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Invited Review

Id genes in nervous system development

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Summary. Id genes encode helix-loop-helix proteins that function to mediate processes important for normal development including cellular differentiation. proliferation and apoptosis. Id proteins act as negative regulators of other transcription factors, which are essential for cell determination and differentiation in diverse cell types, and interact with proteins important for cell cycle regulation. Studies of Id gene expression in the nervous system and in neural cells in culture indicate that Id proteins contribute to the regulation of mammalian nervous system development. Also, recognition of a wide variety of proteins with which Id transcription factors are capable of interacting suggests that it will be possible to understand more precisely their specific functions and importantly how these are integrated.

Key words: Id, Helix-Loop-Helix, Dominant-negative transcription factor, Central Nervous System

Introduction

The complexity of the central nervous system (CNS) presents major challenges for understanding how its functional components are formed and organized. Recent studies have been especially fruitful for the identification of molecules that mediate cell differentiation. One of the best examples is the identification of basic helix-loop-helix (bHLH) transcription factors. Basic HLH transcription factors play an important role in the regulation of cell determination and differentiation in many cell lineages (for review see Campos-Ortega, 1993; Jan and Jan, 1993; Weintraub, 1993; Lee, 1997). Basic HLH factors have been designated as Class I and Class II proteins based upon their being ubiquitously expressed in many different cell types (Class I) or being specifically expressed in cells of a single or a few lineages (Class II). Recently, a number of genes encoding bHLH factors have been cloned and shown to convert cells destined to non-neural fates to a neural fate (for review see Lee, 1997).

During the last decade, Id proteins, a group of helix-loop-helix (HLH) transcription factors that differ from bHLH proteins in lacking the basic amino acid domain necessary for DNA binding, have also been identified (Benezra et al., 1990; for review see Norton et al., 1998). A distinctive structural feature of Id proteins, the HLH region, allows them to inhibit the functional activity of bHLH proteins following heterodimerization with a bHLH partner. As Id proteins do not bind to DNA, they function primarily as dominant-negative regulators of bHLH proteins through the formation of nonfunctional Id-bHLH heterodimers. Data identifying a functional activity of the N-terminal region of Id proteins suggests that Id proteins may also function through mechanisms other than the direct interaction with bHLH proteins (Florio et al., 1998). Id genes seem to have a role in cellular events important for normal development and are expressed during nervous system development. Understanding the biological activity and function of Id genes in the nervous system may provide important insights into the processes by which the nervous system develops.

Brief history of discovery of Id genes

In 1990, work in the laboratory of Harold Weintraub identified a novel murine gene which encoded a protein that later would be the mammalian prototype of a subfamily of HLH transcription factors (Benezra et al., 1990). This subfamily is characterized by the lack of basic amino acids located N-terminal to the HLH motif which are found in other members of the HLH transcription factor class (see below). Both the gene and protein were named Id for its functional activity as an inhibitor of DNA binding and an inhibitor of differentiation. Id was found to be structurally similar to the product of *extramacrochaetae*, a *Drosophilu melanogaster* gene that mediates sensory organ

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patterning by antagonizing the neurogenic activity of proneural genes. Later, when other Id genes were identified, this prototype of the Id family was renamed Id1. Four members of the Id gene family have been identified in mammals to date: Id1 (Benezra et al., 1990; Hara et al., 1994; Deed et al., 1994a; Springhorn et al., 1994; Zhu et al., 1995), Id2 (Sun et al., 1991; Biggs et al., 1992), Id3, originally termed HLH462 (Christy et al., 1991) in the mouse and heir-1 (Ellmeier et al., 1992) or HLH 1R21 (Deed et al., 1993) in humans, and Id4 (Riechmann et al., 1994; Pagliuca et al., 1995). In addition to the known mammalian Id genes, homologues in *Xenopus laevis* (Wilson and Mohun, 1995; Zhang et al., 1995), zebrafish (Sawai and Campos-Ortega, 1997) and trout (Rescan, 1997) have also been cloned.

Structure of Id genes and proteins

Id proteins have a HLH dimerization domain that consists of two conserved amphipathic α helices separated by a loop of variable length and sequence (Fig. 1). Basic HLH transcription factors typically contain a domain of basic amino acids N-terminal to the HLH motif (Fig. 2). Following dimerization mediated by their

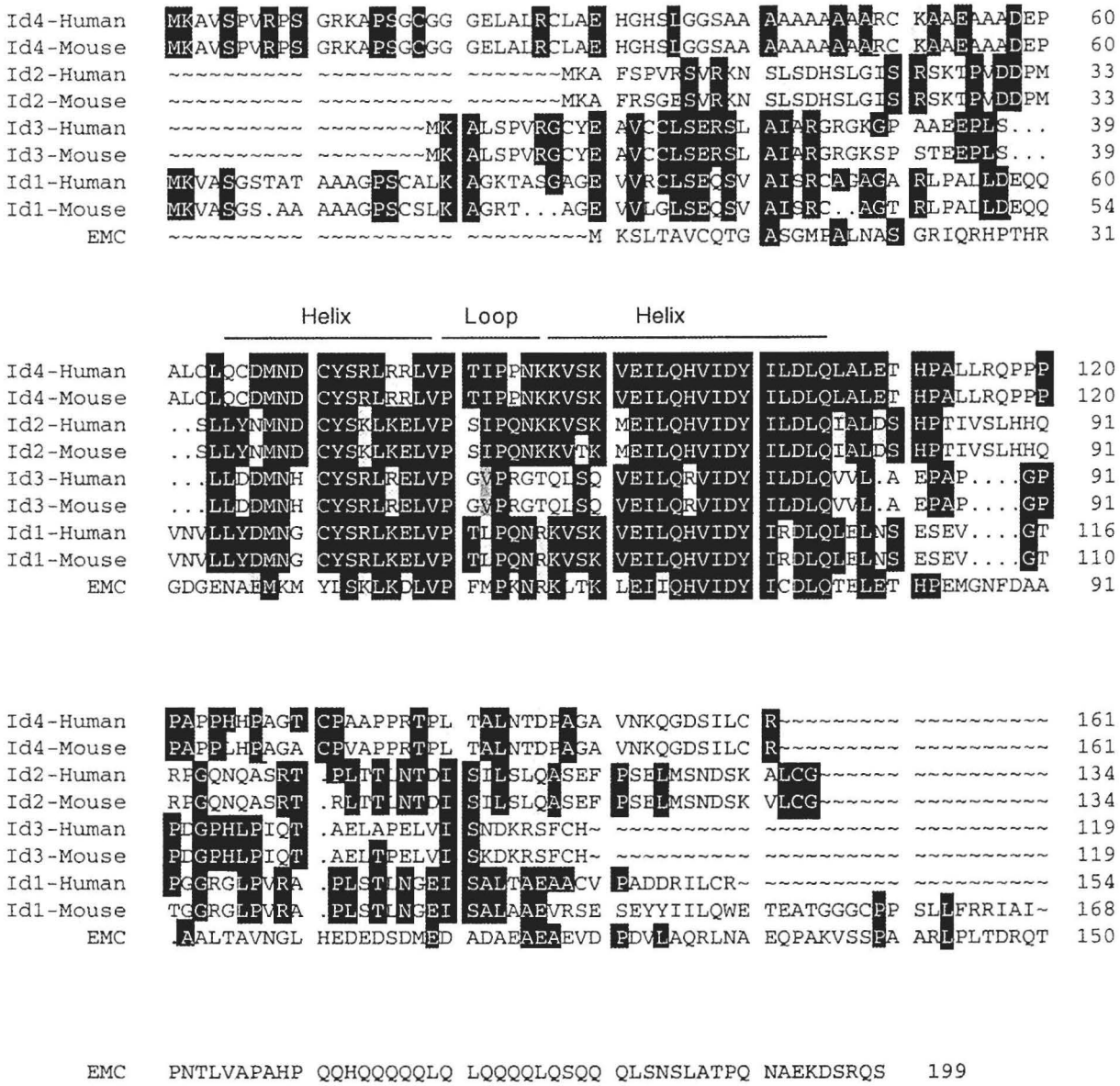

Fig. 1. Amino acid sequence alignment of Id proteins. The predicted amino acid sequences of mouse Id1 (Benezra et al., 1990), human Id1 (Hara et al., 1994), mouse Id2 (Sun et al., 1991), human Id2 (Biggs et al., 1992), mouse Id3 (Christy et al., 1991), human Id3 (Ellmeier et al., 1992), mouse Id4 (Riechmann et al., 1994), human Id4 (Pagliuca et al., 1995), and *Drosophila* EMC (Ellis et al., 1990; Garrell and Modolell, 1990) proteins are shown. The amino acid sequences corresponding to the helix-loop-helix domain are indicated. Numbers on the right refer to the last amino acid respectively for each protein. Amino acids are shown in single-letter code.

