-8} M 1α,25-(OH)$_2$-vitamin D$_3$ (*lane 4*), 10^{-7} M estradiol (*lane 5*), 10^{-8} M estradiol (*lane 6*), 5×10^{-8} M growth hormone (*lane 7*), 5×10^{-9} M growth hormone (*lane 8*), 2×10^{-7} M T$_3$ (*lane 9*), or 10^{-6} M dexamethasone (*lane 10*). Northern blots were sequentially hybridized to a PLP and cyclophilin (*Cy*) probes (see "Experimental Procedures"). Membranes were exposed for 3 days. Bands corresponding to mature PLP mRNAs (3.2 and 1.6 kb) and the control gene cyclophilin mRNA (1.1 kb) are pointed out. No expression of the third (2.4 kb) PLP mRNA was observed in C6 cells. Additional minor bands of 5.5 and 4.5 kb corresponding probably to precursor PLP mRNAs were usually detected (see Campagnoni and Macklin, 1988).

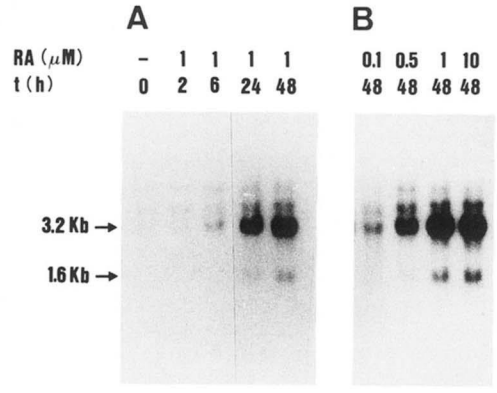

FIG. 2. **Time course and dose dependence of PLP mRNA up-regulation by RA in C6 cells.** At the day of confluence, cell monolayers were exposed for the times indicated to 1 μM RA (*panel A*) or for 48 h to various concentrations of RA (*panel B*). Northern analysis was as described under "Experimental Procedures." Membrane was hybridized with the PLP probe. Exposure time was 3 days. Identical results were obtained in four independent experiments.

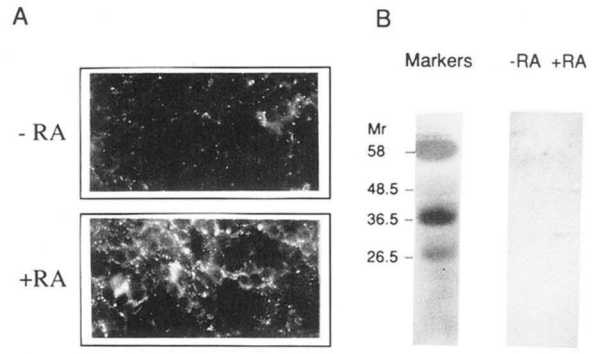

FIG. 3. **Increase by retinoic acid of the immunoreactive PLP in C6 cells.** *Panel A*, immunofluorescence analysis of the PLP. C6 cells treated without (*upper*) or with 1 μM RA (*lower*) for 48 h were subjected to immunofluorescence using an anti-PLP antiserum as described under "Experimental Procedures." *Panel B*, Western blot analysis of the PLP induction by RA. Details of the protocol used appear under "Experimental Procedures." *Numbers* on the *left* correspond to the sizes of marker proteins (Sigma) run in parallel.

