DISSERTATIONES BIOLOGICAE UNIVERSITATIS TARTUENSIS
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ROLE OF HELIX-LOOP-HELIX AND NUCLEAR HORMONE RECEPTOR TRANSCRIPTION FACTORS IN NEUROGENESIS

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PRESS

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CONTENTS

1. ORIGINAL PUBLICATIONS

This thesis is based on the following original publications which are referred to in the text by their Roman numerals.

I Soosaar, A., Chiaramello, A., Zuber, M. X., and Neuman, T. (1994) Expression of helix-loop-helix transcription factor ME2 during brain development and in the regions of neuronal plasticity in the adult brain. Mol. Brain Res. 25, 176–180.

II Chiaramello, A., Soosaar, A., Neuman, T., and Zuber, M. X. (1995) Differential expression and distinct DNA-binding specificity of ME1 and ME2 suggest a unique role during differentiation and neuronal plasticity. Mol. Brain Res. 29, 107–118.

III Neuman, T., Soosaar, A., and Nornes, H. O. (1995) Isolation of genes which block neuronal differentiation of teratocarcinoma PCC7 cells. Exp. Cell Res., 217, 363–367.

IV Neuman, K., Soosaar, A., Nornes, H. O., and Neuman, T. (1995) Orphan receptor COUP-TF I antagonizes retinoic acid induced neuronal differentiation. J. Neurosci. Res. 41, 39–48.

V Soosaar, A., Neuman, K., Nornes, H. O., and Neuman, T. (1996) Cell type specific regulation of COUP-TF II promoter activity. FEBS Lett. 391, 95–100.

2. ABBREVIATIONS

bHLH	basic domain helix-loop-helix
bp	base pair
CAS	class A specific
CAT	chloramphenicol acetyltransferase
cAMP	cyclic adenosine 5'-monophosphate
cDNA	complementary deoxyribonucleic acid
cdk	cyclin dependent kinase
CNS	central nervous system
CMV	cytomegalovirus
COUP TF	chick ovalbumine upstream promoter transcription factor
CRE	cAMP response element
C-terminal	carboxyl-terminal
dBcAMP	dibutyryl cyclic-AMP
E-box	immunoglobulin enchancer core consensus sequence
EMSA	electrophoretic mobility shift assay
E1A12S, E1A13S	adenovirus oncogenes
ER-TR	estrogen-thyroid response element
GAP43	growth-associated protein 43
GR	glucocorticoid-progesteron response element
HLH	helix-loop-helix
HRE	hormone response element
KLH	Keyhole Limpet Hemocyanin (carrier)
LH	loop-helix
MAP2	microtubule-associated protein 2
MEF1	muscle creatine kinase enhancer E-box
mRNA	messenger ribonucleic acid
NF-L	neurofilament light subunit
N-terminal	amino-terminal
pRb	retinoblastoma protein
RA	retinoic acid
RAR	retinoic acid receptor
RARE	retnoic acid response element
RNP	regulator of neuronal proliferation
RXR	retinoid X receptor
TR	thyroid hormone receptor
VDR	vitamin D3 receptors

3. INTRODUCTION

Initiation of transcription is considered to be the most crucial step of gene expression. Selective transcription of genes generates specific patterns of regulatory proteins. The nodal point of cell fate determination is regulation of genes in G1 phase of cell cycle. Cell cycle is controlled by a certain set of genes which are required for cell cycle progression. Under certain circumstances a cell may become arrested in G1 phase, exit cell cycle (G0 phase) and undergo terminal differentiation. Differentiation is controlled by set of genes whose role is to block cell proliferation and activate cell-lineage specific genes. Interplay of different transcription factors is a part of these regulatory processes. In addition, differentiation of cells includes a complex mechanism of signaling which integrates and coordinates transcriptional and posttranscriptional control of gene expression. Different types of transcription factors are involved in the control of genes which respond to internal and/or external signal(s). Several groups of transcription factors have been identified and classified on the bases of their protein structure and DNA binding properties.