HLH regions, the basic domains of such transcription factors bind to specific DNA sequences, E-boxes, found in the regulatory regions of their target genes (Murre et al., 1989). However, Id proteins lack the basic amino acid domain necessary for DNA binding and following heterodimerization with a bHLH partner inhibit binding to DNA and transcriptional activation by bHLH factors (Benezra et al., 1990; Sun et al., 1991) (see Fig. 3).

The four mammalian Id proteins are encoded by individual genes. The human *Id1*, *Id2*, *Id3*, and *Id4* genes are located on chromosome 20q11 (Mathew et al., 1995; Nehlin et al., 1997), 2p25 (Mathew et al., 1995), 1p36 (Ellmeier et al., 1992; Deed et al., 1994b), and 6p21.3-22 (Pagliuca et al., 1995) respectively; and the murine *Id1*, *Id2*, *Id3*, and *Id4* genes are located on chromosome 2 (Sun et al., 1991), 12 (Sun et al., 1991; Mantani et al., 1998), 4 (Christy et al., 1991), and 13 (Mantani et al., 1998; van Crüchten et al., 1998) respectively. Despite their unlinked chromosomal localizations, these genes share a similar genomic organization that suggests they are likely to have been derived from a common ancestral *Id* gene (Sun et al., 1991; Deed et al., 1994b; Mantani et al., 1998; van Crüchten et al., 1998). The common ancestor of Id proteins may have possessed a DNA binding region that was subsequently lost during evolution (Atchley and Fitch, 1997). Interestingly, alternative open reading frames have been identified for the *Id1* (Hara et al., 1994; Springhorn et al., 1994; Zhu et al., 1995; Hernandez et al., 1996; Nehlin et al., 1997) and *Id3* (Deed et al., 1996) genes, and a cDNA from an *Id2* pseudogene which could encode a peptide corresponding to the first 36 amino acids of Id2 has been identified (Kurabayashi et al., 1993). The HLH domain of Id proteins, which is located in the middle region of the molecule, is highly conserved and essential for dimerization with other proteins (Pesce and Benezra, 1993). The functions of the distinctive N-terminal and C-terminal regions of the different Id proteins are largely unknown. The C-terminal portion of Id3 is important for the stabilization of this protein (Chen et al., 1997), although little is known of the mechanisms which regulate Id protein levels in any cell type. Importantly, a role for the N-terminal region of Id2 in mediating the apoptotic response of myeloid progenitor cells to growth factor withdrawal has recently been identified (Florio et al., 1998), suggesting that multiple different domains of these proteins will be of functional significance.

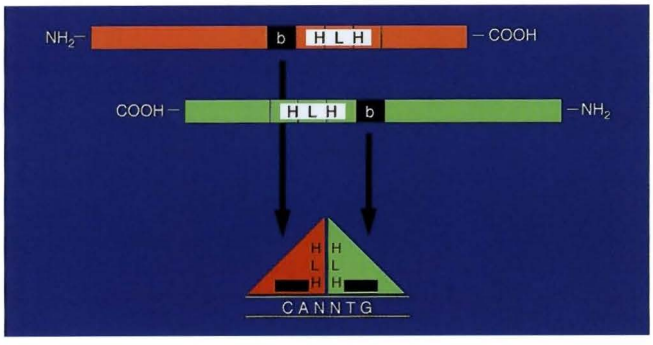

Fig. 2. Basic helix-loop-helix (bHLH) transcription factors bind to specific DNA sequences in target genes. Schematic representation of a Class I (green) and a Class II (orange) bHLH proteins. Following heterodimerization mediated by their helix-loop-helix (HLH) regions, the basic domains (b) bind to consensus E-box regulatory sequences (CANNTG) in their target genes. N: any nucleotide.

Known function of Id genes

The best characterized function of Id encoded proteins is to inhibit lineage specific gene expression. Most of the data supporting this view come from studies in cultured cells in which the introduction of Id expression vectors modulates lineage specific gene expression and inhibits differentiation. Id protein heterodimerization with bHLH transcription factors and the consequent inability of these factors to bind DNA

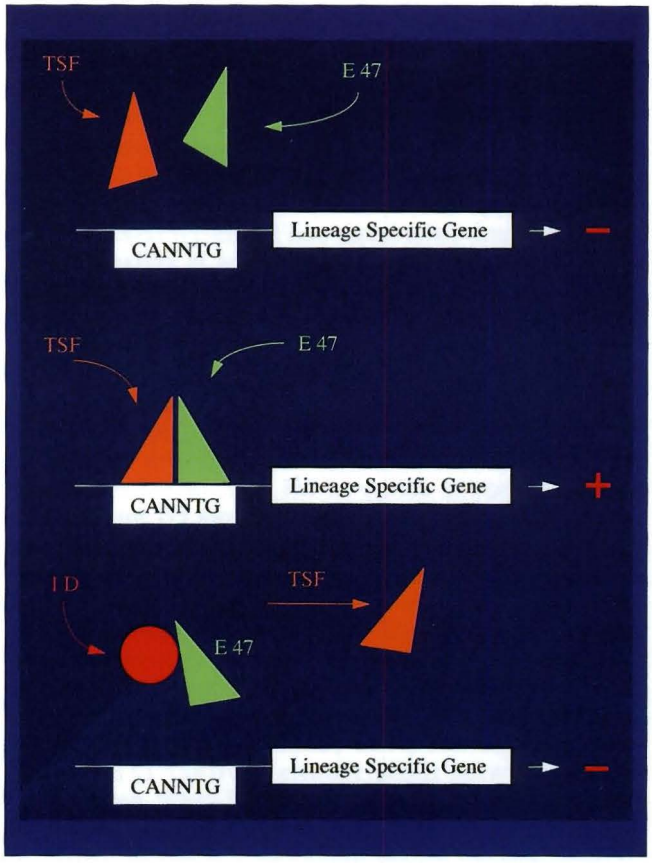

Fig. 3. Id proteins antagonize bHLH proteins and regulate transcription. Schematic representation of the general mechanism of action of Id proteins. In this example, a Class II tissue specific bHLH factor (TSF) heterodimerizes with E47 (a Class I ubiquitously expressed bHLH factor) and activates transcription of a lineage specific gene. Id protein (ID) competes with TSF for E47 dimerization. Because Id proteins lack the basic amino acid domain necessary for DNA binding, they inhibit binding to DNA and transcriptional activation by bHLH factors.

and regulate transcription of lineage specific target genes seem to be the mechanism responsible for the inhibition of cell differentiation attributed to Id proteins. Id proteins inhibit differentiation in a variety of cell lineages including muscle cells (Jen et al., 1992; Gundersen and Merlie, 1994; Atherton et al., 1996; Melnikova and Christy, 1996), myeloid cells (Kreider et al., 1992), lymphoid cells (Wilson et al., 1991; Sun, 1994; Heemskerk et al., 1997; Blom et al., 1999), erythroid cells (Shoji et al., 1994; Lister et al., 1995), mammary epithelial cells (Desprez et al., 1995), and adipose cells (Moldes et al., 1997).

The first *in vivo* evidence for Id proteins as negative regulators of differentiation came from genetic studies in *Drosophila melanogaster*. In *Drosophila*, the *extramacrochaetae* gene product (a protein structurally similar to Id) acts as a negative regulator of bHLH proteins which are encoded by the *achaete-scute* complex genes and are important in neural fate determination (Ellis et al., 1990; Garrell and Modolell, 1990). In transgenic mice, the constitutive overexpression of *Id1* or *Id2* genes in the lymphoid lineage impairs the development of B cells (Sun, 1994). The constitutive overexpression of Id1 in differentiated muscle cells leads to muscle atrophy (Gundersen and Merlie, 1994). Recently, it has been reported that the overexpression of Id2 in the chick embryo surface ectoderm not only impairs the epidermal development of the ectoderm overlying the neural tube but influences ectodermal precursors toward neural crest and neurogenic fates (Martinsen and Bronner-Fraser, 1998).