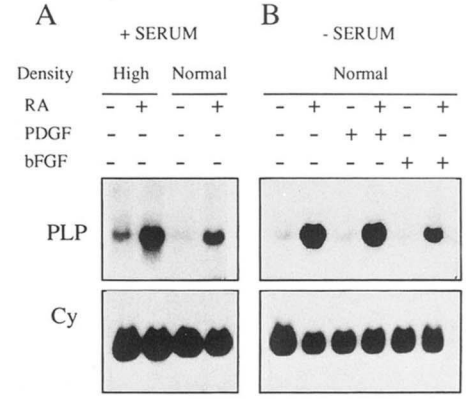

FIG. 4. **Effect of cell density, serum, and growth factors on the RA-mediated induction of PLP mRNA levels.** *Panel A*, effect of cell density. Glioma C6 cells were incubated in serum-containing growth medium (see "Experimental Procedures") in the presence or absence of 1 μM RA for 24 h at either normal (2×10^7/150-mm dish) or high (7×10^7/150-mm dish) density. *Panel B*, effect of serum and growth factors. C6 cells at normal (2×10^7/150-mm dish) density were incubated in the absence of serum with or without RA (1 μM), PDGF (5 ng/ml), or bFGF (5 ng/ml) for 24 h as indicated. Poly(A)$^+$ RNA was isolated and analyzed in Northern blots as described under "Experimental Procedures" and in the legend to Figs. 1 and 2. Membranes were hybridized sequentially with PLP and cyclophilin (*Cy*) probes. Two separate experiments were carried out giving similar results.

added 30 min before RA), which caused a more than 95% inhibition in overall protein synthesis, prevented completely the RA-induced increase in PLP mRNA levels observed at 6 and 8 h (not shown).

Retinoic Acid Increases Proteolipid Protein Content in C6 Cells—To determine whether the observed increase in PLP mRNA levels by RA caused a concomitant induction in the level of PLP, we performed immunofluorescence and Western analyses. As shown in Fig. 3, *panel A*, RA markedly increased the immunoreactive intensity of the PLP in immunofluorescence analyses using a specific anti-PLP antiserum. The kinetics of PLP increase was similar to that of PLP mRNA, being the maximum immunoreactive intensity observed after exposing the cells to RA for 48 h. Also, by Western blotting (Fig. 3, *panel B*), a band of the expected size (around 30 kDa) for the mature PLP was detected only in extracts from RA-treated cells. An additional unknown protein of higher size was also found.

Influence of Cell Growth Conditions on Retinoic Acid Action—Recent work has shown that PLP gene expression in C6 cells increases at high cell density (Nave and Lemke, 1991). These authors also reported that removal of serum leads to a further increase in PLP mRNA, suggesting the presence of inhibitory factors in serum (PDGF and bFGF possible candidates) (Bögler *et al.*, 1990). We wished to determine whether the RA induction of PLP gene expression could be affected by these conditions or growth factors. First, we observed that PLP mRNA levels were equally increased (10-fold in both conditions) by RA at either normal (2×10^7 cells/150-mm dish) or high (7×10^7 cells/150-mm dish) cell density (Fig. 4, *panel A*). It was also confirmed that basal levels of PLP mRNA was higher in denser cultures.

To investigate the possible effect of serum and growth factors, cells cultured in normal growth medium containing high serum or in serum-free medium supplemented or not with PDGF or bFGF were exposed to RA for 24 h. RNA analysis showed that the presence of serum (10% horse serum

+ 2.5% fetal calf serum) does not greatly affect the increase in PLP mRNA levels by RA (Fig. 4, *panel B*). Also, recombinant PDGF (2 and 5 ng/ml) failed to block either the low basal levels or the RA-induced levels of PLP mRNA expression in C6 cells. In contrast, bFGF was able to inhibit RA action partially (Fig. 4, *panel B*). A 14–45% inhibition of the RA-induced increase in PLP mRNA level was observed in two independent experiments with bFGF doses ranging from 2 to 15 ng/ml.

