The basic helix-loop-helix transcription factor c-Myc is a potent trigger of programmed cell death when overexpressed during late oligodendrocyte development in transgenic mice. Here we provide evidence that c-Myc can act synergistically with the Pit, Oct, Unc homeodomain transcription factor Oct-6 to produce myelin disease pathogenesis in transgenic mice. More than 70% of c-myc/Oct-6 bitransgenic mice, obtained from crosses between phenotypically normal heterozygous mice of various My (c-Myc) and Oc (Oct-6) transgenic strains that express c-myc and oct-6 transgenes under transcriptional control of the myelin basic protein gene, developed severe neurological disturbances characterized by action tremors, recurrent seizures, and premature death. Affected bitransgenic mice exhibited multiple hypomyelinated lesions in the white matter that did not stain with myelin-specific antibodies against myelin basic protein, proteolipid protein, CNPase, and myelin-associated glycoprotein. The mice also exhibited a larger number of terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end-labeling positive cells in the white matter as well as ultrastructural evidence of glial cell death and astrogliosis. These observations indicate that the myelin lesions observed in the c-myc/oct-6 bitransgenic mice result from the untimely programmed cell death of oligodendroglia and that the c-myc and oct-6 transgenes act synergistically in producing the lesions.

Myelin surrounds the axons of many of the nerve cells in the mammalian nervous system and is essential for the conduction of nerve impulses (1, 2). In the central nervous system (CNS), a single oligodendrocyte can form myelin internodes on between 1 and 30 different axons depending on their location within the CNS (3). The structure of myelin is determined by a number of proteins that are expressed specifically within the myelin-forming cells. The myelin basic protein (MBP) gene, for example, is essential for the compaction of the cytoplasmic membrane surfaces of myelin within the CNS (4). Myelin internodes degenerate in portions of the white matter (disseminated plaques) of patients with the acquired myelin disorder multiple sclerosis. The clinical manifestations of disruptions of this type are complex in that they often simultaneously involve the optic nerves, the corticospinal tract, the cerebellar pathways, the brain stem, the ocullomotor pathways, the subcortical white matter, and the dorsal columns of the spinal cord. There is now much evidence to suggest that multiple sclerosis is an autoimmune disorder (5) and that the molecular pathogenesis that results in the destruction of myelin in this disease involves the FAS/APO-1/CD95 cell death pathway (6–10). The FAS cell death pathway involves the activation of FADD, which leads to the activation of a caspase protease cascade and ultimately to the death of the affected cells (11, 12). There is increasing evidence that the helix-loop-helix transcription factor c-Myc plays a role in sensitizing cells to FAS-mediated programmed cell death (PCD) and that c-Myc-mediated PCD can be prevented by caspase inhibitors (13–18).

Previously, we developed a transgenic mouse model to study the physiological consequences of overexpressing c-myc in oligodendroglia in the intact CNS (19, 20). In this transgenic model, expression of a human c-myc transgene was under transcriptional control of the MBP gene. Affected MBP/c-myc transgenic mice developed neurological disturbances, which coincided with an increased rate of untimely oligodendrocyte PCD. To identify genes that potentiate c-myc-mediated PCD of oligodendroglia, we have subsequently investigated the possibility that c-myc acts synergistically with the Pit, Oct, Unc (POU) homeodomain transcription factor Oct-6/SCIP/Tst-1 (21–23) in the pathogenesis of oligodendrocyte PCD. This POU transcription factor is transiently expressed in the promyelin cells of both oligodendrocyte and Schwann cell lineages (24, 25). Although Oct-6 is expressed transiently in promyelin oligodendrocytes, absence of the POU factor does not affect myelination in the rodent CNS (26, 27), presumably because oligodendroglia express Oct-6-related POU factors (28). We have previously shown that transgenic mice overexpressing Oct-6 under transcriptional control of the MBP gene develop a nonapoptotic structural myelin abnormality, indicating that developmental regulation of Oct-6 expression is involved in the normal assembly of myelin in the CNS (29). In this study, we show that the majority of c-myeloct-6 bitransgenic mice, obtained from crossing unaffected heterozygous mbpc-myc and mbploct-6 transgenic animals, develop severe neurological disturbances, which coincide with the presence of multiple hypomyelinated lesions in the white matter as well as with an increased number of apoptotic cells in the brain. These findings indicate that c-Myc
and Oct-6 transcription factors act synergistically in the intact CNS to trigger PCD in oligodendroglia.

