Cell-Intrinsic Timers and Thyroid Hormone Regulate the Probability of Cell-Cycle Withdrawal and Differentiation of Oligodendrocyte Precursor Cells

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INTRODUCTION

In many vertebrate cell lineages, precursor cells divide a limited number of times before they stop and terminally differentiate into postmitotic cells. It is not known what limits cell proliferation and causes the cells to stop dividing when they do. The stopping mechanisms are important because they influence both the timing of cell differentiation and the number of terminally differentiated cells generated.

We have been studying the mechanisms that stop precursor cell division and initiate differentiation in the oligodendrocyte cell lineage in the rat optic nerve. Oligodendrocyte precursor cells migrate into the developing nerve from the brain, beginning before birth (Small et al., 1987). After a period of proliferation, they stop dividing and terminally differentiate into oligodendrocytes, which myelinate the axons in the nerve. The first oligodendrocytes appear in the nerve around the day of birth and then increase in number for the next 6 weeks (Skoff et al., 1976; Miller et al., 1985; Barres et al., 1992).

The normal timing of oligodendrocyte development can be reconstituted in cultures of dissociated perinatal optic nerve cells: as long as the oligodendrocyte precursor cells are stimulated to proliferate by either astrocytes (Raff et al., 1985) or platelet-derived growth factor (PDGF) (Raff et al., 1988), oligodendrocytes begin to appear at the equivalent of the day of birth. Clonal analyses of either single (Temple and Raff, 1986) or purified (Barres et al., 1994a) precursor cells isolated from postnatal day 7–8 (P7–8) rat optic nerve suggest that both a cell-intrinsic program and extracellular signals play important parts in determining when the precursor cells stop dividing and differentiate. In the presence of appropriate signaling molecules, the precursor cells di-
vide up to eight times before they stop and differentiate, and the progeny of an individual precursor cell tend to stop dividing and differentiate at about the same time (Temple and Raff, 1986; Barres et al., 1994a). Moreover, when the two daughter cells of an individual precursor cell are separated and cultured on astrocyte monolayers in separate microwells, they tend to differentiate more or less synchronously, suggesting that an intrinsic mechanism operates in the precursor cells to cause them to withdraw from the cell cycle and differentiate after a certain period of time or number of cell divisions (Temple and Raff, 1986). When precursor cells are cultured at 33°C rather than 37°C, they divide more slowly but differentiate sooner, after fewer cell divisions, suggesting that the intrinsic mechanism does not operate by simply counting cell divisions but instead measures elapsed time in some other way (Gao et al., 1997).

One part of the timer seems to involve the gradual accumulation of the cyclin-dependent kinase inhibitor p27kip1 (Durrand et al., 1997).

At least two kinds of extracellular signals seem to be required for the timer to operate normally—the mitogen PDGF (Noble et al., 1988; Raff et al., 1988; Richardson et al., 1988) and hydrophobic signals such as thyroid hormone (Barres et al., 1994a; Ibarrola et al., 1996; Ahlgren et al., 1997; Baas et al., 1997). In the absence of mitogen, cultured precursor cells prematurely stop dividing and differentiate into oligodendrocytes within 2 days (Noble and Murray, 1984; Temple and Raff, 1985), whether or not hydrophobic signals are present (Barres et al., 1994a; Ahlgren et al., 1997). In the presence of mitogen but in the absence of hydrophobic signals, most of the precursor cells tend to keep dividing and do not differentiate for at least 16 days; if thyroid hormone is added to such cultures after 8 days, however, most of the precursor cells stop dividing and differentiate within 4 days, suggesting that a timing mechanism of some kind continues to operate in the absence of hydrophobic signals (Barres et al., 1994a). These findings suggest that the intrinsic timer consists of at least two components: a timing component that counts elapsed time and is not regulated by hydrophobic signals such as thyroid hormone and an effector component that is regulated by hydrophobic signals and stops cell proliferation and initiates differentiation when the timing component indicates it is time. Bügler and Noble (1994) independently provided evidence for distinct timing and effector components, using a combination of PDGF and basic fibroblast growth factor (FGF-2), rather than an absence of thyroid hormone, to inhibit differentiation.

Thyroid hormone has also been shown to play a role in regulating oligodendrocyte differentiation in the developing optic nerve in vivo. Hypothyroid rats (Ibarrola et al., 1996) and mice (Ahlgren et al., 1997) have reduced numbers of oligodendrocytes in the optic nerve, at least during the first week of postnatal development.

In the present study, we have analyzed clonal cultures of oligodendrocyte precursor cells purified from embryonic day 18 (E18) rat optic nerves. We show that the first oligodendrocytes develop at the equivalent of the day of birth, just as in unpurified embryonic optic nerve cell cultures (Raff et al., 1985) and in vivo (Miller et al., 1985), but only if thyroid hormone is present, providing direct evidence that a cell-intrinsic, thyroid-hormone-regulated timer controls the initial development of oligodendrocytes in culture. Unlike oligodendrocyte development in clonal cultures of P7–8 optic nerve precursor cells, however, which occurs more or less synchronously within clones (Temple and Raff, 1986; Barres et al., 1994a), we find that much of the initial oligodendrocyte development in the cultures of E18 cells occurs asynchronously within clones; moreover, an increasing proportion of precursor cells stop dividing and differentiate with time in culture, even in the absence of thyroid hormone. These findings, together with previous evidence for asynchronous oligodendrocyte development within clones in culture (Vaysse and Goldman, 1990; Lubetzki et al., 1992; Zhang and Miller, 1995; Ibarrola et al., 1996), suggest that stochastic processes, as well as an intrinsic timer, can influence the timing of oligodendrocyte differentiation. We propose that both the timing component of the timer and thyroid hormone regulate the probability that a precursor cell will stop dividing and differentiate, such that the probability increases with both time and thyroid hormone stimulation.