Overexpression of the Thyroid Receptor/c-erbA α-1 Gene Is Required for Thyroid Hormone to Increase PLP mRNA Levels in C6 Cells—Since the negative result obtained with T_3 in C6 cells was in conflict with the hormonal dependence of PLP gene expression observed *in vivo*, we studied the endogenous expression of thyroid receptor genes in these cells. In agreement with published data (Bahouth, 1991) and similar to results obtained in several other cell lines of neural origin studied by us in the last years,[3] C6 cells expressed low levels of TRα-1 RNA, whereas TRβ RNA could not be detected. Higher levels of RNA corresponding to the nonbinding TRα-2 form were found (not shown). Since several groups have reported an inhibition by TRα-2 of the *trans*-activating activity of TRα-1 in transiently cotransfected cells (Koenig *et al.*, 1989; Lazar *et al.*, 1989; Rentoumis *et al.*, 1990), we decided to investigate whether this could be the reason for the apparent T_3 unresponsiveness of the C6 cells. To this end, we used a previously described retroviral vector encoding the chicken c-*erbA*/TRα-1 gene (Muñoz *et al.*, 1990) to overexpress the TRα-1 form and in this way increase the number of active thyroid receptors in the cells. In parallel, and for comparison, we also infected C6 cells with another retrovirus encoding the mutant viral v-*erbA* protein.

Northern blot analysis of PLP mRNA expression in the three C6 cell types showed two effects (Fig. 5). First, neither c-*erbA*/TRα-1 nor v-*erbA* overexpression inhibited the RA induction of PLP mRNA. Second, c-*erbA*/TRα-1 overexpression made the C6 cells sensitive to thyroid hormone, *i.e.* T_3 treatment increased PLP mRNA levels in c-*erbA*/TRα-1 cells but not in control or v-*erbA* cells. The kinetics of this action is very similar to that of RA (an induction was detected 12–16 h after T_3 exposure) (not shown).

Expression of Other Glial-specific Genes in C6 Cells—Next we investigated whether RA and T_3 affected other glial genes in C6 cells. First, expression of myelin basic protein and myelin-associated glycoprotein genes, coding for the two other predominant myelin proteins, were undetectable in C6 cells under any of the conditions tested in agreement with results by others (Nave and Lemke, 1991) (not shown). High levels of cyclic nucleotide phosphodiesterase mRNA were found in untreated C6 cells. However, in contrast to the results obtained with PLP, these high basal levels of cyclic nucleotide phosphodiesterase mRNA were only slightly up-regulated by RA (Fig. 5). Also, in contrast to its reported action *in vivo*, T_3 had no major effect on cyclic nucleotide phosphodiesterase expression in C6 cells, probably a result of the loss in T_3 responsiveness of this gene observed after long term culture *in vitro* (Shanker *et al.*, 1985). In addition, v-*erbA* did not affect cyclic nucleotide phosphodiesterase expression. Another gene studied was vimentin, a marker gene for glial O2A precursors in the rat optic nerve which is down-regulated upon differentiation to oligodendrocytes (Dubois-Dalcq, 1987). As shown in Fig. 5, vimentin mRNA levels were slightly reduced upon RA treatment in both control and c-*erbA*/TRα-1 cells. The addition of thyroid hormone resulted in a significant inhibition of the level of vimentin mRNA in c-*erbA*/TRα-1-overexpressing cells but not in control or v-*erbA* cells. In these cells, RA and T_3 seemed to cooperate in the decrease of vimentin mRNA levels (Fig. 5). As seen before, c-*erbA*/TRα-1 overexpression appeared to be required for C6 cells to respond to T_3. In contrast, none of these effects was observed in v-*erbA*-expressing cells. v-*erbA* seemed to block both RA and T_3 inhibition of vimentin mRNA levels.