**EXPERIMENTAL PROCEDURES**

**Transgenic Animals, DNA Blotting, RNA Blotting, and PCR Analy-

ses—**The generation and the characterization of the transgenic strains used in this study, My5, My23, Oc215, and Oc216, have been described in detail elsewhere (20, 29). Transgenic mice were identified by slot blots, Southern blots, and PCR of DNA samples taken from the tail. For PCR analysis, the following program was used in the thermocycler: one cycle at 96 °C for 5 min; 30 cycles each at 96 °C for 30 s, 55 °C for 30 s, and 74 °C for 3 min; and one cycle at 74 °C for 8 min. The following primers were used: Mbp promoter primer, sense: 5'-GGG CCC CGC GGC TAA CTG G-3'; Oct-6 primer, antisense: 5'-CCT CCG CCT ACC CAC CAG G-3'; and c-myc primer, antisense: 5'-CCT CCG CCT ACC CAA CAC G-3'. Primers for the mouse immunophilin gene muFKP78 (30) were used as positive controls for the genomic DNA template (sense: 5'-CGG ATG AAG ACA CTG GTC-3' and antisense: 5'-CAT GAG CGG GAC ACT GAG-3').

**For Northern blotting, total brain RNA was extracted by the isothiocyanate method (31), 9°-DNA template (sense: 5'-CGG AGG CGA CCA ACG ACG-3').**

**Tissue samples were postfixed in 1% osmium, dehydrated, and embedded in Epon. Ultrathin sections were mounted on mesh grids and stained with uranyl acetate and lead citrate.**

**Electron Microscopy—**The brains of transgenic and nontransgenic mice were perfused intracardially with 1% paraformaldehyde. The brains were removed, embedded in OCT compound (Tissue Tek), and frozen with CO2 gas. Sections (10 μm) were cut with a cryostat microtome, collected on glass slides, and fixed with methanol. The sections were incubated with anti-rabbit immunoglobulin G (diluted 1:100 in Hanks' buffer), CNPase (Roche Molecular Biochemicals) in terminal deoxynucleotidyltransferase buffer. After three to five washes in Hanks' buffer, the sections were incubated for 1 h at 37 °C in Vectastain elite ABC peroxidase staining solution (Vector Laboratories), rinsed twice in Hanks' buffer, and stained for 10–15 min at room temperature using the aminohexylcarbazole substrate kit for horseradish peroxidase (Vector Laboratories). The sections were counterstained with Mayer's hematoxylin and mounted with Aqua-Poly/Mount (Polysciences, Inc.).

**Immunohistochemistry—**For immunohistochemistry, the brains and optic nerves were removed from transgenic and control mice and immediately frozen in N2. Tissue sections (10 μm) were cut with a cryostat microtome, collected on glass slides, and fixed with methanol. The sections were incubated with anti-rabbit immunoglobulin G (diluted 1:100 in Hanks' buffer), CNPase (Roche Molecular Biochemicals) (diluted 1:100 in Hanks' buffer), and MIP (Biogenics) (diluted 1:100 in Hanks' buffer) antisera for 1 h at 37 °C in a moist chamber. Following several washes in Hanks' buffer, the sections were incubated with appropriate secondary rhodamine-conjugated antisera (Dako, Denmark) (diluted 1:50 in Hanks' buffer) for 30 min at 37 °C in a moist chamber. The sections were extensively washed in Hanks' buffer, mounted, and visualized with a Leica DMRB fluorescent microscope. Photographs were taken with a rhodamine filter.