Transcription factors of helix-loop-helix (HLH) family (c-myc, E2F, MyoD, Id etc.) have been demonstrated to carry important function during development and cell differentiation. Role of E2F is well established during cell cycle progression. Also, the function of MyoD during myogenesis is described as a regulator of lineage specific gene expression. Several other HLH family transcription factors such as ubiquitously expressed basic helix-loop-helix (bHLH) transcription factors have been demonstrated to regulate transcription of target genes as partners of tissue specific transcription factors (MyoD). However, the exact role of these transcription factors is still obscure.

Nuclear hormone receptor transcription factors are required for activation of target genes in a hormone dependent manner. Role of the nuclear hormone receptor family transcription factors (retinoic acid receptors, retinoid X receptors, thyroid hormone receptors, steroid hormone receptors etc.) is relatively well-studied during development and differentiation. Nuclear orphan receptors are a subclass of nuclear hormone receptor family and their ligands are unknown. Role of the nuclear orphan receptors in these processes is not well understood so far.

Nervous system is an important part of multicellular organisms (excl. plants). Nerve cells are generated and system of communications between different parts of an organism are created, refined, and remodeled during embryonic development. All neurons are generated once and are not subsequently

replenished. Molecular mechanisms underlying neurogenesis are not well understood yet. However, neuronal determination during embryonic development is relatively well-studied in *Drosophila*. In particular, requirement of HLH transcription factors encoded by genes *daughterless, hairy, Enhancer of split, extramacrochatae* and *achate-scute* as well as an orphan receptor transcription factor encoded by *seven-up* has been well established in the regulation of neuronal determination of progenitor cells in *Drosophila*. To date, vertebrate homologues of these genes are identified. Based on remarkable degree of similarity between several mammalian HLH genes and *Drosophila* proneuronal genes, it is likely that mammalian HLH factors are also involved in regulation of neuronal development.

The main objective of the present thesis work is to characterize the role of HLH and nuclear orphan receptor transcription factors during neurogenesis. The first aim of this work is to describe expression of mouse bHLH transcription factors ME1a and ME2 during neurogenesis, to characterize DNA-binding and dimerization properties of these factors, and to demonstrate their ability to regulate transcription in neuronal cell lines. The second aim is to characterize HLH and orphan receptor (COUP TF I) transcription factor which block neuronal differentiation of teratocarcinoma cells. The third aim is to localize basal promoter of the orphan receptor transcription factor COUP TF II and to characterize specific effects of this promoter in response to RA and cAMP treatment in different cell lines.

4. REVIEW OF THE LITERATURE

4. 1. CELL CYCLE AND DIFFERENTIATION

Initial differentiation requires suppression of cell cycle progression genes. Negative regulators of cell cycle progression, such as retinoblastoma proteins (pRb, p107, p130), have been identified. These proteins share a specific domain called the pocket domain that is required for binding of other cellular proteins such as E2F, cyclins, MyoD and Id. Interaction between pRb family members and these proteins results in the timely transcription of genes encoding factors essential for cell cycle progression (E2F, [Cao *et al.,* 1992]) or induction of the differentiated state (MyoD [Gu *et al.,* 1993]). These complexes are cell cycle phase-dependent and may be critical in cell-growth regulation (Ikeda *et al.,* 1996; Zwicker *et al.,* 1996).