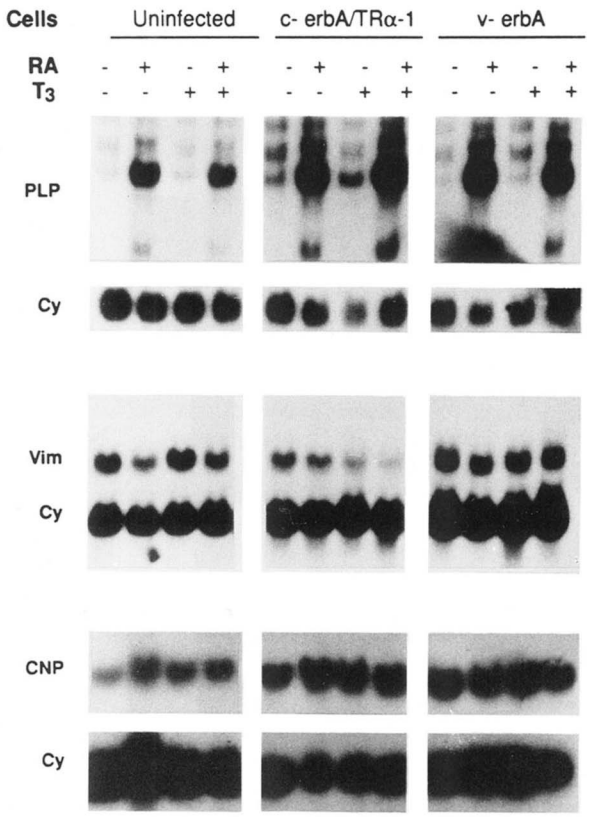

FIG. 5. **Regulation by RA and thyroid hormone of PLP and other glial-specific mRNAs in uninfected and c-*erbA*/TRα-1- and v-*erbA*-overexpressing glioma C6 cells.** Confluent monolayers of uninfected and c-*erbA*/TRα-1- or v-*erbA*-overexpressing C6 cells were treated for 48 h with 1 μM RA, 150 nM T_3, both together or left untreated. RNA analysis, blotting, and hybridization with PLP, vimentin (*Vim*), and cyclic nucleotide phosphodiesterase (*CNP*) probes were as described under "Experimental Procedures" and in the legend to previous figures. Each membrane was reprobed with cyclophilin (*Cy*) for control of RNA loading. Exposure times: PLP/cyclophilin, 3 days and overnight, respectively; vimentin/cyclophilin, 2 days for both; cyclic nucleotide phosphodiesterase/cyclophilin, 1 day and 2 days, respectively. Two independent experiments gave the same results.

Mechanism of Retinoic Acid Increase of PLP mRNA Levels—The RA-induced increase in PLP mRNA steady-state levels could be a result of a transcriptional effect, an effect on PLP mRNA stability, or a combination of both. To determine whether RA increased the rate of transcription of the PLP gene we performed nuclear run-on experiments. Nuclei from control or RA- or T_3-treated cells were isolated and nascent transcripts hybridized to filter-bound plasmid probes as described under "Experimental Procedures." Neither RA nor T_3 had an effect on the transcription rate of the PLP gene measured at 2, 4 (Fig. 6), 8, or 16 h (not shown), suggesting a posttranscriptional effect of RA.

We next assessed whether RA treatment affected the stability of the PLP mRNA (Fig. 7 and Table I). Since the basal expression of PLP gene in C6 cells was low, we first exposed C6 cells to RA for 24 h to increase PLP mRNA levels. Retinoic

[3] A. Muñoz, T. Iglesias, S. Llanos, A. Rodríguez-Peña, and J. Bernal, unpublished results.

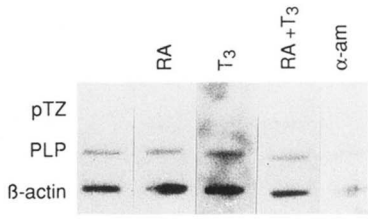

FIG. 6. **Transcription rate of PLP gene in RA- and T_3-treated C6 cells.** Nuclei isolated from confluent monolayers of C6 cells untreated or treated for 4 h with 1 μM RA, 150 nM T_3, or both together were assayed for transcription as described under "Experimental Procedures." Labeled RNAs (10^7 cpm) were hybridized to nylon membranes containing 5 μg of linearized PLP cDNA, β-actin as an internal control, and pTZ as a background control. The RNA polymerase II inhibitor α-amanitin (2 μg/ml) was used as a control of the reaction. Similar results were obtained with 2-, 8-, or 16-h treatments in two separate experiments.