A second function of Id proteins is enhancing cell cycle progression. The hypothesis that Id proteins could have a role in cell proliferation and cell cycle regulation was proposed initially for Id3 (Christy et al., 1991). Id3 gene expression is induced in mouse 3T3 cells during the immediate early transcriptional response to growth factors. Subsequently, the induction of expression of other *Id* genes by serum and isolated growth factors was described, and the ability of Id1, Id2, or Id3 expression to enhance the progression of cells from G1 into S phase was characterized. Antisense oligonucleotides against Id1, Id2, and Id3 mRNAs delayed the reentry of growth-arrested cells into the cell cycle elicited by stimulation with serum or growth factors (Barone et al., 1994). Moreover, Id2, but not Id1 or Id3, can reverse the inhibition of cellular proliferation mediated by pRb (Iavarone et al., 1994). Id2 can also bind the pRb related proteins p107 and p130 (Lasorella et al., 1996). In agreement with these results, neither Id1 nor Id3 expression affects the arrest of cell cycle progression mediated by pRb. The growth-suppressive activities of cyclin-dependent kinase inhibitors p16 and p21 are also efficiently antagonized by high levels of Id2 but not by Id1 or Id3 (Lasorella et al., 1996). These data provide evidence for Id2-mediated effects on regulatory events important for cell cycle progression, and they identify distinctive roles for different *Id* gene products. Consistent with this interpretation, Id1 overexpression in NIH 3T3 cells can inhibit p21 expression and accelerate cell growth (Prabhu et al., 1997). Recently, it has been reported that Id proteins bind to the ETS DNA-binding domain and disrupt the formation of DNA-bound complexes between ternary complex factors (TCFs) and the serum response factor (SRF) on the c-fos serum response element (SRE). Id proteins cause down-regulation of the transcriptional activity mediated by the TCFs and thereby block MAPK signaling to SREs (Yates et al., 1999). These findings show a novel facet of Id function in the coordination of mitogenic signaling and cell cycle entry.

A role for Id proteins in mediating apoptosis has also been identified. Transfection of expression vectors carrying the Id1, Id2, or Id3 coding regions into primary rat embryo fibroblasts under serum free conditions enhances the transition of cells into S phase, and this same population of cells contain cells which display apoptotic nuclei (Norton and Atherton, 1998). The conditional expression of Id3 in stable transfectants of this same cell type also induces apoptosis in serum-deprived cells. In both cases, apoptosis was suppressed in the presence of serum. In 32D.3 murine myeloid progenitor cells, the overexpression of Id2 and Id1, but not of Id3, enhances apoptosis following withdrawal of the survival factor IL-3. This activity of Id2 resides in its N-terminal region and is associated with the enhanced expression of the proapoptotic gene BAX (Florio et al., 1998). In cardiac myocytes, the overexpression of Id1 leads to the induction of apoptosis through a redox-dependent mechanism (Tanaka et al., 1998). Id proteins also mediate apoptosis in cells from the nervous system. The overexpression of Id2 induces apoptosis in neuronal progenitor cells (El-Bizri and Miller, 1998) and the overexpression of Id4 induces apoptosis in U373 MG, an astrocytoma-derived cell line (Andres-Barquin et al., 1999).

Finally, data currently available from in vivo *Id* gene-targeting studies show that Id1 and Id3 single knockout mice are healthy, fertile, and phenotypically normal (Yan et al., 1997). However, Id1/Id3 double-knockout mutants are not viable indicating that at least in mammals, Id functions are indispensable for survival and their loss cannot be compensated for by other classes of dominant-negative bHLH antagonists (Yan et al., 1997). Interestingly, in crosses of mutant mouse lines, disruption of the *Id1* gene partially rescues the neonatal lethality occurring in E2A-knockout mice. This finding provides additional evidence that the Id1 protein is indeed involved in E2A-mediated regulatory pathways at certain stages of development (Yan et al., 1997). In contrast to Id1 and Id3 single knockout mice, Id2 null mutant mice do not develop normally. Id2 knockout mice lack lymph nodes and Peyer's patches, and show a greatly reduced population of natural killer cells, indicating that Id2 has an essential role in the development of peripheral lymphoid organs and natural killer cells (Yokota et al., 1999).

Studies in neural cells in culture

A variety of cell culture systems has been used to study the regulation of *Id* gene expression during neuronal differentiation. Rat pheochromocytoma PC12 cells, a well-characterized cell culture model of neuronal differentiation, undergo differentiation to a sympathetic neuronal phenotype in response to nerve growth factor (NGF) treatment. Upon induction of PC12 cell differentiation, Id1, Id2, and Id3 mRNA steady-state levels, all detectable in the undifferentiated cells, initially increase several fold and subsequently decrease (Nagata and Todokoro, 1994; Einarson and Chao, 1995). Gel mobility shift assays using PC12 cell nuclear extracts demonstrate that active bHLH complexes exist throughout differentiation, and addition of exogenous Id1 protein disrupts complexes formed by these cell nuclear extracts on an E-box consensus oligonucleotide (Einarson and Chao, 1995). The NGF-induced decrease of Id1, Id2, and Id3 mRNA steady-state levels in PC12 cells was blocked in cells treated with the DNA methyltransferase inhibitor 5-azacytidine, and this effect was concomitant with the inhibition of NGF-mediated neuronal differentiation induced by 5-azacytidine (Persengiev and Kilpatrick, 1997). These findings indicate that Id proteins are a downstream target of the NGF transduction pathway and their inhibition is closely associated with neuronal differentiation. Furthermore, they suggest a role for DNA methylation in Id expression during NGF-induced neuronal differentiation (Persengiev and Kilpatrick, 1997). Phosphorylation studies have shown that the Id1 protein is phosphorylated in NGF-induced PC12 cell lysates but not in the unstimulated cell lysates (Nagata et al., 1995). This suggests that Id protein phosphorylation may modulate Id gene activity during neuronal differentiation.

During the retinoic acid-induced neuronal differentiation of SMS-KCNR neuroblastoma cells the level of Id2 mRNA decreased (Biggs et al., 1992). Down-regulation of Id2 expression also occurs during neuronal differentiation of neuroblastoma-glioma hybrid NG108 cells, whereas no change and an increase in the levels of Id2 mRNA were observed during neuronal differentiation of neuroblastoma N18 cells and teratocarcinoma PCC7 cells respectively (Neuman et al., 1993). These divergent findings in different cell lines, and the pattern of *Id2* gene expression during neurogenesis (see below), suggest that Id2 is not a general inhibitor of differentiation in these neuronal cell types and that Id proteins have different functions in different cell types during neurogenesis (Neuman et al., 1993). In primary cultures of neuronal progenitor cells, Id2 overexpression induced apoptosis (El-Bizri and Miller, 1998).

Recent studies have addressed the question whether *Id* genes are expressed in astrocytes and have a functional role in this major cellular component of the CNS. Studying NSE Hip2-28 murine astrocyte progenitors, it has been found that the four known members of the *Id* gene family are expressed and regulated during astrocyte differentiation (Andres-Barquin et al., 1997). Upon induction of NSE Hip2-28 differentiation, at a time when glial fibrillary acidic protein (GFAP) expression becomes detectable, the expression of all four Id genes initially increases, and subsequently decreases. A similar pattern of expression has been observed in primary cultures of mouse forebrain astrocytes following the induction of differentiation by activation of the cAMP-dependent signal transduction pathway in serum-deprived cultures (Andres-Barquin et al., 1999). In the presence of serum, cAMP induced a potent and selective inhibition of Id4 but not Id1, Id2 and Id3 expression which was accompanied by astrocyte differentiation, suggesting that the cAMP pathway acts as an inhibitor of Id4 gene expression in astrocytes and that Id4 is strategically positioned in the chain of molecular events regulating astrocyte differentiation. These observations contrast sharply with the long-standing view that high levels of Id mRNA in proliferative and undifferentiated cells decrease as they are induced to differentiate (Benezra et al., 1990; Sun et al., 1991; Kreider et al., 1992; Kawaguchi et al., 1992), although high levels of Id genes have been found in hematopoietic cells following the induction of differentiation as well (Ishiguro et al., 1996). This suggests that Id molecules may also inhibit the expression of inhibitors of differentiation or promotors of cell growth in some lineages. Interestingly, overexpression of Id4 in an astrocyte-derived cell line can induce death by apoptosis (Andres-Barquin et al., 1999).

Id gene expression has been examined also in primary cultures of mouse forebrain astrocytes under experimental conditions in which astrogliosis was elicited by mechanical injury (Andres-Barquin et al., 1998). After injury, at a time when astrocytes develop the characteristic phenotypic changes of astrogliosis, Id4 expression decreased dramatically, while Id1, Id2, and Id3 mRNA levels did not change significantly. This suggests that Id4 expression plays a role in astrogliosis. Conditioned medium from injured astrocytes induced a decrease of Id4 mRNA expression in astrocyte cultures. Also, blockade of gap junction-mediated intercellular communication between astrocytes before and after injury did not prevent the decrease in the levels of Id4 mRNA observed after injury. These findings suggest that soluble factors released in response to injury may trigger Id4 downregulation.