4.1.1. Retinoblastoma proteins regulate cell differentiation

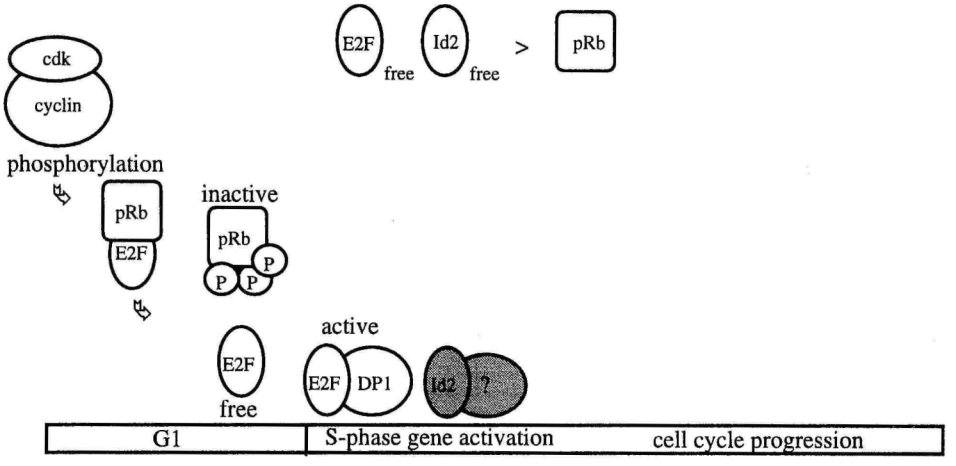
The retinoblastoma tumor suppressor protein (pRB) is a transcriptional repressor that regulates gene expression by physically associating with transcription factors of E2F family members.

Transgenic animals lacking pRb1 or expressing mutant pRb1 gene have been analyzed to address the function of pRb proteins. These animals either died or showed a number of abnormalities in neuronal and haematopoietic development (Jacks *et al.,* 1992; Clarke *et al.,* 1992).

A variety of cyclins and cyclin dependent kinases (cdk) have been demonstrated to be involved in the modulation of pRb function. G1 cyclins function as regulatory subunits of cdk which phosporylate pRb (p107) and inactivate its growth-inhibitory function (Pagano *et al.,* 1992; Hall *et al.,* 1993; Ohtsubo *et al.,* 1995). Hyperphosphorylated pRb releases active E2F which switches on genes required for S-phase initiation and progression. (Fig. 1). Several proteins are able to inhibit cdk's and suppress cell-cycle progression by maintaining pRb protein hypophosphorylated. These proteins have been divided into two groups (1) universal inhibitors such as p21 (Shiyanov *et al.,* 1996), and (2) specific inhibitors for cyclin D-dependent kinases, like the INK proteins (Chan *et al.,* 1995) (Fig. 1).

The most extensively characterized targets of pRB are the members of E2F transcription factor family.

proliferation



cell cycle block and differentiation

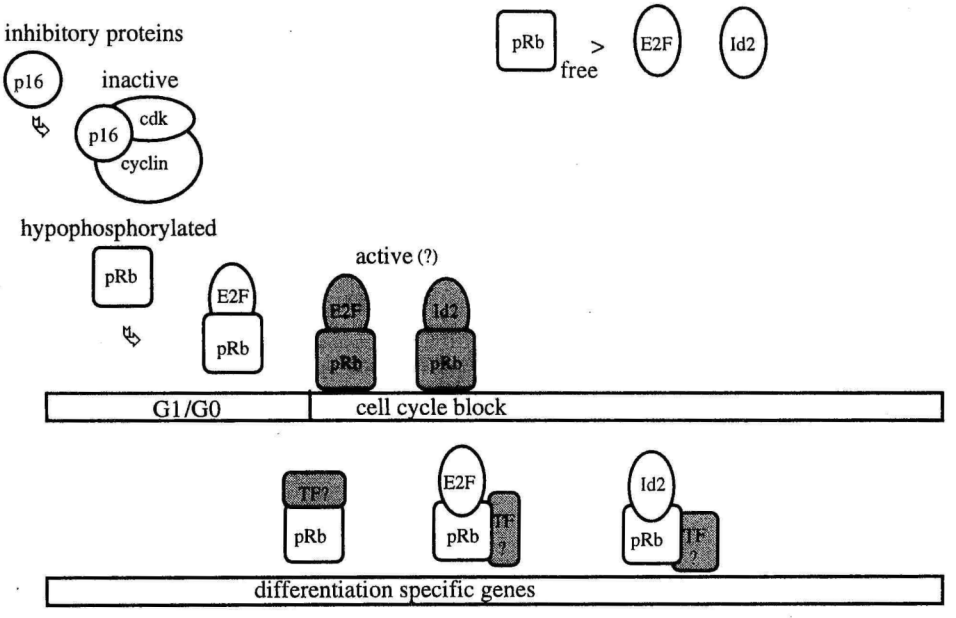


Figure 1. Hypothetical model of pRb, E2F and Id2 in cell proliferation and differentiation (explanations in the text).