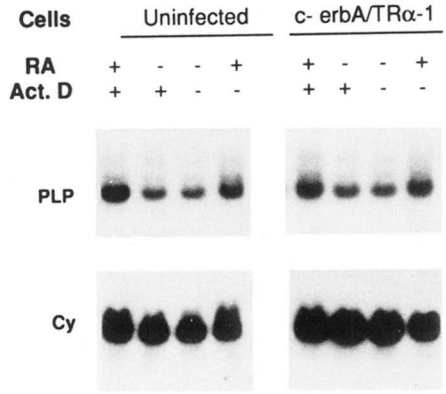

FIG. 7. **Effect of RA on the stability of PLP mRNA in glioma C6 cells.** Uninfected and c-*erbA*/TRα-1-overexpressing C6 cells were cultured in serum-free medium containing 1 μM RA. After 24 h media were changed, and parallel dishes were cultured for an additional 8 h in the presence of RA (1 μM), actinomycin D (*Act. D*; 3 μg/ml), RA + actinomycin D, or plain medium as indicated. Ten μg of poly(A)$^+$ RNA corresponding to each treatment was analyzed, blotted, and hybridized with PLP and cyclophilin (*Cy*) cDNA probes as described under "Experimental Procedures." Exposure times: PLP, 3 days; cyclophilin, overnight.

TABLE I

Effect of RA on PLP mRNA half-life in C6 cells

Uninfected C6 cells were pretreated with RA for 24 h as described in Fig. 7. The medium was changed, and the cells were treated for different times with either actinomycin D alone or actinomycin D + RA as in Fig. 7. PLP mRNA levels were estimated in Northern blots using PLP and cyclophilin probes. The data represent individual percentage values of actual PLP mRNA levels over those existing after the initial 24-h treatment with RA (time 0) in three independent experiments after normalization to cyclophilin levels.

	Time				
	0 h	3 h	5 h	9 h	12 h
			%		
Exp. 1					
−RA	100	100	70	40	30
+RA	100	100	95	90	85
Exp. 2					
−RA	100	100	80	45	30
+RA	100	100	90	85	80
Exp. 3					
−RA	100	100	85	40	30
+RA	100	100	100	95	90

acid was then removed, and parallel dishes were incubated in the presence of the transcription inhibitor actinomycin D plus or minus RA, left untreated, or treated with RA alone (Fig. 7). PLP mRNA levels were lower in actinomycin D-treated cells in the absence of RA than in its presence, suggesting an effect of RA on the stability of the PLP message. Cells kept in the presence of RA contained more PLP mRNA than those from which RA was removed after the initial 24-h treatment. Similar results were obtained with c-*erbA*/TRα-1 cells. In three independent experiments using duplicate plates (Table I), we observed consistently that RA stabilizes the PLP mRNA. The half-life of PLP mRNA increases from 8 h in untreated cells to more than 12 h in RA-treated cells. Because of the impossibility of keeping the cells in the presence of actinomycin D for longer than 12 h, the exact magnitude of PLP mRNA half-life in the presence of RA could not be determined. This increase in PLP mRNA half-life may be sufficient to account for the observed increase in the PLP mRNA steady-state levels which become apparent 16–24 h after RA exposure.

The lack of transcriptional effect of RA and T_3 on the PLP gene was further studied by analyzing the available 1.2 Kb fragment of promoter sequences described by Nave and Lemke (1991) for the presence of binding sites for RA or T_3 receptors. Using vaccinia virus-infected HeLa cells extracts enriched in either RAR-α or c-*erbA*/TRα-1 proteins we failed to detect any specific binding site for these receptors in band-shift experiments (not shown). Our results indicated that neither RAR not c-*erbA*/TR act at the PLP promoter level.