Primary astrocytes obtained from different areas of mouse brain seem to express variable levels of Id mRNA (Andres-Barquin et al., 1998). Primary cultures of astrocytes prepared from olfactory bulbs, forebrain, cerebellum, and quadrigeminal bodies all express Id1, Id2, Id3 and Id4 mRNAs. Id1, Id2 and Id3 genes are expressed in astrocyte cultures from olfactory bulbs and forebrain at higher levels than in astrocyte cultures from cerebellum and quadrigeminal bodies, whereas Id4 is more abundantly expressed in astrocyte cultures prepared from forebrain, cerebellum and quadrigeminal

bodies (Andres-Barquin et al., 1998). Id1, Id2 and Id3 expression has also been examined in rat secondary astrocytes where serum stimulation induced Id3 expression and addition of Id3 antisense oligonucleotides blocked DNA synthesis in serum stimulated cells (Tzeng and de Vellis, 1997). Figure 4 shows Id expression in primary cultures of mouse forebrain astrocytes.

All four Id proteins are expressed in the Schwann cell lineage in culture (Stewart et al., 1997). In the precursor cells, Id2 and Id4 were located in the nucleus whereas Id1 and Id3 were predominantly cytoplasmic. In Schwann cells, Id1, Id2 and Id3 localized to the cytoplasm whereas Id4 was present in the nucleus. After dibutyryl cAMP treatment, a treatment which induces myelin formation and differentiation in Schwann cells, Id2 mRNA was up-regulated. Also, Id1 and Id2 mRNAs were up-regulated when Schwann cells progressed through the cell cycle following mitogen stimulation.

Finally, the expression levels of Id1, Id2, Id3, and Id4 mRNA have been examined in a number of cell lines derived from nervous system tumors of neuronal and astrocytic origin (Biggs et al., 1992; Ellmeier et al., 1992; Zhu et al., 1995; Andres-Barquin et al., 1997; for review see Israel et al., 1999). In these cell lines one or more Id genes was found to be expressed. Interestingly, the nervous system cell lines in which Id4 is highly expressed are also characterized by high levels of Id2 expression, and cell lines in which the level of Id4 expression is low also have relatively low levels of Id2 expression (Andres-Barquin et al., 1997). The similar expression pattern of Id2 and Id4 in nervous system cell lines suggest that these different Id genes may have functional similarities in some neural cells.

bHLH proteins are involved in neurogenesis

Basic HLH transcription factors are known today to play an important role in the regulation of cell determination and differentiation in numerous cell lineages and recently, important advances in the understanding of the regulation of vertebrate neurogenesis by bHLH proteins have been made (for review see Guillemot, 1995; Kageyama et al., 1995; Lee, 1997).

Studies in *Drosophila* indicate that HLH transcription factors play an essential role in neural development. The HLH factors encoded by the proneural genes, *achaete-scute* complex (AS-C), *atonal*, and *daughterless* are positive regulators of neurogenesis, while those encoded by *hairy*, *Enhancer of split* [*E(spl)*], and *extramacrochaetae* (*emc*) are negative regulators. These HLH factors regulate each other at the transcriptional level following protein-protein interaction, and the balance between the positive and negative regulators is important for neural development (for review see Kageyama et al., 1995). A number of vertebrate neural bHLH genes have been cloned as orthologs of the *Drosophila* proneural genes (for review see Lee, 1997). The nervous-system-specific genes cloned in this manner include, among others, MASH (mammalian *achaete-scute* homolog), XASH (*Xenopus achaete-scute* homolog), MATH/NEX, and NeuroM (homologs of *atonal*), HES (mammalian *hairy* and *E(spl)* homolog), neuroD2, neurogenin (ngn)-1/neuroD3, ngn2 (same as MATH-4A), ngn3, neuroD4 (same as MATH-3), and neuroD5. Other genes, such as neurological stem cell leukemia NSCL-1 and NSCL-2, have been cloned first in mammals. Recently, using the yeast two-hybrid system which detects proteins by their interactions, investigators cloned NeuroD/Beta2, and MATH-4A (ngn2) as genes encoding proteins that interact with Daughterless/E12/E47 and MASH-1, respectively. Three gene products, NeuroD, Ngn1/NeuroD3 and NeuroD2, have been shown to mediate the development of ectopic neurons in *Xenopus* injection assays (Lee et al., 1995; Ma et al., 1996; McCormick et al., 1996).

Pattern of Id gene expression in the nervous system

Early studies of Id gene expression using Northern blot analysis in a variety of mouse or human tissues revealed the presence of Id1, Id2, Id3, and Id4 transcripts in CNS tissues (Benezra et al., 1990; Christy et al., 1991; Biggs et al., 1992; Riechmann et al., 1994). Subsequent investigations using *in situ* RNA hybridization provided a systematic analysis of *Id* gene expression during mouse development. These investigations showed that the expression of all four known *Id* gene family members is independently regulated during nervous system development (Duncan et al., 1992; Wang et al., 1992; Evans and O'Brien, 1993; Neuman et al., 1993; Ellmeier and Weith, 1995; Riechmann and Sablitzky, 1995; Zhu et al., 1995; Jen et al., 1996, 1997). Outside the nervous system, Id1, Id2, and Id3 are expressed in multiple tissues.

Id1

Id1 is expressed in undifferentiated mouse neural precursors of the ventricular zone, the proliferative neuroepithelial layer of the CNS, which gives rise to diverse neuronal and glial populations. It is not expressed in their differentiated derivatives (Duncan et al., 1992; Evans and O'Brien, 1993; Zhu et al., 1995). At embryonic day 8.5 (E8.5) during mouse development, Id1 mRNA can be detected in the neural folds prior to neural tube closure (Wang et al., 1992; Jen et al., 1997). At E12.5, Id1 is expressed in the forebrain, where the choroid plexus and infundibular floor region show more intense labeling than the ventricular zone of the ganglionic eminences, septum and eminentia thalami (Zhu et al., 1995). Id1 expression is detectable in both the dorsal and ventral ventricular zones of the midbrain, hindbrain, and spinal cord (Duncan et al., 1992). Id1 expression is also detectable in the dorsal root ganglia (Duncan et al., 1992) and the trigeminal ganglion (Zhu et al., 1995). These are two neural crest derivatives

where all Ids are expressed (Riechmann and Sablitzky, 1995; Zhu et al., 1995; Jen et al., 1997).

Later, as cells of the ventricular zone cease dividing and undergo migration and differentiation, Id1 mRNA expression diminishes (Duncan et al., 1992). At E14.5, expression is detectable along the medial telencephalic wall (Duncan et al., 1992), the choroid plexus, and its attachment taeniae (Zhu et al., 1995). By E16.5, Id1 expression is localized to the hippocampus (Duncan et al., 1992), the choroid plexus, and some periventricular