4.1.2. E2F transcription factors have dual role in cell cycle regulation

E2F family bHLH transcription factors directly regulate transcription of a diverse set of genes involved in DNA replication and cell growth control, including E2F gene itself (Hsiao *et al.,* 1994). E2F1 expression is sufficient to induce entry into S phase of the cell cycle. E2Fs require DPs as dimerization partners to activate transcription of cell cycle progression genes (Wu *et al.,* 1996). A number of known cell cycle regulators such as pRb, p53, cdk-2, cdk-4 and certain cyclins appear to exert their effects by altering E2F activity. These regulators are commonly mutated in cancer cells and play important role in coordination of cell cycle (Adams and Kaelin, 1995; Ohtsubo, *et al.,* 1995; Xu *et al.,* 1994). Interaction of pRb and E2F leads to the inhibition of E2F/DP1 heterodimer-mediated transactivation (Helin *et al.,* 1993). Also, it has been suggested that pRb/E2F active complex may block transcription directly by binding to target gene promoter (Fig. 1). pRb in this complex blocks the basal transcription machinery by inactivating surrounding transcription factors (Weintraub *et al.,* 1995).

Recently, mice homozygous for a nonfunctional E2F1 allele has been characterized. Mice lacking E2F1 are viable and fertile, but developed a broad spectrum of tumors (Yamasaki *et al.,* 1996). This unexpected result demonstrates that E2F1 may function as a tumor suppressor.

Also, it has been demonstrated that the above-mentioned cell cycle factors are involved in the process of cell differentiation. pRb and related proteins have been proposed to function as a possible partners involved in the modulation of E2F activity during cell differentiation. *In situ* hybridization analysis have shown presence of pRB and E2F transcripts in proliferating as well as differentiating cells during neuronal development of spinal cord (Zhao *et al.,* 1995). E2F plays dual role during differentiation of P19 cells: proliferating cells contain free E2F and E2F complex with cycline A. In contrast, differentiated cells do not contain detectable amount of free E2F and contain a specific complexes which do not contain cyclin A (Reichel, 1992). Teratocarcinoma P19 cell differentiation is accompanied by the changes in the cdk-activities, pRB expression and E2F DNA-binding (Kranenburg *et al.,* 1995). In the G1 phase of the proliferating cells amount of pRB proteins appears to be limited and E2F may form transcriptionally active complexes. As these cells exit the cell cycle, amount of hypophosphorylated pRb forms exceed the amount of E2F, and causes consequently repression of S-phase specific genes (Ikeda *et al.,* 1996).

These data indicate that E2Fs regulate cell cycle progression genes and are also involved in the regulation of cell differentiation genes. In either case different partners may be recruited (Fig. 1).

4.2. HELIX-LOOP-HELIX TRANSCRIPTION FACTORS

Helix-loop-helix transcription factors are involved in the regulation of cell proliferation and differentiation as well as in the control of various developmental pathways (Jan and Jan, 1993; Weintraub, 1993). HLH Transcription factors have been grouped into different classes depending on their expression patterns and structural characteristics (Fig. 2).

4.2.1. Class A bHLH transcription factors

4.2.1.1. Structure and expression

Subclass A consists of the HLH transcription factors which contain basic domain adjacent to the helix-loop-helix motif (bHLH). These proteins contain at least five functional domains.