Expression of RA Receptors in C6 Cells—Since the first step of RA action is binding to specific receptors (RARs), we investigated the presence of the different RA receptors in the C6 cells. A single 3.3-kb band was seen hybridizing with the RAR-β probe (Fig. 8, *panel A*). The low basal expression of RAR-β was lower than that of RAR-α and rapidly induced by RA in a cycloheximide-resistant fashion as reported for other cell types (de Thé *et al.*, 1989; Hu and Gudas, 1990; Martin *et al.*, 1990; Clifford *et al.*, 1990). Cycloheximide alone was also able to increase the level of RAR-β mRNA. The higher increase observed in cells treated with RA + cycloheximide is probably the consequence of the additive effects of both agents. In the mouse all three RAR genes give rise to multiple isoforms, some of them containing RAREs in their regulatory region (de Thé *et al.*, 1990, Sucov *et al.*, 1990; Vivanco Ruiz

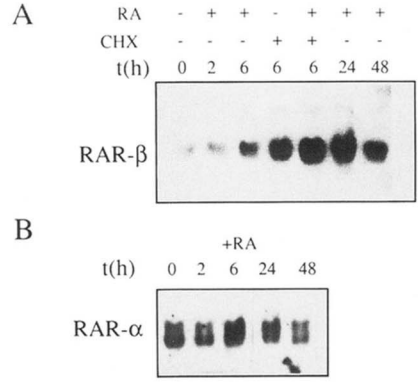

FIG. 8. **RAR expression in control and RA-treated C6 cells.** Poly(A)$^+$ RNA (10 μg) was isolated from cells cultured in the absence or presence of 1 μM RA as indicated. Blotting and hybridization to RAR-β (*panel A*) and RAR-α (*panel B*) cDNA probes were as described under "Experimental Procedures." Cycloheximide (*CHX*) treatment (8 μg/ml) was initiated 30 min before the addition of RA. Exposure times: RAR-α, 2 days; RAR-β, 3 days. Three independent experiments were done.

et al., 1991; Leroy *et al.*, 1991; Lehmann *et al.*, 1992). The fact that RA induced a rapid and cycloheximide-resistant increase in RAR-β mRNA levels suggested a transcriptional effect on any of the RAR-β isoforms in C6 cells. However, the lack of sequence information for the rat isoforms made it impossible to use specific probes to elucidate the particular isoform involved. Using the corresponding cDNA as probe we detected high basal levels of the expected two bands (3.8 and 2.8 kb) for the RAR-α mRNAs (Fig. 8, *panel B*). As in many other cell types, the expression of RAR-α was unaffected by RA treatment and was also independent on c-*erbA*/TRα-1 or v-*erbA* overexpression (not shown). Finally, no hybridization signals were obtained using the RAR-γ cDNA.

The expression of receptors (RXRs) for the RA-derivative 9-*cis* retinoic acid in C6 cells was also investigated. A single 5.4-kb species of RXR-α mRNA whose expression was unaffected by RA, T_3, or dexamethasone was detected after overnight exposure. The expression of RXR-β mRNA (two bands of 2.4 and 2.6 kb) was much lower, detected only after 2 weeks of exposure, and equally unchanged (not shown).

DISCUSSION

PLP is essential for the critical process of myelination in the central nervous system. PLP is not only the most abundant myelin protein but also seems to play an essential role in the initial steps of myelin formation and in the compaction of mature myelin. In spite of the high clinical importance of demyelinating diseases, including multiple sclerosis and many others, very little is known about the mechanisms and factors regulating the expression of PLP and other myelin genes.

In this study we demonstrate that the morphogen RA is a potent regulator of PLP mRNA and protein expression in C6 glioma cells. RA induces an stabilization of PLP mRNA. In contrast, the rate of PLP gene transcription is unchanged by RA. The increase in mRNA stability leads to a 10–20-fold increase in PLP mRNA steady-state levels after 48 h of treatment. As a result, the amount of PLP in C6 cells increases.