Barres et al. (1994a) showed previously that P14 oligodendrocyte precursors are more sensitive to thyroid hormone than P1 precursor cells, which correspondingly express fewer β2 thyroid hormone receptors. These findings raised the possibility that the intrinsic timer may involve, in part at least, an increase in thyroid hormone sensitivity, which may, in turn, reflect a progressive increase in β thyroid hormone receptors. In the present study we provide evidence that supports this possibility. We show that both the sensitivity of purified E18 precursor cells to thyroid hormone and the level of β1 thyroid hormone receptors increase in parallel with time in culture in the absence of thyroid hormone. Moreover, the level of these receptors increases faster at 33°C than at 37°C, as expected if the increase is part of the timer, which runs faster at the lower temperature (Gao et al., 1997).

**EXPERIMENTAL METHODS**

**Animals and Chemicals**

Sprague-Dawley rats were obtained from the Animal Facility at University College London. Chemicals were purchased from Sigma, except where indicated. Recombinant human PDGF-AA and neurotrophin-3 (NT-3) were purchased from Peprotech.

**Preparation of Optic Nerve Cells**

Optic nerve cells were prepared as previously described (Barres et al., 1992). In brief, optic nerves were removed from E18 or postnatal rats and incubated at 37°C for 60 min in papain solution (100 units, Boehringer Mannheim) in Hepes-buffered minimal Eagle's medium (MEM/Hepes). Cells were dissociated by trituration using a Gilson Pipetman set at 500 μl. They were washed sequentially...
in a low concentration of ovomucoid and bovine serum albumin (BSA) (1.5 mg/ml of each) in MEM/Hepes and then in a high concentration of ovomucoid and BSA (6 mg/ml of each).

Purification of Oligodendrocyte Precursor Cells

Oligodendrocyte precursor cells were purified by immunopanning, as described previously (Barres et al., 1992). In brief, dissociated optic nerve cells were resuspended in 10 ml of L15 air medium (GIBCO), containing 0.2 mg/ml BSA and 10 μg/ml bovine insulin, and plated on a 100-mm bacteriological petri dish (Falcon) coated with a monoclonal anti-GC antibody (Ranscht et al., 1982, supernatant, diluted 1:4). The cells that did not adhere were then incubated on a dish coated with the A2B5 monoclonal antibody (Eisenbarth et al., 1979, ascites fluid, diluted 1:2000) for 45 min. For the purification of E18 precursor cells the anti-GC dish was omitted. The dish was then washed five to eight times with 6 ml of MEM/Hepes, and the remaining adherent oligodendrocyte precursor cells were dislodged by trypsin treatment (0.012% in Eagle’s balanced salt solution) and washed in L15 air medium containing 20% fetal calf serum (FCS).

Clonal Cultures of Precursor Cells

The purified oligodendrocyte precursor cells were counted and plated at clonal density (4000–4000 cells in 3 ml of culture medium) in a poly-o-lysine (PDL)-coated 25-cm² flask (Falcon). In some cases the cells were cultured on PDL-coated glass coverslips in 24-well Falcon culture dishes (2000 cells per coverslip). The cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) containing bovine insulin (10 μg/ml), human transferrin (100 μg/ml), BSA (10 μg/ml), progesterone (60 ng/ml), putrescine (16 μg/ml), sodium selenite (40 ng/ml), N-acetylcysteine (60 μg/ml), forskolin (5 μM), trace minerals (GIBCO), PDGF (usually at 10 ng/ml), neurotrophin-3 (NT-3) (5 ng/ml), and penicillin and streptomycin (GIBCO). In some experiments triiodothyronine (T3, 30 ng/ml) was also added.

Cultures were maintained in a 5% CO₂ incubator at 37°C. If cultures were maintained for longer than 4 days, 50% of the medium was replaced with freshly made medium every 4 days, or every 2 days in long-term cultures where the cell density was greatly increased. Oligodendrocytes and oligodendrocyte precursor cells in each clone were identified by their characteristic morphologies in an inverted phase-contrast microscope (Temple and Raff, 1986). To be certain that our ability to distinguish oligodendrocytes and precursor cells by morphology was accurate, we immunostained some cultures with a monoclonal anti-GC antibody to identify oligodendrocytes (Raff et al., 1978; Ranscht et al., 1982) and the A2B5 monoclonal antibody to identify the precursor cells (Raff et al., 1983). The assessments by morphology and immunostaining were always consistent. After 4 days in culture, the total number of nonoligodendrocyte lineage cells in a 25-cm² flask was fewer than 2 cells for P7 cultures and fewer than 50 cells for E18 cultures, and these cells did not proliferate.

In some experiments cells in 25-cm² flasks were passaged after 7–11 days. The cells were treated with 0.005% trypsin (in Eagle’s balanced salt solution) for ~5 min at 37°C, removed from the flask by gentle pipetting after adding 5 ml of ovomucoid and BSA (1.5 mg/ml of each) in MEM/Hepes, washed in 5 ml of MEM/Hepes containing 20% FCS, and recultured in 25-cm² flasks at clonal density (1000 cells per flask) or low density (10,000 cells per flask).