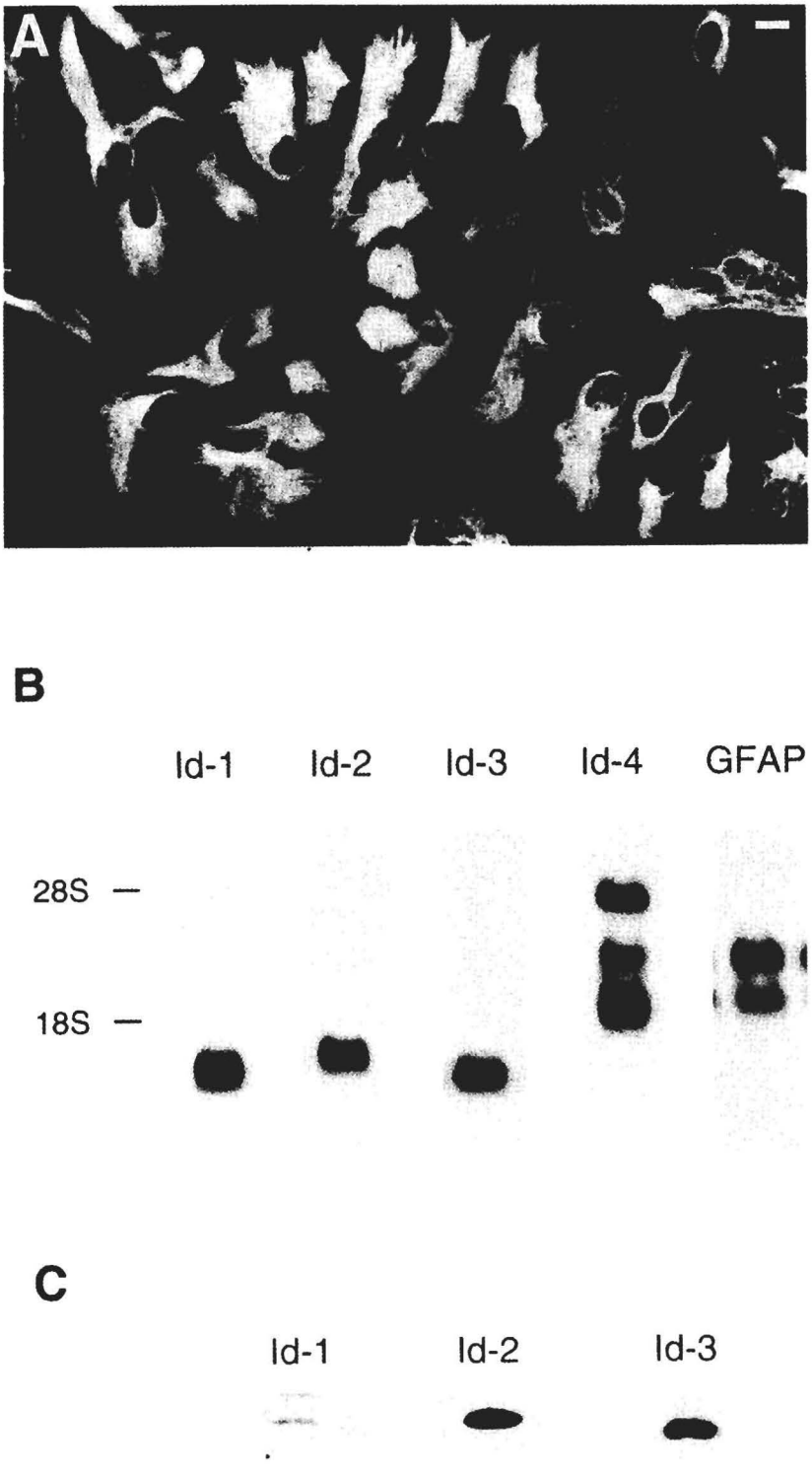

Fig. 4. Id expression in primary cultures of mouse forebrain astrocytes. **A.** Fluorescence photomicrograph of astrocytes immunostained at day 7 of culture for GFAP. GFAP was detected by indirect immunofluorescence with an anti-GFAP antibody followed by a rhodamine-conjugated secondary antibody. Scale bar: 10 μm. **B.** Id1, Id2, Id3, Id4, and GFAP mRNAs were detected by Northern blot analysis. Total RNA was isolated from cells at day 14 of culture. Id4 film was exposed approximately twice as long as Id1, Id2 and Id3 films. Numbers on the left indicate the positions of the 28S and 18S ribosomal RNAs. **C.** Immunoblotting analysis of Id1, Id2, and Id3 proteins. Id1 and Id3 proteins were detected with anti-Id1 and anti-Id3 polyclonal antibodies (M.-C. Hernandez, unpublished) respectively, and Id2 with a commercial anti-Id2 polyclonal antibody.

areas (Zhu et al., 1995). Prior to birth, Id1 expression decreases greatly except in the meninges (Wang et al., 1992). Interestingly, Id1 has been found to be transiently expressed in cells of the upper internal granule layer of the postnatally developing cerebellum that have recently completed their migration from the external granule layer and are starting to differentiate (Duncan et al., 1997). Surprisingly, Id1 is not seen in the external granule layer, which contains undifferentiated, proliferative cells. These observations suggest that Id1 may have region-specific roles in neuronal differentiation. At postnatal days 1 and 5 (P1 and P5) during rat development, Id1 is expressed in the CA1-4 layers of the hippocampus, the corpus callosum, and the ventricular/subventricular zone (Tzeng and de Vellis, 1998). These are areas of the rat brain in which *Id2* and *Id3* genes are also expressed. The choroid plexus at P1, and the internal granule cell layer of the cerebellum at P5, also express Id1 mRNA. These are regions in which Id2 and Id3 mRNA are also detectable.

Id2

At early stages of mouse nervous system development, Id2, like Id1, is expressed in the ventricular zone. However, as development progresses, Id2 expression decreases in neuroepithelial cells and is high in presumptive neurons which are undergoing maturation (Neuman et al., 1993; Zhu et al., 1995; Jen et al., 1997). At E8.5, Id2 expression can be detected in the neural folds (Jen et al., 1997). At E12.5, the neocortex expresses Id2 in a rostro-caudal gradient (Zhu et al., 1995). The infundibulum, olfactory bulb and septal primordia express Id2. In the secondary prosencephalon, Id2 expression extends from the eminentia thalami into the supraoptic paraventricular area. Expression can also be detected in the mammillary area and in the archicortex. In the diencephalon, mantle regions including the pretectal area, dorsal thalamus and ventral thalamus show weak expression. In the midbrain, the alar plate and the roof plate express Id2. In the hindbrain, there are several distinct boundaries of Id2 expression recognizable in the ventricular zone and the mantle. Also, Id2 expression is strong in the tela choroidea, as well as in the isthmocerebellar plate. In this region there is expression in the isthmic area and rhombic lip, the anlage of the cerebellar nuclei. In the spinal cord, Id2 is expressed throughout the ventricular zone and the mantle in a dorso-ventral gradient, with strong expression in the roof plate (Zhu et al., 1995).

At E14.5, Id2 expression in the neocortex is detectable in the mantle zone, with little or no expression in the proliferative zone (Neuman et al., 1993; Zhu et al., 1995) (Fig. 5A). The neocortex primordium expresses Id2 in rostro-caudal and latero-medial maturational gradients (Zhu et al., 1995). The olfactory bulb has high levels of expression. Within the cerebellum, Id2 is expressed in the ventricular zone only in the midline areas. More laterally, cerebellar expression is found in the primordia of the cerebellar nuclei and Purkinje cells.

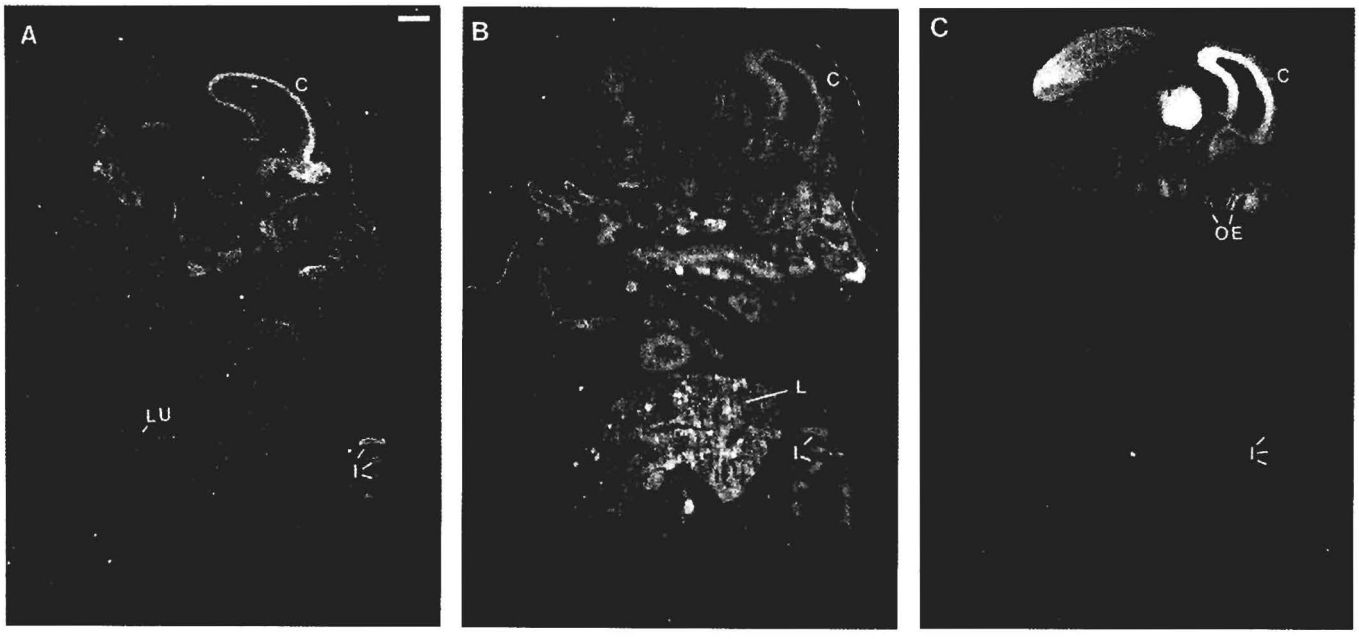

Fig. 5. Expression pattern of Id2, Id3, and Id4 genes detected by *in situ* RNA hybridization to sagittal sections of an E14.5 mouse embryo. Radiolabelled antisense riboprobes specific for Id2, Id3, and Id4 were used in the corresponding section. Id2 expression is shown in **Panel A**; Id3 expression is shown in **Panel B**; and Id4 expression is shown in **Panel C**. In the neocortex, Id2 expression is high in the mantle zone and low in the proliferative zone, whereas Id4 expression is much higher in the proliferative zone than in the mantle zone. Id3 is diffusely expressed in all cell layers. The dorsal thalamus and the olfactory epithelium express high levels of Id4 mRNA. Note that in the intestine, the layer which expresses Id4 is not the same as the layer which expresses Id2. C: cortex; DT: dorsal thalamus; I: intestine; L: liver; LU: lung; OE: olfactory epithelium. Scale bar: 600 μm.