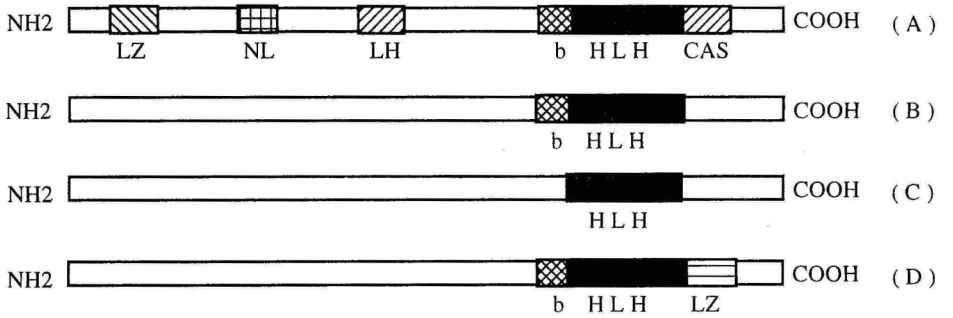


Figure 2. Schematic representation of differences between the functional domains of HLH proteins. (A) class A bHLH transcription factors represented after ME1a; common and restrictive features of class B (B), Id- like (C), and class C (D) transcription factors.

Highly conserved helix-loop-helix domain is responsible for protein-protein interaction and basic domain is the DNA-binding module of the protein (Murre *et al.,* 1989a; Murre *et al.,* 1989b; Lassar *et al.,* 1989; Davis *et al.,* 1990; Neuman *et al.,* 1993b). Nuclear localization region (Klein *et al.,* 1993) and an additional loop helix (LH) motif, which is involved in transcriptional activation (Davis *et al.,* 1990; Henthorn *et al.,* 1990; Quong *et al.,* 1993), are also clearly defined. Class A bHLH transcription factors contain a class A specific domain (CAS). CAS is believed to form a loop and a helix and can assumingly mediate a specific interaction between class A and tissue-specific transcription factors (Zhang Y. *et al.,* 1991) (Fig. 2).

Several factors of the subclass are described from *Drosophila* to mammals: Da encoded by *Drosophila daugtherless* (Caudy *et al.,* 1988), mouse E2A (E12, E47) (Murre *et al.,* 1989a), ME1(ME1a, ME1b) (Neuman *et al.,* 1993a), human HEB, (Hu *et al.,* 1992) and HTF4 (Zhang *et al.,* 1991), rat REBβ, REBα (Klein *et al.,* 1993), chick CTF4 (Tsay *et al.,* 1992) and G1 (Neuman *et al.,* 1993a), Akv murine leukemia virus ALF1b, ALF1a (Nielsen *et al.,* 1992), human E 2-2, ITF2 (Henthorn *et al.,* 1990), mouse ME2 (Neuman *et al.,* 1993a), and MITF2 A and B (Skerjanc *et al.,* 1996).

Based on the expression patterns it has been postulated that class A bHLH proteins are ubiquitously expressed (Murre *et al.,* 1989a, b). All these factors are expressed in a nontissue-specific manner and have dynamic and partially overlapping expression patterns. The exact role of these proteins has been difficult to determine. To regulate transcription, bHLH proteins form homo- and heterodimers and bind specifically to a DNA consensus sequence CANNTG, known as an E-box (Ephrussi *et al.,* 1985; Murre *et al.,* 1989a, b).

Functional redundancy of an individual class A transcription factor is suggested. It has been shown that a double knockout of the E2A gene in mouse embryonic stem cells had no effect on differentiation of muscles, erythrocytes, neurons and cartilage (Zhuang *et al.,* 1992). Block of expression of individual class A bHLH transcription factors had no detectable effect on proliferation and differentiation of neural tube cells. At the same time, simultaneous blocking of these factors in cultured neural tube cells resulted in the reduction of differentiating neurons (Suda *et al.,* 1994).

4.2.1.2. Dimerization

An important role of class A proteins as necessary dimerization partners for tissue-specific bHLH proteins during cell differentiation has been shown in variety of systems. The E2A gene products heterodimerize with tissue specific class B (MyoD) transcription factors(chapter 4.2.2.). These active heterodimers turn on expression of tissue-specific genes and induce myogenesis (Davis *et al.,* 1987; Pinney *et al.,* 1988; Lassar *et al.,* 1989; Lassar *et al.,* 1991; Bain *et al.,* 1994; Zhuang *et al.,* 1994). Recently, a splice variants of the class A transcription factor mITF2 has been described. These proteins have been demonstrated to form MyoD/ mITF2 heterodimers *in vitro*. Alternatively spliced forms of mITF2 could either activate or have no influence on the MyoD regulated transcription of target gene. (Skerjanc *et al.,* 1996). The presence of different sets of class A proteins, which have different DNA-binding specificities, suggests selective interactions with different binding sites of target genes. The ratio of different class A bHLH proteins during differentiation is probably important for the right timing of cell-type specific gene expression.