Immunofluorescence Assays

Immunofluorescence assays were carried out with cells on either PDL-coated coverslips or PDL-coated slide flasks. For staining surface antigens, the cells were fixed with 2% paraformaldehyde for 2 min at room temperature and incubated for 30 min in blocking solution, consisting of 50% goat serum in Tris buffer (pH 7.4) containing 1% BSA and 150 mM l-lysine. The cells were then incubated in either anti-GC antibody (Ranscht et al., 1982, supernatant, diluted 1:1), followed by Texas Red-conjugated goat anti-mouse IgG (Jackson ImmunoResearch, diluted 1:100), or A2B5 antibody (Eisenbarth et al., 1979; ascites fluid, diluted 1:100), followed by fluorescein-conjugated goat anti-mouse IgM (Jackson ImmunoResearch, diluted 1:100). For detection of thyroid hormone receptors, the cells were fixed with 2% paraformaldehyde as above, permeabilized with 0.1% Triton in phosphate-buffered saline (PBS), and then incubated in rabbit anti-thyroid hormone receptor antibodies (which recognize both α1 and α2 receptors) or monoclonal anti-β thyroid hormone receptor antibodies (Santa Cruz; 20 μg/ml), followed by biotin-conjugated goat anti-rabbit (or mouse) IgG and then fluorescein-conjugated streptavidin (both from Amersham, diluted 1:100). The coverslips were mounted in Citifluor mounting medium (Citifluor, UK), sealed with nail varnish, and examined with a Zeiss Axioskop fluorescence microscope.

Quantitative Confocal Microscopy

Quantitative confocal microscopy was carried out on a Bio-Rad MRC 1000 confocal laser-scanning fluorescence microscope as previously described (Durand et al., 1997). Briefly, individual cells immunostained for β thyroid hormone receptor antibodies were selected randomly, and the brightness of fluorescence staining in the nucleus of the cells was determined using settings that were kept the same for all the measurements in each experiment. The average fluorescence intensities were expressed in arbitrary units (pixels).

Time-Lapse Video Recording

Purified oligodendrocyte precursor cells were cultured at clonal density in 25-cm² flasks, as described above. Individual clones were continuously followed on a heated stage of a Zeiss inverted phase-contrast microscope, coupled to a Sony CCD video camera and time-lapse video cassette recorder.

RESULTS

Purified E18 Precursor Cells Differentiate on Schedule in Clonal Culture

When we cultured purified E18 precursor at clonal density in the presence of T3, the first oligodendrocytes, identified either by their characteristic morphology (Temple and Raff, 1985) or by staining with anti-galactocerebroside (GC) antibody (Raff et al., 1978; Ranscht et al., 1982), developed after 3–4 days (Table 1), equivalent to around the time of birth, as occurs in vivo (Miller et al., 1985). Moreover, the percentages of oligodendrocytes after 3–4 and 6 days in culture (Table 1) were similar to the percentages found previously in unpurified embryonic optic nerve cell cultures (Raff et
TABLE 1
The Timing of Oligodendrocyte Differentiation in Clonal Cultures of Purified E18 Oligodendrocyte Precursor Cells
in the Presence or Absence of Thyroid Hormone

<table>
<thead>
<tr>
<th>Days in culture</th>
<th>Experiment 1</th>
<th></th>
<th>Experiment 2</th>
<th></th>
<th>Experiment 3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>+T3</td>
<td>-T3</td>
<td>+T3</td>
<td>-T3</td>
<td>+T3</td>
</tr>
<tr>
<td>2</td>
<td>0/125 (0%)</td>
<td>0/376 (0%)</td>
<td>0/242 (0%)</td>
<td>0/250 (0%)</td>
<td>0/206 (0%)</td>
</tr>
<tr>
<td>3–4</td>
<td>6/507 (1.2%)</td>
<td>0/1844 (0%)</td>
<td>16/1241 (1.4%)</td>
<td>0/1525 (0%)</td>
<td>19/1177 (1.6%)</td>
</tr>
<tr>
<td>6</td>
<td>ND</td>
<td>ND</td>
<td>144/4618 (3.1%)</td>
<td>0/6554 (0%)</td>
<td>121/3654 (3.3%)</td>
</tr>
<tr>
<td>8</td>
<td>ND</td>
<td>ND</td>
<td>266/1504 (17.7%)</td>
<td>2/3487 (0.06%)</td>
<td>ND</td>
</tr>
</tbody>
</table>

Note. The results are shown as oligodendrocytes/oligodendrocytes + precursor cells (% oligodendrocytes). All of the cells in each flask were counted, except at day 8, when randomly selected areas were counted. ND, not done.

The First Oligodendrocytes Appear Asynchronously within Clones

The way in which the first oligodendrocytes developed in these E18 clones, however, often differed from the way oligodendrocytes have been found to develop in P7–8 clones. In clonal analyses of P7–8 precursor cells, most of the cells within a clone tend to stop dividing and differentiate at around the same time (Temple and Raff, 1986; Barres et al., 1994a; Zhang and Miller, 1995). In E18 clones, by contrast, only rare clones where most of the cells had become oligodendrocytes were seen after 3–4 days. Only 2 of 154 clones (2 of 12 oligodendrocyte-containing clones) analyzed at this time point showed this type of behavior: in 1 of them, 9 of 13 cells had become oligodendrocytes; in the other 13 of 18 cells had done so. In most of the oligodendrocyte-containing clones at 3–4 days, only a small minority of the cells within the clone were oligodendrocytes; of the 154 clones we examined at this stage, 8% contained oligodendrocytes, on average these contained 17 ± 1 cells, of which 4 ± 1 were oligodendrocytes (n = 12, mean ± SEM). The few oligodendrocytes seen in such non-synchronous clones tended to be clustered together within the clone. At 6 days, 37% of the clones examined contained oligodendrocytes; on average these contained 49 ± 4 cells, of which 5 ± 1 were oligodendrocytes (n = 45). Thus, even 2–3 days after the first oligodendrocytes appeared in a clone, most of the cells in the clone remained precursor cells.