Id2 is not expressed in the rhombic lip-derived external granule cells of the cerebellum (Zhu et al., 1995). In the midbrain and the hindbrain, Id2 is expressed in many postmitotic neurons (Zhu et al., 1995; Jen et al., 1997). At E16.5, the neocortical and paleocortical plates as well as the subplate and intermediate zones strongly express Id2, while the ventricular zone does not (Neuman et al., 1993; Zhu et al., 1995). The olfactory bulb continues to express high levels of Id2 at this time. The diencephalon expresses Id2 in the dorso-medial periventricular areas of the pretectum (Zhu et al., 1995). The epithalamus also expresses Id2. Id2 is also expressed at high levels in the differentiating Purkinje cells of the cerebellum. At P4 and P7, Id2 is expressed at high levels in two layers of the neocortex, one in the upper region of the cortical plate and the other in layers 5 and 6 (Neuman et al., 1993). Between P7 and adulthood, the expression is reduced in the outer layer of the cortical plate which forms layers 2 and 3 of the neocortex in the adult. The highest expression of Id2 in the adult neocortex is in the large pyramidal cells of layer 5. Id2 expression continues to be high in the olfactory bulb and in Purkinje cells, during the postnatal development and in the adult (Neuman et al., 1993). As in the mouse, Id2 mRNA is also expressed in the cortical plate and layer 5,6 of the rat neocortex at P1 and P5, and in the Purkinje cells of the adult rat cerebellum (Tzeng and de Vellis, 1998).

Id3

Id3 mRNA is detectable at E8.5 in the neural folds and in the neural groove (Jen et al., 1997). At E11.5, Id3 is expressed on the medial sides of the telencephalic vesicles, located laterally to the IIIrd ventricle. This is the region from which the future hippocampus is derived. Spinal cord expression is observed in the roof plate, the ventral portion, and the floor plate of the ventricular zone. Id3 is the only Id gene expressed in the floor plate of the spinal cord (Jen et al., 1997). At E12.5, the general expression pattern of Id3 in the telencephalon is similar to that observed at E11.5. Id3 expression is detectable in the hindwall, an area between the parietal cortex and the dorsal subpallial sulcus (Riechmann and Sablitzky, 1995). At this time, Id3 is expressed in the pons and the ventricular zone of the IVth ventricle, including the posterior choroid plexus. Id3 is also expressed by cells of the dorsal thalamus and the tectum (Riechmann and Sablitzky, 1995). In the spinal cord, high expression of Id3 continues at this stage of development and decreases at later stages (Jen et al., 1997). At E14.5, Id3 is expressed in the hippocampal region of the forebrain, the floor of the midbrain, and in specific areas of the metencephalon and myelencephalon (Ellmeier and Weith, 1995). The hindwall of the telencephalon and the ventricular zone of the IVth ventricle continue to show expression (Riechmann and Sablitzky, 1995). We too have observed Id3 to be diffusely expressed in the neocortex at this stage of mouse development (Fig. 5B). Interestingly, a mutually exclusive expression of Id3 and the proto-oncogene N-myc has been described, during the development of the forebrain and in other tissues outside the nervous system in the mouse (Ellmeier et al., 1992; Ellmeier and Weith, 1995).

Id4

Unlike other Ids, Id4 is expressed primarily in the nervous system (Riechmann et al., 1994; Riechmann and Sablitzky, 1995; Jen et al., 1996). Throughout mouse nervous system development, the expression of Id4 increases and seems to be restricted to specific cell types (Riechmann and Sablitzky, 1995). At E11.5, Id4 expression is detectable throughout the telencephalic vesicles (Jen et al., 1997). Id4 can be also found in the presumptive motor neurons of the metencephalon and the spinal cord. In the spinal cord, Id4 is expressed both in the ventral portion of the intermediate zone where future motor neurons will reside, and in the ventral one-fourth of the ventricular zone near the lumen. Scattered expression is also detected in the alar plate of the spinal cord, in both the intermediate zone and the ventricular zone away from the lumen. At E12.5, Id4 is expressed in the frontal and parietal cortex of the telencephalon; the

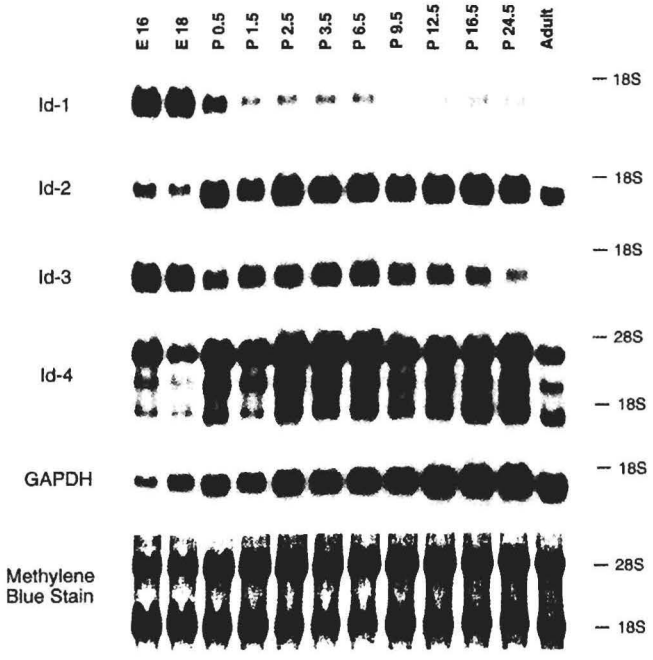

Fig. 6. Northern blot analysis of Id1, Id2, Id3, and Id4 mRNAs in murine brain tissue. The four known members of the *Id* gene family are expressed during postnatal development of murine brain. Total RNA was isolated from embryonic heads and postnatal brains at the indicated times of development. 15 micrograms of total RNA were loaded in each gel lane. The hybridization signal of a GAPDH probe and the methylene blue staining of ribosomal RNAs on the blot used as an RNA loading and transfer control are shown. Id4 film was exposed approximately three times as long as Id1, Id2 and Id3 films. Numbers on the right indicate the positions of the 28S and 18S ribosomal RNAs. E: embryonal day; P: postnatal day.

epithalamus; the preoptic, supraoptic, and postoptic areas; and the optic recess (Riechmann and Sablitzky, 1995). The superior cerebellar peduncle, the pontine flexure, the pyramidal tract, the medulla, and the spinal cord also express Id4. Two days later, Id4 mRNA is detectable in both the ventricular zone and cortical plate of the telencephalon (Jen et al., 1997) (Fig. 5C). The subventricular zone in the ganglionic eminence also expresses Id4. Id4 is expressed in the mantle and marginal layers of the thalamus, and in postmitotic nuclei in the midbrain and the hindbrain (Riechmann and Sablitzky, 1995; Jen et al., 1997). The expression pattern of Id4 at E16.5 is very similar to that observed at E14.5 (Jen et al., 1997). Neurons expressing Id4 are located in the hypothalamus, thalamus, and brainstem. At E17.5, Id4 expression is still detectable, although at low levels, in the telencephalon and the metencephalon (Riechmann and Sablitzky, 1995). Id4 expression is also detectable during postnatal development of murine brain (see below).

Id genes have distinct expression patterns in the nervous system

Comparative studies of Id expression in the mouse show that each member of the *Id* gene family has a unique expression pattern in the nervous system (Figs. 5-8 and our unpublished data) (Riechmann and Sablitzky, 1995; Zhu et al., 1995; Jen et al., 1996). For example, in the telencephalon, Id1 and Id2 seem to have mutually exclusive expression patterns (Zhu et al., 1995). Id2 is expressed at high levels in the neocortex, paleocortex, archicortex, and olfactory bulb, while Id1 expression cannot be distinguished in these regions from background levels. In contrast, Id1 is expressed in basal telencephalic primordia (medial ganglionic eminence and lateral ganglionic eminence), where Id2 is not detectable at levels above background. In the hindbrain, a more complex pattern of *Id1* and *Id2* gene expression