On the other hand, formation of homodimers of class A transcription factors has been observed during haematopoiesis. The ratio, of two different bHLH transcription factors E2A/E2-2, changes and these proteins have different DNA-binding properties during B-cell development. The level of E2A proteins increases while the level of E2-2 decreases during B-cell maturation. Binding of E2-2 to the E-box has been detected only in pre-B cells but not in mature B-cells (Bain *et al.,* 1993). E2A null-mutant mice fail to generate mature B-cells while other haematopoietic lineages are intact. No class B specific heterodimerization partners have been found from tested B cells which indicates that these E2A homodimers are capable specifically direct B cell differentiation (Bain *et al.,* 1994; Zhuang *et al.,* 1994). Also, variable expression pattern has been shown for two alternatively spliced E2A gene transcription factors E12 and E47 in different organs and cell lines (Watada *et al.,* 1995).

The presence of different sets of class A proteins which have different DNA-binding specificities suggests selective interactions with different binding sites of target genes. The ratio of different class A bHLH proteins during differentiation is probably important for the right timing of cell-type specific gene expression. The various spatial and temporal expression pattern of class A proteins suggest the possibility that, under certain circumstances, these homodimers appears to be cell-type specific and carry responsibility of cell fate determination during development. Thus, differently spliced forms of class A bHLH transcription factors as well as proteins coded by different genes may either dimerize with the same class B tissue-specific transcription factor or homodimerize and activate or repress transcription.

In addition, class A transcription factors form inactive complexes with Id family transcription factors.

4.2.2. Class B bHLH transcription factors

Tissue-specific bHLH proteins (Fig. 2) comprise the largest family of HLH transcription factors which include extensively characterized myogenic proteins (MyoD and myogenin) that regulate muscle differentiation (Davis *et al.,* 1987; Pinney *et al.,* 1988) Class B transcription factors have tissue and cell type specific expression pattern and they are believed to bind DNA as heterodimers with class A bHLH proteins (Murre *et al.,* 1989a, b; Lassar *et al.,* 1989; Lassar *et al.,* 1991). They also form functional homodimers (Nielsen *et al.,* 1992; Klein *et al.,* 1993).

The family consists of the following proteins identified so far: (1) *Drosophila* products of the genes *achaete-scute* AS-C, *hairy* and *Enchancer of split* (Ghysen and Dambly-Chaudiere, 1989; Campos-Ortega and Jan, 1991; Cabrera, 1992). (2) vertebrates myogenic factor MyoD (Davis *et al.,* 1987) (3) neural specific factors NeuroD (Lee *et al.,* 1995), BETA2 and 3 (Peyton *et al.,* 1996),

MASH1 and 2, (Johnson *et al.,* 1990), XASH (Ferreiro *et al.,* 1995), NSCL1 and 2, (Lipkowitz *et al.,* 1992), HES1 and 3 (Sasai *et al.,* 1992).

4.2.2.1. Involvement of class B bHLH transcription factors in regulatory processes

To date, MyoD is described as a nodal point for activation of several downstream muscle regulators during the muscle differentiation. MyoD requires class A (E12/E47) transcription factors to form transcriptionally active heterodimers. These heterodimers have been demonstrated to bind to the E-box sequences of genes which regulate transcription of muscle differentiation specific genes (chapter 4.2.1.2.).

MyoD and Id HLH proteins form transcriptionally inactive heterodimers. Dominantly negative Id transcription factor abolishes DNA binding properties of these complexes. This results in inhibition of lineage specific gene expression and differentiation (Benezra *et al.,* 1990; Wilson *et al.,* 1991).