The Timely Differentiation of Embryonic Precursor Cells Requires Thyroid Hormone

When we cultured purified E18 precursor cells at clonal density in the absence of T3, no oligodendrocytes developed during the first 6 days (Table 1), indicating that the normal timing of the initial differentiation of oligodendrocytes depends on thyroid hormone, at least under these culture conditions. Small numbers of oligodendrocytes, however, began to appear after 8 days in these cultures, but the percentage

FIG. 1. Oligodendrocyte production in clonal cultures of purified E18 precursor cells. Precursor cells were cultured at clonal density in the presence of T3, and 60 clones were followed.
of oligodendrocytes at this time was always more than 100 times less than the percentage that developed at the same time in the presence of thyroid hormone (Table 1).

**The Probability of Differentiation in the Absence of Thyroid Hormone Progressively Increases**

When we cultured purified P7 precursor cells at clonal density in the absence of T3, small numbers of oligodendrocytes developed within 2 days and their numbers increased with time, so that by 4 days about 3% of the cells were oligodendrocytes (Fig. 2). By contrast, when we cultured purified E18 precursor cells at clonal density in the absence of thyroid hormone for 4–6 days, no oligodendrocyte developed (Table 1). These findings indicate that the probability of precursor cell differentiation in the absence of thyroid hormone increases with maturation.

To determine if the probability of precursor cell differentiation in the absence of thyroid hormone continues to increase with time in culture even after the time when most precursor cells would have differentiated had thyroid hormone been present, we cultured purified P7 precursor cells for 11 or 20 days at clonal density without T3, removed them from the flask with trypsin, and recultured them at clonal density for 4 days with or without T3. The precursor cells at day 20 had been passaged once or twice before they were studied. As seen in Fig. 3, in the presence of T3 almost all the recultured cells differentiated by 4 days, whereas in the absence of T3 about 7% of the cells passed after 11 days differentiated, and about 12% of the cells passed after 20 days differentiated. Thus the probability of differentiation in the absence of T3 continued to increase with time even after most of the cells would have differentiated had T3 been present.

At all times tested, the percentage of oligodendrocytes in these P7 cultures was at least 5–10 times less in the absence of T3 than that in its presence and, in the absence of T3, was always much greater than that seen with E18 precursor cells in the absence of T3 at the same time point (see Fig. 2 and Table 1). In the absence of T3, oligodendrocyte development in both E18 and P7 cultures was usually asynchronous within clones.

**E18 Precursor Cells Become Sensitive to Thyroid Hormone with Time Even in the Absence of Thyroid Hormone**

Whereas some P7 precursor cells stop dividing and differentiate rapidly when cultured in the presence of T3 (see Fig. 2), E18 precursors under these conditions do not do so until 3–4 days (see Table 1), suggesting that E18 precursors may only become sensitive to the differentiation-promoting effects of thyroid hormone with maturation. To determine whether purified E18 precursor cells acquire this sensitivity even in the absence of thyroid hormone, we cultured them at clonal density and added T3 for the first time after various periods. As seen in Fig. 4, the cells rapidly differentiated when T3 was added after 6 or 9 days in culture; the percentage of oligodendrocytes in cultures treated with T3 from
Timing in Oligodendrocyte Development

After T3 was added and differentiated without dividing by 1 day, the majority divided once or twice over 1–3 days before differentiating, so that by 3 days almost all of the cells of the clone within the microscopic field had differentiated (Fig. 5). Thus, although T3 acted quickly in some cells to stop division and initiate differentiation, even within the same clone the cells did not all stop dividing and differentiate at the same time. Occasional cells in the clone kept dividing even after 2 weeks in the presence of T3 (a total of 4 weeks in culture), although by this time their cell-cycle times and migration rates were very slow (not shown).

Thyroid Hormone β1 Receptors Increase with Maturation in the Absence of Thyroid Hormone

One possible explanation for the finding that the sensitivity of E18 precursor cells to thyroid hormone increases with time is that the cells acquire one or more types of thyroid hormone receptors (TRs) as they mature. Vertebrate cells have two genes that encode homologous TRs, called TRα and TRβ; alternative RNA splicing creates two variants of each to generate four TRs—α1, α2, β1, and β2 (Hodin et al., 1990; Brent et al., 1991; Chin, 1994). To study the expression of TRs we used indirect immunofluorescence to stain purified E18 and P7 precursor cells after 1 day in culture, using antibodies against either TRα (reactive with both α1 and α2) or TRβ1. Whereas all oligodendrocyte lineage cells seemed to express about equal levels of TRα at both ages (not shown), this was not the case for TRβ1, which was expressed at high levels in all P7 oligodendrocytes and most P7 precursor cells (Fig. 6A) but was not

The Response Time to the Delayed Addition of Thyroid Hormone Is Variable within Clones

To determine more accurately how quickly and uniformly thyroid hormone acts to help stop cell division and initiate differentiation within clones once the timing component of the intrinsic timer reaches its end point, we cultured purified P7 precursor cells at clonal density in the absence of T3 for 11 days; by this time the timer would be expected to have triggered differentiation in most precursor cells had T3 been present from the start (Barres et al., 1994a). We then added T3 and followed the behavior of cells within a clone by time-lapse video recording. Although some cells within the clone stopped migrating at around 10 h after T3 was added and differentiated without dividing by 1 day, the majority divided once or twice over 1–3 days before differentiating, so that by 3 days almost all of the cells of the clone within the microscopic field had differentiated (Fig. 5). Thus, although T3 acted quickly in some cells to stop division and initiate differentiation, even within the same clone the cells did not all stop dividing and differentiate at the same time. Occasional cells in the clone kept dividing even after 2 weeks in the presence of T3 (a total of 4 weeks in culture), although by this time their cell-cycle times and migration rates were very slow (not shown).