In agreement with a posttranscriptional action of RA are the slow kinetics and the dependence on new protein synthesis. Since transcriptional activation by RA has been reported to be inhibited by unliganded c-*erbA*/TRα-1 (Brent *et al.*, 1989; Barettino *et al.*, 1993), the lack of inhibition of RA action in c-*erbA*/TRα-1-overexpressing cells also supports this mechanism.

Taken together, the increase in PLP expression and the vimentin down-regulation by RA point to an oligodendrocyte differentiation-inducing action of RA. However, the finding that RA did not greatly affect the expression of cyclic nucleotide phosphodiesterase, a very early myelin marker, indicated that the drastic up-regulation of PLP expression was not the result of a general induction of oligodendrocyte genes. Likewise, the expression of the glycerol phosphate dehydrogenase gene, another oligodendrocyte marker, was basically unchanged by RA (results not shown). It may be that other factors in addition to RA are required to induce oligodendrocyte differentiation. It is also possible that this partial effect of RA in C6 cells is because of an intrinsic inability of these cells to undergo terminal differentiation or the necessity for longer treatments. On the other hand, bFGF partially blocked RA action. This result suggested that bFGF could be involved in the inhibition of oligodendrocyte differentiation caused by serum (Bögler *et al.*, 1990).

Among other hormones studied only thyroid hormone had an effect, albeit much reduced, that was only seen when the low levels of endogenous thyroid receptors were elevated by exogenous expression of an c-*erbA*/TRα-1 gene. Our results suggest that the thyroid hormone dependence *in vivo* of PLP expression at a pretranslational level during the neonatal period (Muñoz *et al.*, 1991) is not the result of a direct transcriptional effect of the hormone.

In this study, other hormones including dexamethasone did not affect PLP gene expression. This result is compatible with the transient decrease in PLP mRNA induced by dexamethasone in postnatal rat brain (Tsuneishi *et al.*, 1991) and in contrast with the reported 2–3-fold increase in PLP mRNA levels by hydrocortisone in primary rat glial cell cultures (Kumar *et al.*, 1989). Cell type-dependent, indirect mechanisms could account for these discrepancies.

As for other genes described as posttranscriptionally regulated by RA either positively or negatively (Glick *et al.*, 1989; Antras *et al.*, 1991; Gellersen *et al.*, 1992), one has to assume that the mechanism of PLP regulation involves the control of one or more genes whose product(s) are responsible for the stability of PLP mRNAs. Our data on PLP and RAR expression support such an indirect mechanism taking place in RA-treated C6 glioma cells. The shorter kinetics of RAR-β induction by RA makes possible the idea that RAR-β induction may be required for the increase in PLP mRNA levels, perhaps by controlling the expression of the putative protein(s) responsible for it.

The increase in PLP mRNA levels during development has been found to be much higher than that of transcription, suggesting the possibility of regulated posttranscriptional steps in PLP gene expression (Gardinier *et al.*, 1986; Macklin *et al.*, 1991). Our results support this hypothesis and indicate that RA can be proposed as responsible for such posttranscriptional control, perhaps in cooperation with thyroid hormone or other uncharacterized agents. Given the key role assigned to PLP in myelin formation possibly participating in the regulation of the expression of other myelin genes (Macklin *et al.*, 1991) and in the functionality of the mature myelin sheath, if this was true, RA should be considered as an important regulator of oligodendrocyte differentiation. Furthermore, the presence of relatively high PLP mRNA levels in adult animals (Gardinier *et al.*, 1986) also suggests the possibility of a late developmental RA control of PLP gene expression. The severe effects of myelin deficiencies and alterations in brain function make the study of RA and T_3 effects on oligodendrocytes of obvious great interest.

Acknowledgments—We thank those colleagues who appear under "Experimental Procedures" for supplying cDNA probes and the anti-PLP antiserum and monoclonal antibody, Dr. K.-A. Nave for a plasmid containing 1.2 kb of the rat PLP gene promoter sequences, and Dr. Angel Zaballos for critically reading the manuscript. We also acknowledge the expert technical assistance of Margarita González and Gloria Chacón.

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