4.2.2.2. Class B transcription factors in neurogenesis

In *Drosophila,* AS-C (Campos-Ortega and Jan, 1991) bHLH transcription factors have been described as a positive regulators of sensory organ formation. *Hairy* and *Enhancer of split* gene products function as transcriptional repressors during *Drosophila* neural determination.

HES1 is expressed at high levels throughout ventricular zone which consist of neural precursor cells but not in the outer layers where differentiated cells are present in the developing CNS of mammals (Sasai *et al.,* 1992). Overexpression of HES1 results in repression of differentiation of retinal progenitor cells and accordingly, HES1 null-mutant mice show acceleration of the retina differentiation (Tomita *et al.,* 1996). The *hairy* related transcription factors (HES) form active homodimers, which may repress transcription of various genes, including their own genes by direct binding to N-box (CACNAG) sequences (Tietze *et al.,* 1992). However, the possible mechanism of repression by *hairy*-related proteins (HES1) has been assumed to be direct binding to DNA (N-box) in target gene promoter rather than interfering with activator proteins (Takebayashi *et al.,* 1994).

NeuroD (Lee *et al.,* 1995) and MASH1(Johnson *et al.,* 1990), neural-specific bHLH proteins, are expressed transiently in mammals during neuronal differentiation. MASH1 is induced in differentiating PC12 cells (Johnson *et al.,* 1990). MASH1 null-mutation mice died at birth, and showed severe losses in olfactory and autonomous neurons (Guillemot, 1995). Thus, NeuroD has been demonstrated to act as neuronal determination factor and MASH1 as a deter-

mination factor for olfactory and autonomous neurons but assumingly it is not essential for development of most of the CNS.

4.2.3. Id like HLH transcription factors

4.2.3.1. Structural difference with other HLH proteins

Id-like proteins, which contain the HLH motif but lack the basic domain, (Fig. 2) may interact with above-described class A and class B bHLH transcription factors. Those heterodimers do not bind DNA (Benezra *et al.*, 1990). Id proteins appear to dimerize without DNA stabilization because of better packed hydrophobic core (Wibley *et al.*, 1996).

4.2.3.2. Involvement in the regulatory processes

Id family consists of a number of structurally related members, Id1-4. Analysis of functions and spatial-temporal expression patterns show differences between these family members: Id2, unlike Id1 and Id3, binds to pRB related proteins (Lasorella *et al.*, 1996) and is involved in pRB mediated cell cycle block. The role of Id2 as a partner of the class A and B bHLH transcription factors during myogenesis has been studied. Expression of dominant negative Id2 gene inhibits the ability of these bHLH transcription factors to carry out their functions (Benezra *et al.*, 1990).

The inhibitory role of Id proteins has been described during haematopoiesis. Id proteins are expressed only in B-lymphoid progenitor cells but not in mature B cells. High levels of Id transcription factors repress the activity of class A bHLH transcription factors during B cell differentiation *in vivo* (Wilson *et al.*, 1991). The Id2 and Id3 show distinct and variable expression patterns in T and B cell lines during human lymphocyte differentiation (Ishiguro *et al.*, 1995) and Id1 transcription factor has been demonstrated to block erythrocyte terminal differentiation (Lister *et al.*, 1995).

The inhibitory role of different Id transcription factors have been described during neuronal development and established in neuronal cell lines. The Id1 and E2-2 (class A bHLH transcription factor) proteins form transcriptionally inactive heterodimers in neuronal cells (Einarson and Chao, 1995). The Id2 is expressed in the ventricular zone of neuroepithelium in the rapidly dividing cell population during early neurogenesis. After the first neuronal populations are born, the expression of Id2 is down-regulated in the neuroepithelial cells. However, the expression remains high in some areas of the developing brain

(Neuman *et al.,* 1993b). Id transcription factors are not expressed in the sympathetic nervous system and adrenal medulla, where precursor cells differentiation and proliferation occur simultaneously (Duncan *et al.,* 1992). The Id2 expression is cell line specific in neuronally differentiating cells. The level of the Id2 mRNA is upregulated (PCC7), downregulated (NG108) or unchanged (N18) during differentiation (Neuman *et al.,* 1993b). Consistent with their role as inhibitors of differentiation, expression of Id genes is high in undifferentiated cells. However, variable expression patterns of Id transcription factors in different cells suggests that these factors may have different functions in different cell types and during different stages of differentiation.