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FIG. 6. Immunofluorescence staining of β1 thyroid hormone receptors in culture. Purified P7 (A), E18 (B and C), or P3 (D) precursor cells were cultured in the absence of T3, either at low density for 1 day (A and B) or at clonal density either for 10 days (C) or for various times (D). The cells were fixed, stained with anti-TRβ1 antibodies, and then either photographed (A–C) or analyzed (D) in a confocal laser-scanning fluorescence microscope. The results in D are expressed as means ± SEM of 20–30 cells for each time point. div, days in vitro.

detectably expressed in E18 precursor cells (Fig. 6B), suggesting that TRβ1 increases with precursor cell maturation.

When we cultured purified E18 precursor cells in the absence of T3 for 10 days, they had the same level of TRβ1 as P7 precursor cells cultured for 1 day (Fig. 6C), suggesting that the acquisition of TRβ1 is an intrinsic property of the precursor cells and does not depend on thyroid hormone. We determined the level of TRβ1 expression by quantitative confocal microscopy at various times in cultures of purified P3 precursor cells growing in the absence of T3. As shown in Fig. 6D, the average level of TRβ1 expression reached a plateau value after 12–14 days, which is about the time that most of the cells would have stopped dividing and differentiated had T3 been present.

**TRβ1 Levels Increase Faster at 33°C Than at 37°C**

We showed previously that the intrinsic timer that controls the onset of oligodendrocyte differentiation in culture runs faster at 33°C than at 37°C (Gao et al., 1997). If the increase in TRβ1 is part of the timer, then the levels of TRβ1 should increase faster at the lower temperature. This
was indeed the case when purified P1 precursor cells were cultured at the two temperatures in the absence of T3 and assayed for TRβ1 by quantitative confocal fluorescence microscopy after 5 days: whereas the average intensity of fluorescence in the nucleus was 207,700 ± 10,200 arbitrary units (pixels) at 37°C, at 33°C it was 289,600 ± 18,400 units (mean ± SEM, n = 20 and 27, respectively); the difference between the two results is significant (P < 0.001) when analyzed by Student’s t test.

Precursor Cells Eventually Stop Dividing after Extensive Proliferation in the Absence of Thyroid Hormone

To determine the fate of precursor cells that are stimulated to divide in culture for a prolonged period in the absence of thyroid hormone, we cultured purified P7 precursor cells under these conditions and passed them when the cultures became crowded. With increasing time in culture, cell proliferation progressively slowed and the number of oligodendrocytes gradually increased, even though half of the culture medium and mitogens were replaced every 2 days. The death of precursor cells also increased with time. By 30 days and 3–4 passages, cell division largely ceased, and the vast majority of the cells had either died or differentiated into oligodendrocytes. When we cultured purified E18 precursor cells in the same way, they behaved similarly to P7 cells, except that oligodendrocytes increased more slowly in the cultures, and it took at least an additional week or more for most of the cells to stop dividing and either differentiate or die than it took for the P7 cells.

In two of two cases where the cells were maintained without T3 for more than 30 days, apparently immortalized precursor cells took over the culture. These cells had the morphology and cell-cycle times of immature oligodendrocyte precursor cells, but, unlike normal precursors, they tended to adhere to one another. Like normal precursor cells (Raff et al., 1983), they differentiated into oligodendrocytes when cultured without PDGF and into type 2 astrocytes when cultured in 10% FCS (not shown). Unlike normal precursor cells, however, they did not senesce with multiple passages and did not differentiate into oligodendrocytes when T3 was added in the presence of PDGF for 10 days in culture (not shown).

DISCUSSION

We previously analyzed the roles of a cell intrinsic timer and thyroid hormone in controlling the proliferation and differentiation of oligodendrocyte precursor cells purified from P7–8 rat optic nerve. In the present study we have extended the analysis to embryonic precursor cells purified from E18 rat optic nerve. We show for the first time that oligodendrocyte differentiation occurs on the same schedule in clonal cultures of purified E18 precursor cells as it does in both cultures of unpurified embryonic optic nerve cells (Raff et al., 1985, 1988) and the developing optic nerve in vivo (Miller et al., 1985), suggesting that this timing is intrinsic to the cells themselves, as originally suggested (Raff et al., 1985). The way in which oligodendrocytes initially develop in the cultures of embryonic precursor cells, however, is different from the way they develop in cultures of P7–8 precursor cells.