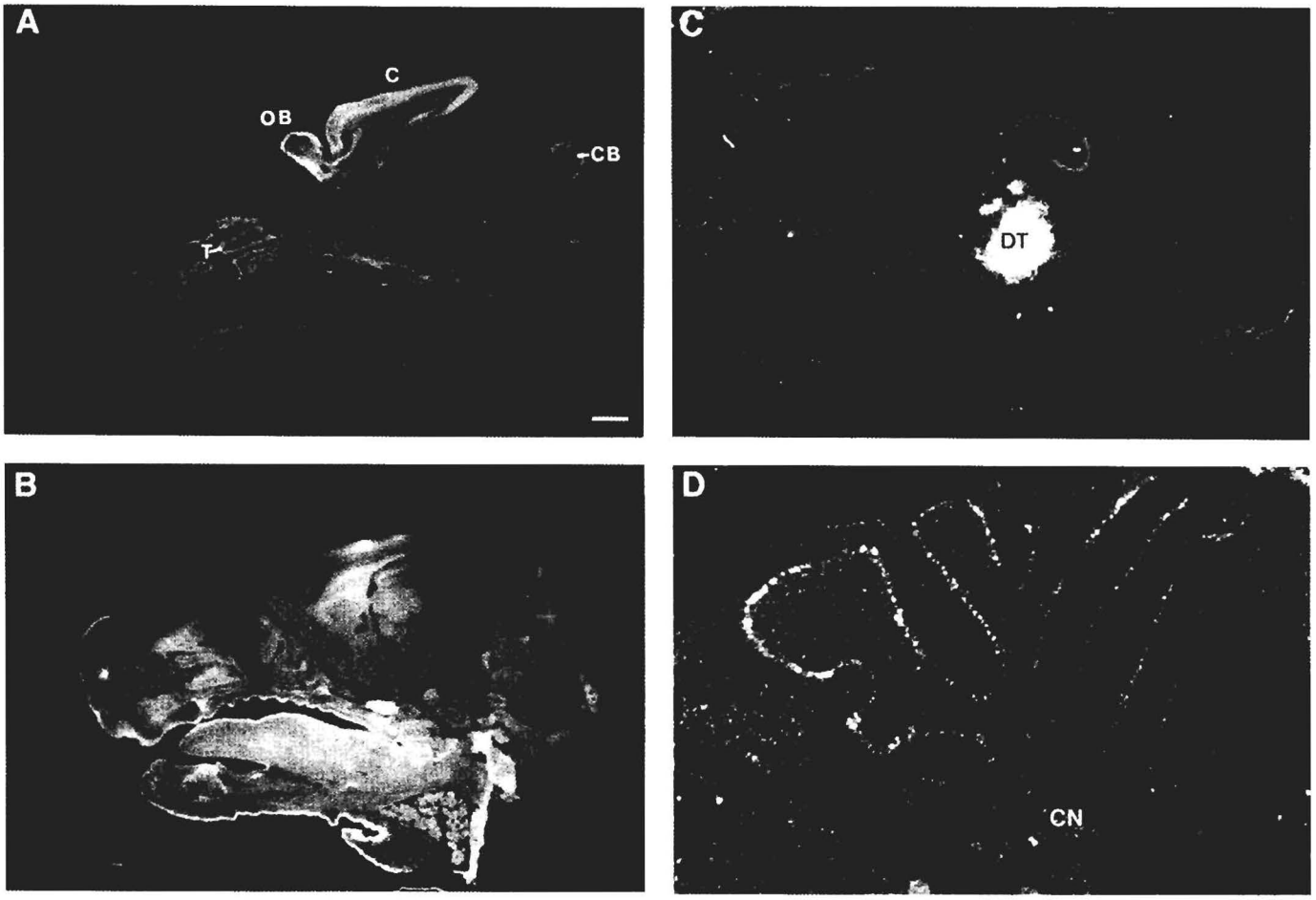

Fig. 7. Expression of *Id2* and *Id3* genes in the postnatal mouse brain detected by *in situ* RNA hybridization. **A and B.** Sagittal sections of a P0.5 mouse head. **C.** Sagittal section of an adult mouse brain. **D.** High magnification showing a detail of the cerebellum from panel C. Radiolabelled antisense riboprobes specific for Id2 and Id3 were used in the sections shown in Panel A and Panels B-D respectively. At P0.5, Id2 is highly expressed in the postmitotic neocortex, whereas low levels of Id3 mRNA are detectable throughout the neocortex. In the adult brain, Id3 expression is high in the thalamus and in the Purkinje cells of the cerebellum, and low in the neocortex. Please notice that the head sections in panels A and B face to the left and the brain section in panel C face to the right. C: cortex; CB: cerebellum; CN: cerebellar nuclei; OB: olfactory bulb; DT: dorsal thalamus; T: tooth. Scale bar: A and B, 900 μm; C, 700 μm; D, 200 μm.

exists (Zhu et al., 1995). Id1 and Id2 are expressed in similar antero-posterior and dorso-ventral regions, but differ in their expression along the medio-lateral axis. Id1 expression is restricted to the ventricular zone, whereas Id2 is expressed both in the ventricular zone and in the postmitotic mantle layer. This is also true in the rhombic lip and its derivatives. Id1 is expressed in the proliferative zone, whereas Id2 is expressed in a subset of rhombic lip derivatives (e.g. pontine nuclei). This suggests that in the hindbrain Id1 and Id2 may play a role in proliferative, undifferentiated cells, and that Id2 may also function in regulating the maturation of the postmitotic migrating cells into the mantle. Also, Id2 is expressed in postmitotic differentiating cells of the cerebral cortex, olfactory bulb and the cerebellum.

In the developing brain, Id4 and Id3 have different expression patterns and their expression frequently seems to be mutually exclusive, which suggests that their function might be distinct during development of the brain (Riechmann and Sablitzky, 1995). Sharp borders between Id4 and Id3 expression are seen in the neuroectoderm of the telencephalon. Whereas Id4 expression is present in the frontal and parietal cortex, Id3 expression is found in the hindwall of the telencephalon (Riechmann and Sablitzky, 1995). At E12.5, Id4 expression is present in the epithalamus, whereas Id3 expression is found in the dorsal thalamus. Also, Id4 expression is high in the serotonergic nuclear complex and absent in the developing pons whereas the inverse is observed for Id3. Id3 is highly expressed in the ventricular zone of the IVth ventricle, including the choroid plexus, and weakly expressed in the tectum, although neither area exhibits Id4 expression. The optic areas, cerebellar peduncle, pontine flexure, and pyramidal tract express Id4 but not Id3. At E14.5, Id4 is highly expressed in the preoptic area, mesencephalon, serotonergic nuclear complex, metencephalon, and the spinal cord, all regions that exhibit low Id3 expression.

Id genes present different patterns of expression along the dorso-ventral axis of the neural tube. For example, in the early stages of spinal cord development, Id1 and Id2 are expressed in the roof plate, whereas Id3 is expressed both in the roof and the floor plates (Jen et al., 1997). As embryonic development progresses, the expression of both Id1 and Id3 is detected in dividing neuroblasts, whereas Id2 and Id4 are expressed in presumptive neurons which are undergoing maturation. The expression of Id2 and Id4 in more mature neurons of the CNS is reiterated in some regions of the peripheral nervous system and in neurons of some sensory organs (Jen et al., 1997). This indicates that the expression of *Id2* and *Id4* genes is not just limited to undifferentiated neurons and suggests that in this cell lineage Id2 and Id4 do not act simply as inhibitors of differentiation.

Although a remarkable number of studies have focused on the analysis of *Id* gene expression during mouse embryonic development, there are few studies detailing the expression of *Id* genes during postnatal development and in the adult. Significant levels of Id1 (Benezra et al., 1990; Duncan et al., 1992; Hernandez et al., 1996), Id2 (Neuman et al., 1993), Id3 (Christy et al., 1991), and Id4 (Riechmann et al., 1994) mRNA have been detected by Northern blot analysis in the brain of adult mouse. A detailed comparative analysis of the steady-state levels of Id1, Id2, Id3 and Id4 mRNA by Northern blot analysis of RNA prepared from mouse brains at different stages of postnatal development and in adulthood shows that the four *Id* genes are highly expressed during postnatal development of murine brain (Fig. 6 and our unpublished data). *In situ* RNA hybridization demonstrated that during the first day of life (P0.5) in the mouse brain, Id2 is expressed at high levels in postmitotic cells of the neocortex, whereas low levels of Id3 mRNA are diffusely expressed in this region. In the adult, Id2 expression continues to be high in the neocortex. Id3 expression is very high in the thalamus and in the Purkinje cells of the cerebellum and low in the neocortex (Fig. 7 and our unpublished data). Id4 mRNA is also detectable by *in situ* hybridization in the postnatal and adult mouse brain. At P0.5, Id4 expression is low in the neocortex and high in the thalamus and the ependyma. In the adult, Id4 expression is detectable in the Purkinje cells and, at low levels, in the neocortex (Fig. 8 and our unpublished data).

Finally, Id1, Id2, and Id3 mRNAs have been detected in S100α positive and GFAP positive astrocytes in the postnatal rat brain (Tzeng and de Vellis, 1998). Id2 mRNA was also observed in neurofilament (NF) positive neurons. All four Id mRNAs have been identified in Schwann cells of rat and mouse (Stewart et al., 1997). After nerve transection, a weak and transient up-regulation in the mRNAs for Id1 and Id2 but not Id3 and Id4 was observed in the distal stump using semi-quantitative RT-PCR (Stewart et al., 1997).