**RESULTS**

**c-Myc and Oct-6 Transgenic Mice—**For a previous series of studies designed to investigate the pathological consequences of overexpression of either c-Myc or Oct-6 proteins during late oligodendrocyte development, we generated strains of mbp/c-myc transgenic mice, designated My (20), and strains of mbp/Oct-6 transgenic mice, designated Oc (29, in which c-myc and oct-6 transgenes, respectively, are expressed under transcriptional control of the mbp gene. Backcrossing transgenic mice from the c-transgenic strains, My5 and My23, and the Oct-6 strains, Oc215 and Oc216, with nontransgenic animals of the same genetic background resulted in offspring with no neurological disturbances (Table I). Only homozygous mice from strains My5 and Oc215, which comprised about 25% of the offspring from heterozygous crosses (Table I), developed neurological disturbances. These included the development of severe action tremors around postnatal day 10, recurrent seizures, and premature death during the third to fifth postnatal weeks. A lower incidence of neurological disturbances was observed in homozygous mice of the My23 strain, in that only about 13% of the mice derived from heterozygous matings were affected. There was no evidence of disease in either heterozygous or homozygous mice from the Oc216 strain.

**c-Myc/Oct-6 Bitransgenic Mice—**To investigate the possibility of a synergism between c-Myc and Oct-6 in the pathogenesis of myelin disease in the intact CNS, homozygous mice from various My and Oc strains were crossed to generate c-Myc/Oct-6 bitransgenic mice (Fig. 1B). In contrast to the effects observed when My and Oc transgenic mice are backcrossed with nontransgenic controls, most of the bitransgenic animals developed neurological disturbances similar to those observed in the homozygous My and Oc mice described above (Table II). Intermatings between My5 and Oc215 heterozygous mice produced offspring with distinct transgene genotypes (Table II). Approximately 80% of bitransgenic mice that contained both the mbp/c-myc and the mbp/oct-6 transgenes developed neurological disturbances, whereas single mbp/c-myc and mbp/oct-6 transgenic animals were unaffected. A similar high incidence of neurological disease among c-Myc/Oct-6 bitransgenic mice derived from crosses between unaffected homozygous My5 and Oc216 parents as well as from crosses between unaffected heterozygous My23 and Oc215 parent animals was also observed (Table II). Notably c-Myc/Oct-6 bitransgenic mice from My5 and Oc216 crosses developed disturbances that were comparable in severity to those observed in bitransgenics of My5 and Oc216.
Western blots indicated that the levels of CNPase in affected My/Oc animals was notably lower than those of affected My and Oc mice (Fig. 3). Ultrastructural analyses were carried out on selected regions of the central nervous system of c-Myc/Oct-6 bitransgenic mice. Fig. 4, A–C shows the ultrastructural features of lesions in the optic nerves of an affected c-Myc/Oct-6 bitransgenic mouse. As shown in the low power image in Fig. 4A, the axons in the lesion were unmyelinated and there was a pronounced astrogliosis. In addition, degenerating glial cells with pyknotic nuclear chromatin (Fig. 4B) and abnormal myelin profiles (Fig. 4C) were occasionally observed in the lesions. The affected c-Myc/Oct-6 bitransgenic mice also exhibited a pronounced decline in the levels of expression of both MBP and proteolipid protein mRNAs in the brain. These levels were comparable to those observed in age-matched affected homozygous My5 transgenic mice (Fig. 4D). Four to five times more TUNEL positive nuclei were observed in the brains of bitransgenic animals than in age-matched controls (Fig. 4E). Taken together, these results suggest that the myelin disorder observed in bitransgenic animals resulted from the untimely PCD of myelin-forming oligodendroglias.