Synchronous versus Nonsynchronous Oligodendrocyte Differentiation

Previous clonal analyses of single (Temple and Raff, 1986) or purified (Barres et al., 1994a) P7–8 oligodendrocyte precursor cells showed that cells within a clone tended to stop dividing and differentiate at around the same time—a behavior that we shall refer to as synchronous differentiation, even though all the cells within a clone do not stop dividing and differentiate at exactly the same time. By contrast, the first oligodendrocytes that develop in clonal cultures of purified E18 precursor cells do not generally show such synchronous differentiation, although rare clones do: in most cases the first oligodendrocytes appear in clones in which most of the cells are still precursor cells; moreover, 2–3 days after the first oligodendrocytes appear, most oligodendrocyte-containing clones still consist of mainly precursor cells. These findings indicate that the first oligodendrocytes tend to develop asynchronously within clones, and they raise the possibility that, whereas an intrinsic timer seems to ensure that oligodendrocyte differentiation does not begin before the time of birth, or the equivalent time in culture, some other factor(s) determines which cells begin to differentiate around this time. Interestingly, when two or more oligodendrocytes are seen in such a nonsynchronous clone, they tend to be clustered together, suggesting that either local environmental factors can influence the time of differentiation or siblings or close cousins within a clone tend to behave more similarly than less closely related cousins, perhaps because a stochastic event occurred in the cell that gave rise to the early differentiating, closely related cells.

Relatively few oligodendrocytes develop in the first 6 days in these cultures, however. The great majority develop from 9 days onward, and during this time most oligodendrocyte-containing clones consist mainly of oligodendrocytes, suggesting that differentiation at these times is more synchronous within clones. Thus most of the oligodendrocytes that develop in these cultures in the first 2 weeks are produced by the synchronous mode of differentiation.

Asynchronous oligodendrocyte differentiation within clones in culture has been reported previously—by Vaysse and Goldman (1990) in cultures of the neonatal rat striatum, by Lubetzki et al. (1992) in cultures of newborn rat brain, by Zhang and Miller (1995) in cultures of embryonic rat spinal cord, and by Ibarrola et al. (1996) in cultures of both embryonic rat cortex and purified P1 rat optic nerve precursor cells. It seems likely that all of these cases can be explained by the general rule that immature oligodendrocyte precursor cells tend to differentiate asynchronously within...
clones, while more mature oligodendrocyte precursor cells tend to differentiate more or less synchronously within clones. Ibarrola et al. (1996) discuss the possible implications of asynchronous oligodendrocyte differentiation at length. We shall postpone our discussion of the possible implications until we have discussed our other findings.

**Thyroid Hormone and the Development of the First Oligodendrocytes**

We find that the timely development of the first oligodendrocytes in clonal cultures of purified E18 precursor cells depends on thyroid hormone, in that the timing is delayed by 3–4 days if thyroid hormone is omitted from the culture medium. Even after 8 days, very few oligodendrocytes develop in these cultures in the absence of thyroid hormone. Similar results were obtained in cultures of unpurified E18 optic nerve cells (Ahlgren et al., 1997). These findings suggest that thyroid hormone plays an important part in timing the initial development of oligodendrocytes, as proposed previously (Barres et al., 1994a).

By contrast, Ibarrola et al. (1996) reported that the presence of thyroid hormone had little effect on the timing of the initial generation of oligodendrocytes in cultures of unpurified cells dissociated from embryonic rat cortex or in cultures of oligodendrocyte precursor cells purified from the neonatal rat optic nerve: although thyroid hormone greatly increased the number of oligodendrocytes that developed at the various time points they studied, it did not increase the probability of an individual clone generating at least one oligodendrocyte. It seems likely that these different results reflect differences in the cell preparations and culture conditions. When Ibarrola et al. added neurotrophin 3 (NT-3) and forskolin to their cultures, as we do, both of which they found tended to decrease oligodendrocyte development, their results were more similar to ours. It remains to be seen which conditions most closely resemble those in vivo. Anti-NT-3 antibodies decrease oligodendrocyte precursor cell proliferation in the developing rat optic nerve, suggesting that NT-3 normally helps drive the proliferation of these cells in vivo (Barres et al., 1994b). We add forskolin to our purified cell cultures because it greatly improves cell survival at clonal density, but it is not known if this activates or mimics a signaling pathway that is normally activated in oligodendrocyte lineage cells in vivo. It will be important to determine whether the development of the first oligodendrocytes is delayed in the optic nerve of thyroid-deficient pups born from thyroid-deficient mothers. Ibarrola et al. (1996) reported that the percentage of oligodendrocytes in the optic nerves of such thyroid-deficient rats is fourfold lower at P2 than that in euthyroid rats at the same age, and Ahlgren et al. (1997) found a threefold difference at P7 in such thyroid-deficient mice. Thus there is little doubt that thyroid hormone is a potent promoter of oligodendrocyte differentiation both in vivo and in vitro. Because thyroid hormone promotes the differentiation of many other cell types as well, it seems likely that it serves to coordinate the timing of differentiation in a number of tissues throughout the body, much as it coordinates the events of metamorphosis in amphibians (reviewed in Shi et al., 1996).

**Acquisition of Both Thyroid Hormone β Receptors and Thyroid Hormone Sensitivity with Maturation**

Two lines of evidence suggest that oligodendrocyte precursor cells acquire sensitivity to the proliferation-stopping and differentiation-promoting action of thyroid hormone with maturation: (1) whereas thyroid hormone acts to decrease DNA synthesis in P14 rat optic nerve precursor cells, it does not do so in P1 precursor cells (Barres et al., 1994a); (2) whereas some P7 precursor cells stop dividing and differentiate rapidly when cultured in the presence of mitogens and thyroid hormone (Temple and Raff; 1985; Barres et al., 1994a), embryonic precursor cells under the same conditions do not do so until the equivalent of the time of birth (Raff et al., 1985; Raff et al., 1988; Ahlgren et al., 1997; this study). Our finding that most E18 precursor cells stop dividing and differentiate within 1–3 days when thyroid hormone is added for the first time after 9 days in clonal cultures of purified cells suggests that responsiveness to thyroid hormone can be acquired in the absence of thyroid hormone and in the virtual absence of other cell types. These studies also suggest that thyroid hormone acts directly on the embryonic precursor cells to promote their differentiation, as shown previously for P7–8 precursor cells (Barres et al., 1994a).