Future directions for understanding Id function during nervous system development

Studies in cultured cells obtained from a variety of tissues, including those of the nervous system, suggest that *Id* genes can function as regulators of differentiation, proliferation and apoptosis. The molecular mechanisms that mediate these different functions need to be further elucidated. Id proteins seem to be a target of molecular pathways important in the regulation of neural cell biology including the NGF transduction pathway and the cAMP-dependent signal transduction pathway which suggest that Ids may function during the development of the nervous system. Also, although the role of Id genes in cell fate determination has not been extensively studied, recent experiments of Id2 overexpression in chick-embryo surface ectoderm indicate that Id2 converts ectodermal precursors to neural crest and neurogenic fates suggesting for the first time an effect of *Id* genes on promotion of vertebrate neurogenesis. These findings indicate a novel biologic function for Ids, and it is possible that Id proteins have functions other than those

already identified. For example, injury experiments in astroglial cultures suggest that Id4 expression may play a role in the process of astrogliosis.

All four Id genes are expressed during nervous system development, but they have different spatio-temporal expression patterns. In sharp contrast to Id1, Id2, and Id3 genes, which are also expressed in multiple tissues outside the nervous system during mouse development, the expression of Id4 seems to be largely limited to the central and peripheral nervous system. Therefore, Id4 studies may be of particular importance in gaining insight into the role of *Id* genes in the nervous system. Loss-of-function analyses of *Id* genes in the mouse show that mice in which the *Id1* or *Id3* gene have been homozygously deleted are essentially normal, whereas Id1/Id3 double-knockout mice are embryo lethal (see above). More gain-of-function and loss-of-function analyses of *Id* genes, individually and in

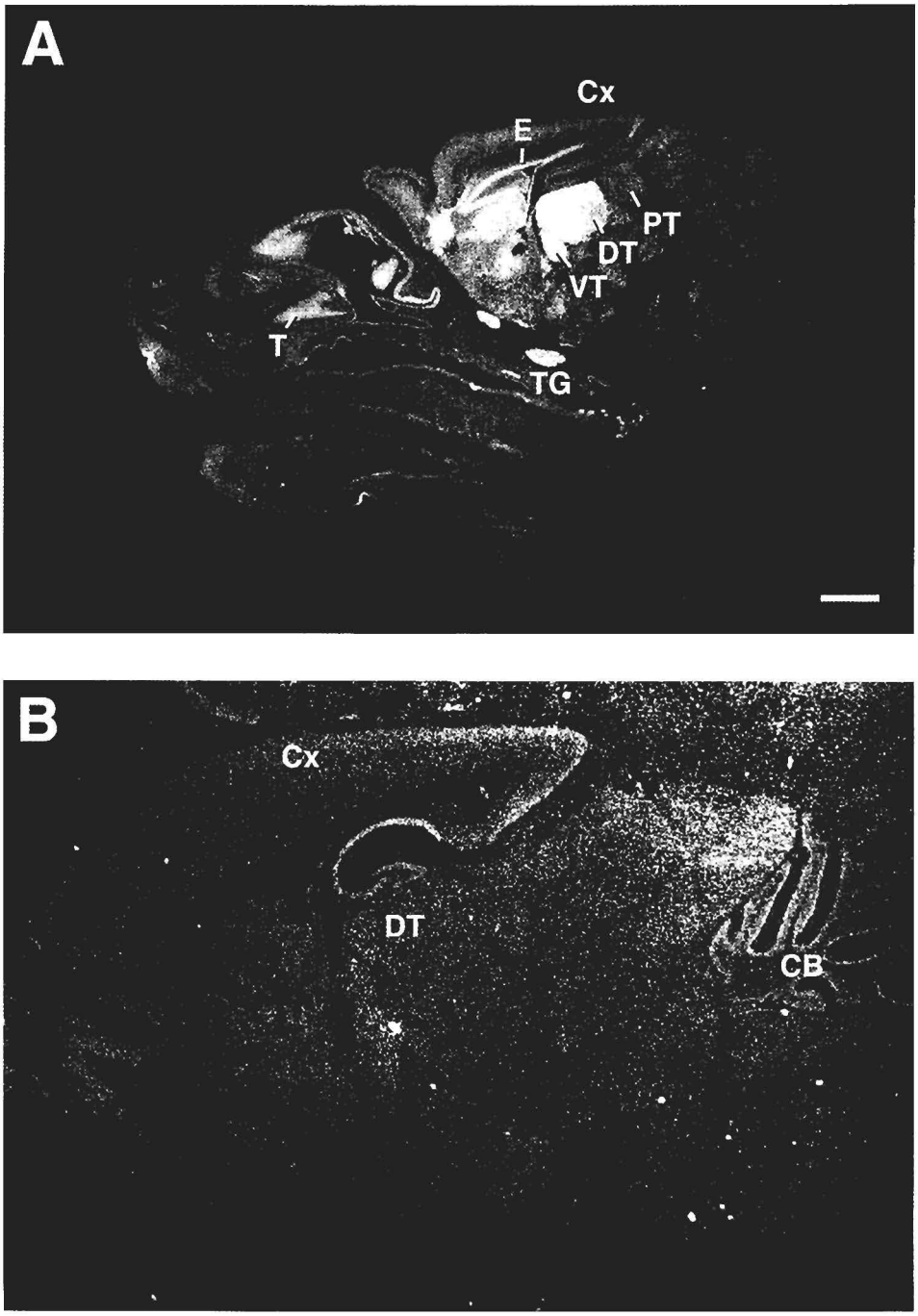

Fig. 8. Expression of *Id4* gene in the postnatal mouse brain detected by *in situ* RNA hybridization. A radiolabelled antisense riboprobe specific of Id4 was used. **A.** Sagittal section of a P0.5 mouse head. Id4 is highly expressed in the ependyma and the thalamus. Low levels of Id4 mRNA are detectable in the neocortex. **B.** Sagittal section of an adult mouse brain. Id4 is weakly expressed in the neocortex. Purkinje cells in the cerebellum also express Id4. Cx: cortex; CB: cerebellum; DT: dorsal thalamus; E: ependyma; VT: ventral thalamus; PT: pretectal area; TG: trigeminal ganglion; T: tooth. Scale bars: A, 900 μm; B, 700 μm.

combination, and conditional gene-targeting strategies, are likely to be necessary to clarify the function of *Id* genes in the nervous system.

Most of the data on Id expression available to date describes Id mRNA analysis. The biologic activity of Ids may be regulated not only at the transcriptional and post-transcriptional level, but also by translational and post-translational mechanisms. Therefore, it is important to obtain more information on the subcellular localization, the biochemical properties, and the expression of Id proteins. Also, the physiological role of different Id proteins in specific cell types of the nervous system remains to be clarified.

The precise molecular events mediated by Id proteins during nervous system development may be very complex. Potentially, Id proteins have the ability to heterodimerize with bHLH proteins which can either enhance or inhibit neurogenesis. The distinctly different expression patterns of *Id1*, *Id2*, *Id3*, and *Id4* genes, and the increasing number of bHLH factors expressed primarily in the nervous system suggest that there is a complex network of HLH proteins that function in the regulation of nervous system development. An important aspect of clarifying the physiological functions of Id family members in the nervous system is identifying the bHLH proteins that function in neural cells. Also, while the HLH regions of the different Id proteins are likely to mediate interactions with a similar array of bHLH proteins, understanding the functional roles of the unique N-terminal and C-terminal regions of each Id protein should provide important insights into defining the role of Ids in mammalian nervous system development and in the biology of neural cells.

Conclusions

The proliferation, differentiation, and programmed cell death of neural cells are fundamental features of vertebrate nervous system development. *Id* genes are known to function as regulators of cell differentiation in a variety of cell lineages, and experimental observations suggest that *Id* genes can act not only as inhibitors of differentiation but also in the control of cell proliferation and apoptosis. *Id* genes are widely expressed in the nervous system, and each member of the *Id* gene family has a unique spatio-temporal expression pattern, during nervous system development. *Id* gene expression is regulated during neuronal, astroglial and Schwann cell differentiation in culture. Overexpression of Id2 induces apoptosis in neuronal progenitor cells, and overexpression of Id4 induces apoptosis in astrocytic cells. Also, overexpression of Id2 in the ectoderm and open neural plate directs ectodermal precursors toward neural crest and neurogenic fates. Given these observations, *Id* genes are strong candidates for playing a key role in the regulation of vertebrate nervous system development.

Note added in proof

After submission of this manuscript, three communications describing new functional activities for Id genes have been published. Alani et al. (1999) described immortalization of primary human keratinocytes overexpressing Id1. Pan et al. (1999) described that the Id3 gene is required to maintain a normal immune response in the mouse. Finally, Lyden et al. (1999) described that Id1 and Id3 genes are required to maintain the timing of neuronal differentiation in the mouse embryo and invasiveness of the vasculature.

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