**DISCUSSION**

The phenotype of the c-Myc/Oct-6 transgenic mice is consistent with the hypothesis that the helix-loop-helix transcription factor c-Myc and the POU homeodomain transcription factor Oct-6 act synergistically to promote inappropriate oligodendrocyte PCD in affected animals. Hallmarks of the disease phenotype of c-Myc/Oct-6 bitransgenic mice include the presence of multiple disseminated lesions of pronounced hypomyelination in the white matter, a general decline in steady-state levels of several major myelin proteins and their mRNAs, an increased number of TUNEL-positive cells, and ultrastructural evidence of apoptotic glial cell death in affected areas. The rationale behind the experiments described in this report was based on the previous finding that Oct-6 is expressed transiently in oligodendroglial cells (24) and that the POU factor has been shown to function synergistically in transcriptional regulation with the large T antigen of the human papovavirus JC (38), a gliotropic virus responsible for the severe myelin disorder referred to as progressive multifocal leukoencephalopathy (37, 38). POU factors are versatile transcriptional regulators that, through the POU domain or the N-terminal transactivation domain, establish associations with specific coactivators present in postmitotic and proliferative cells during development (39, 40). The POU transcription factor Oct-6 is expressed in numerous cell types during mammalian development (21–23, 41–45) and has been implicated in various developmental processes ranging from the regulation of cell division and differentiation to the specification and survival of particular neuronal subtypes (46–48). The finding of c-Myc and Oct-6 synergism in promotion of pathological oligodendrocyte PCD indicates that the Oct-6 transcription factor potentiates the lethal attribute of c-Myc in oligodendroglias in the intact CNS. To our knowledge, this represents the first evidence of a synergistic relationship between these transcription factors in a mammalian disease process.

Assuming that inappropriate expression of c-Myc interferes with cellular regulatory proteins, the induction of pathological PCD appears to be a multistage process similar to the development of neoplasm in transgenic mice, in which c-Myc functions synergistically with Bcl-2 and cyclin D1 proteins to promote lymphoid tumors (49–53). c-Myc is a short lived basic leucine zipper transcription factor that harbors an N-terminal transcriptional activation domain and a C-terminal DNA-binding dimerization domain (54). c-Myc functions as a dimer in the transcriptional regulation of target genes.
genes together with the ubiquitously expressed basic hexix-loop-helix-leucine zipper factor Max, and stimulation of both proliferation and apoptosis seems to depend on the transcriptional regulating properties of c-Myc (55). The cooperation between c-Myc and Bcl-2 in neoplastic transformation of hemopoietic cells, for example, relies on the ability of Bcl-2 to suppress c-Myc-induced PCD without compromising the proliferative capacity of these cells (56, 57). In addition, oncogene cooperation between c-Myc and ras transgenes has also been observed in the transformation of hemopoietic cells in transgenic mice (58–60). However, the mechanism by which Ras effects cell viability appears to be more complex in that it has recently been demonstrated that oncogenically activated Ras potentiates c-Myc-induced apoptosis, presumably through a mechanism that involves activation of the Raf kinase pathway (61, 62).

There is an increasing amount of evidence that c-Myc is not a primary trigger of apoptosis itself, but rather facilitates apoptosis induced by the FAS/CD95 pathway (16, 18, 63). Accordingly, it is appropriate to view the destruction of oligodendrocytes in the c-Myc/Oct-6 bitransgenic mice as a consequence of an increased vulnerability of affected cells to a preconfigured FAS/CD95 PCD pathway, which has previously been implicated as a cell death pathway in myelin disease pathogenesis (6, 7). This view is further supported by the observation that the myelin lesions observed here, in the c-Myc transgenics derived from My5 × Oc215 and My5 × Oc216 intermatings.

| Transgenic cross | Genotype of offspring | Onset of Action Tremor (postnatal days) |
|------------------|-----------------------|----------------------------------------|
|                  | c-myc | Oct-6 | Nontransgenic | Double Transgenic<sup>a</sup> |                              |
| My5 × Oc215      | 5/0   | 4/0   | 4/0           | 10/8 (80%)                  | 9–12                      |
| My5 × Oc216      | 6/0   | 7/0   | 5/0           | 14/10 (71%)                 | 9–12                      |
| My23 × Oc215     | 0/0   | 4/0   | 2/0           | 7/5 (71%)                   | 9–12                      |

<sup>a</sup> Number of mice/number with action tremor.

<sup>b</sup> The action tremors that occurred in double transgenic animals from the My23 × Oc215 cross appeared generally milder than those observed in the double transgensics derived from My5 × Oc215 and My5 × Oc216 intermatings.