Three lines of evidence suggest that this acquisition of thyroid hormone sensitivity reflects the acquisition of β thyroid hormone receptors (TRβs). First, Barres et al. (1994a) showed that P14 precursor cells are stained much more intensely than P1 precursors with antibodies against TRβ2. Second, we show here that, whereas P7 and E18 precursor cells stain with about the same intensity with antibodies against TRαs and P7 precursor cells are stained intensely with antibodies against TRβ1, E18 precursors are not stained above background with the latter antibodies. Third, when cultured for 10 days in the absence of thyroid hormone, E18 precursor cells acquire the same level of staining with anti-TRβ1 antibodies as P7 precursor cells, in parallel with their becoming sensitive to the differentiation-promoting action of thyroid hormone. Taken together, these findings suggest that oligodendrocyte precursor cells acquire both TRβ1 and TRβ2 as they mature and that β rather than α thyroid hormone receptors may be responsible for the differentiation-promoting activity of thyroid hormone on these cells. Our findings are consistent with previous observations that the TRα gene is expressed early in development and in many tissues, whereas the TRβ gene is expressed later in development and in a more restricted range of tissues, such as brain and pituitary (Forrest et al., 1990; Mielström et al., 1991; Bradley et al., 1992). It was reported previously in studies of brain glial cells in culture that oligodendrocytes express both TRα and TRβ genes, whereas oligodendrocyte precursor cells express only TRα genes (Baas...
et al., 1994a; Baas et al., 1994b). It is unclear why we find TRβ receptors on precursor cells while Baas et al. did not.

The acquisition of thyroid hormone sensitivity is only one aspect of embryonic precursor cell maturation. The cells also progressively slow their cell-division cycle and migration rate (Gao and Raff, 1997) and acquire a more complex morphology as they mature (Fulton et al., 1992; Gao and Raff, 1997). All of these changes can occur in clonal cultures of purified embryonic precursor cells (Gao and Raff, 1997; this study), suggesting that progressive maturation is an intrinsic property of these cells. It seems likely that many types of precursor cells have built-in maturation programs, which can be entrained by hormones such as thyroid hormone that help coordinate the timing of differentiation in various developing organs.

**TRβ and the Timer**

We previously provided evidence that the accumulation of the cyclin-dependent kinase inhibitor p27kip1 (p27) may be part of the timer that stops the cell cycle and initiates differentiation at the appropriate time (Durand et al., 1997). The finding that p27 levels increased faster at 33°C than at 37°C, in parallel with the speeding up of the timer at the lower temperature, supported this possibility (Gao et al., 1997). In the present study we find that, like p27, TRβ1 increases as perinatal precursor cells proliferate in culture in the absence of thyroid hormone and reaches a plateau at around the time most of the cells would have differentiated in the presence of thyroid hormone, and this increase occurs faster at 33°C than at 37°C. These findings are consistent with the possibility that the increase in TRβ1 (and possibly of TRβ2 as well) is also part of the intrinsic timer.

**Thyroid Hormone and the Effector Mechanism**

It was shown previously (Barres et al., 1994a; Bögl er and Noble, 1994) that the intrinsic timer consists of at least two components—a counting component that measures elapsed time (Gao et al., 1997) and an effector component that stops the cell cycle and initiates differentiation when time is reached. Whereas the counting mechanism operates independently of thyroid hormone, the effector mechanism can be triggered by thyroid hormone. To determine how rapidly thyroid hormone can act to stop the cell cycle and initiate differentiation, we cultured purified P7 precursor cells at clonal density in the absence of T3 for 11 days and then added thyroid hormone and followed cells within a clone by time-lapse video recording. Whereas the first cells stop migrating after 10 h and differentiate without dividing 5–10 h later, most divide once or twice before differentiating; by 3 days almost all of the cells being followed have differentiated. Remarkably, occasional cells within the same clone continue to divide for at least 2 weeks after T3 addition, although, by this time, they divide and migrate very slowly. Thus, even within a clone, the rate at which cells respond to thyroid hormone can vary greatly and is generally slower than we expected. It is unclear what distinguishes the cells that differentiate early from those that differentiate late. The few cells that fail to differentiate even 2 weeks after T3 is added may express a low level of β thyroid hormone receptors because even after the average level of TRβ1 has reached a plateau there are a few precursor cells with very low levels of these receptors (unpublished observations).

**Differentiation in the Absence of Thyroid Hormone**

It was shown previously that retinoic acid (RA) can substitute for thyroid hormone in promoting the differentiation of oligodendrocyte precursor cells in culture (Barres et al., 1994a). But neither thyroid hormone nor RA is required for differentiation: in the absence of mitogens, for example, precursor cells rapidly stop dividing and differentiate whether or not thyroid hormone or RA are present (Barres et al., 1994a; Ahlgren et al., 1997). Even in the presence of mitogens, some oligodendrocytes develop in culture in the absence of thyroid hormone or RA (Barres et al., 1994a; Ibarrola et al., 1996; Ahlgren et al., 1997; this study). In vivo as well, oligodendrocytes develop in the absence of thyroid hormone (Ibarrola et al., 1996; Ahlgren et al., 1997), although it is possible that RA or some other hydrophobic signal helps promote oligodendrocyte differentiation in such hypothyroid animals.

The ability of precursor cells to differentiate in the absence of thyroid hormone (or RA) increases with maturation, just as their tendency to differentiate in the presence of thyroid hormone does (Ibarrola et al., 1996; this study). When the thyroid hormone-independent differentiation of purified P7 precursor cells is compared after 11 and 20 days in culture, we find that the older cells have a greater tendency to differentiate, suggesting that the probability of differentiation continues to increase even after the time when thyroid hormone would have triggered the effector mechanism in the great majority of cells had the hormone been present. This finding raises the possibility that a second timer may operate in the precursor cells to stop their division after prolonged proliferation in the absence of thyroid hormone.

**Limits to Precursor Cell Proliferation**

The second timer could be the one responsible for timing the onset of cell senescence in many kinds of dividing cells in culture (Smith and Pereira-Smith, 1996). Our results are consistent with this possibility. When P7 precursor cells are cultured for prolonged periods in the absence of thyroid hormone and passaged when the cultures become crowded, the cell cycle progressively slows and eventually arrests, with the cells either dying or differentiating. Moreover, when E18 cells are analyzed in the same way, they undergo similar changes, but with a 1-week or so delay. This behavior is reminiscent of cell senescence in fibroblasts (Hayflick, 1965; Goldstein, 1990; Cristofalo and Pignolo, 1993). As in the case of rodent fibroblasts, rare cells seem to spontaneously...
FIG. 7. A tentative model for the timing of oligodendrocyte differentiation. Two cell-intrinsic timing mechanisms are proposed to operate in oligodendrocyte precursor cells. One is regulated by thyroid hormone and is part of the timer that normally limits precursor cell proliferation and times oligodendrocyte differentiation. It is responsible for the increasing probability with time that a precursor cell will withdraw from the cell cycle and differentiate in the presence of thyroid hormone. A second timing mechanism is responsible for the onset of cell senescence; it increases the probability with time that a precursor cell will withdraw from the cell cycle when it is stimulated to divide extensively in the absence of thyroid hormone.

Some dividing oligodendrocyte precursor cells are still present in adult optic nerve (ffrench-Constant and Raff, 1986; Wolswijk and Noble, 1989). Whether this population is maintained by the basal migration of precursor cells into the nerve from the brain or by slow self renewal in the nerve remains uncertain (Wren et al., 1992). It is also not known whether the same types of timers operate in these cells as operate in the precursor cells isolated from the developing optic nerve. Our finding that some cells continue to divide for weeks after thyroid hormone is added to clonal cultures raises the possibility that similar cells in vivo are a source of precursor cells in the adult nerve, as suggested by previous experiments (Wren et al., 1992).

A Model for Oligodendrocyte Development

The synchronous mode of differentiation that occurs within clones of P7-8 oligodendrocyte precursor cells in cultures containing thyroid hormone can readily be explained by the operation of a thyroid-hormone-regulated timer (Temple and Raff, 1986; Barres et al., 1994a). As pointed out by Ibarrola et al. (1996), however, such a timer cannot readily explain the asynchronous mode of differentiation that occurs within clones of embryonic precursor cells in cultures containing thyroid hormone or within clones of perinatal precursors in cultures that do not contain thyroid hormone. Ibarrola et al. (1996) suggested that the two types of differentiation may reflect the operation of two distinct timers, one operating independently of thyroid hormone and coming into play early and the other being regulated by various extracellular signals, including thyroid hormone and NT-3, and coming into play later.

We prefer a simpler model, in which a single thyroid-hormone-regulated timer controls normal oligodendrocyte development by progressively increasing the probability that a precursor cell will stop dividing and differentiate in the presence of mitogens. The timing component of the timer measures elapsed time and passes on its present value to each daughter cell at cell division. In this model (Fig. 7), thyroid hormone further increases the probability of cell-cycle arrest and differentiation, but only after a cell has begun to express a sufficient level of $\beta$ thyroid hormone receptors. Thus, for an immature precursor cell that is stimulated to proliferate by PDGF, the probability of differentiation is very low, even in the presence of thyroid hormone, because its timing component indicates “early.” As the cell matures, the probability of differentiation increases and, when thyroid hormone is present, becomes very high when the timing component reaches its end point. Because the timers should be more or less synchronized within a clone, differentiation within mature clones would be expected to occur by the synchronous mode in the presence of thyroid hormone and the asynchronous mode in the absence of thyroid hormone, as is observed. As discussed earlier, a second timer, which operates independently of thyroid hormone, may control the onset of cell senescence in oligodendrocyte...
precursor cells, as it does in most normal dividing cells, although it is unclear to what extent this mechanism comes into play in vivo.

Several lines of evidence suggest that the thyroid-hormone-dependent timer that operates in oligodendrocyte precursors primarily controls the probability of cell-cycle arrest, with differentiation following as a consequence, rather than vice versa. First, removal of mitogens rapidly leads to differentiation no matter how immature the precursor cell is (Noble and Murray, 1983; Temple and Raff, 1985). Second, if the normal timing of cell-cycle arrest is delayed, either by the absence of thyroid hormone (Barres et al., 1994a) or by the combination of PDGF and bFGF (Bögl and Noble, 1994), then differentiation is also delayed. Third, when cells eventually stop dividing when they are stimulated to proliferate extensively in culture when they are stimulated to proliferate, those that do not die tend to differentiate, even though high levels of mitogens are still present in the culture medium (this study).

It seems likely that the relevance of our findings is not confined to oligodendrocyte development and that the mechanisms that limit cell proliferation and initiate differentiation in the oligodendrocyte cell lineage also operate in many other mammalian cell lineages.

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