Myelin Degenerative Disorder in c-Myc/Oct-6 Transgenic Mice

**FIG. 2. Immunohistochemical localization of neurofilament, CNP, myelin-associated glycoprotein, and MBP in the cerebellum of c-Myc/Oct-6 transgenic mice and controls.** Micrographs of sagittal sections through the cerebellum of a nontransgenic control (A, C, E, and G) and an affected c-Myc/Oct-6 (B, D, F, and H) transgenic mouse at P24. Note the similar distribution of neurofilament (NF) immunoreactivity in the control and the bitransgenic mouse and the blotchy appearance (arrows) of CNPase (D), myelin-associated glycoprotein (MAG) (F) and MBP (H) immunoreactivity in the white matter (w) of the c-Myc/Oct-6 (D, F, and H) transgenics.
the FAS/CD95 receptor (6, 9), it is possible that reactive microglia play a direct role in PCD pathogenesis in c-Myc/Oct-6 bitransgenic animals.

Assuming that c-Myc plays a normal physiological role in regulation of cell proliferation and apoptosis, it is not surprising that this proto-oncogene is frequently activated during oncogenesis by a variety of mechanisms that lead to deregulated expression (54, 65). Our finding that c-Myc induces programmed cell death in oligodendroglia, and not neoplasms in the intact CNS (20), is consistent with the finding that c-Myc activation rarely occurs in oligodendrocytic brain tumors (66). Oligodendroglial tumor cells exhibit the properties of progenitor cells in that they often express the early oligodendroglial differentiation marker Oct-6 (67). The lethal nature of the combined expression of c-Myc and Oct-6 suggests that the activation of c-Myc kills oligodendrocytic tumor cells that are expressing Oct-6. The apparent ability of Oct-6 to potentiate c-Myc cytotoxicity in oligodendroglia raises the possibility that PCD therapy involving the simultaneous overexpression of Oct-6 and c-Myc proteins has potential as a therapeutic means to ablate glial tumor cells in the CNS.

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Fig. 3. Two-dimensional Western blots of brain proteins. Left panels, two-dimensional Western blots obtained by nonequilibrium pH gradient electrophoresis (NEPHGE) of brain proteins of a nontransgenic control mouse (A) and affected c-myc (C), c-myc/Oct-6 (E), and Oct-6 (G) transgenic mice at postnatal day 20. The blots were reacted with a polyclonal anti-CNP antibody and developed (for 15 s) with the ECL procedure. Arrows indicate various CNP isoforms. Note the reduction in the amounts of all isoforms of CNPases in the bitransgenic animal (C). Right panels, Coomassie-stained two-dimensional (NEPHGE) gels of nontransgenic control (B) and affected c-Myc (D), c-Myc/Oct-6 (F), and Oct-6 (H) transgenic mice indicate the relative amount of brain proteins loaded/gel. Note that the relative amounts of proteins analyzed are the same for all four mice.

Fig. 4. Molecular pathological characterization of the disease phenotype. Electron micrographs of optic nerves (A, B, and C) from an affected bitransgenic mouse at P20 showing pronounced hypomyelination (A), a degenerating glial cell with pyknotic chromatin (B), and an axon ensheathed by abnormal "serpentine" myelin profiles (C). Scale bars, 2 μm. D, Northern blot analysis showing markedly reduced levels of expression of MBP and proteolipid protein (PLP) mRNAs in c-Myc/Oct-6 bitransgenic mice. Total brain RNA from affected c-Myc/Oct-6, Oct-6, and c-Myc transgenic mice and from nontransgenic controls was isolated at postnatal day 20 and used in successive hybridizations with MBP, proteolipid protein, and β-actin probes, as described. Hybridization with the β-actin probe indicates the relative amounts of total RNA (10 μg/lane). E, graphic representation of the number of TUNEL-labeled cells observed in three matched sagittal sections approximately 1 mm from the midline, from the brains of a control, and an affected c-Myc/Oct-6 double transgenic mouse at postnatal day 20.

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