Promoter analysis of Id2 gene demonstrated the presence of multiple E-box sequences which suggest that the bHLH proteins have a role in the regulation of Id2 gene expression (Neuman *et al.,* 1995).

4.2.4. Class C bHLH transcription factors

4.2.4.1. Structural differences

Class C bHLH transcription factors contain a basic region helix-loop-helix leucine zipper motif in the C-terminal part. Well-studied members of class C family are myc and related factors which have important regulatory role in many different cell proliferation and differentiation processes. Several other class C factors have been isolated: USF (Gregor *et al.,* 1990), AP-4 (Hu *et al.,* 1990) and TFE3 (Beckmann *et al.,* 1990). The class C bHLH proteins form dimers with other class C protein to activate transcription of target genes. Class C bHLH transcription factors do not form heterodimers with Id family proteins (Sun *et al.,* 1991).

4. 3. HLH TRANSCRIPTION FACTORS ARE INVOLVED IN THE CELL CYCLE REGULATION

Id family HLH proteins have high expression in proliferating cells and their expression is downregulated during differentiation (Ellmeier *et al.,* 1992). Iavarone *et al.,* (1994) have demonstrated that HLH protein Id2 physically associates with the hypophosphorylated form of pRb (Fig 1). Later studies have shown that high levels of Id2 relieved cell cycle arrest induced by cdk inhibitor in the presence of pRb (Lasorella *et al.,* 1996). It has been also demonstrated that pRb proteins may repress transcription independently of E2F proteins. This repression is mediated by the pocket region of pRb proteins. Inhibition is direct

and requires interactions with another protein instead of E2Fs (Bremner *et al.*, 1995). These observations suggest that Id proteins have an important role in the regulatory events of cell cycle.

4. 4. HLH TRANSCRIPTION FACTORS DURING CELL DIFFERENTIATION

Development of a specialized cell type is a two step process. First step is the determination of a cell lineage and second is an activation of set of cell-type specific genes. To date, role of HLH transcription factors has been described in cell-type specific gene regulation at the molecular level by *in vitro* analysis. These observations demonstrate that heterodimers of class A and class B bHLH proteins are involved in regulation of the cell-lineage specific genes during terminal differentiation.

Well studied is synergism of class A and class B bHLH transcription factors during myogenesis. During terminal differentiation of myoblasts, interactions between specific factors and nonspecific class A transcription factors activate differentiation specific genes. Complexes of E12/MyoD and E47/MyoD have been identified in developing muscle cells. On the other hand, competition for E12/E47 by Id dominant negative transcription factor has been postulated to extinguish muscle-specific gene expression by precluding interaction of E12/E47 with MyoD or myogenin under conditions when Id levels are high. Id level decreases in response to differentiation signals. These changes result in the release of free MyoD and E12/E47 which can form heterodimers capable of switching on differentiation specific genes. The class A proteins have been implicated in muscle, lymphoid, pancreatic and neural development, which suggest that these transcription factors may interact with tissue-specific HLH regulators of several cell types. Thus, tissue-specific factors may regulate expression of lineage specific genes and inhibit other developmental programs by sequestration of commonly required dimerization partners of tissue-specific bHLH factors. This competition for commonly required bHLH binding partners may exclude alternative developmental programs (Davis *et al.*, 1987; Pinney *et al.*, 1988; Benezra *et al.*, 1990; Lassar *et al.*, 1991; Neuman *et al.*, 1993a;).

Also, HES1 has been described to heterodimerize efficiently with E12/E47 proteins in differentiating myoblasts (Sasai *et al.*, 1992). Consequently, HES1 may repress differentiation by similar mechanism discussed above (Ishibashi *et al.*, 1994).

Studies in invertebrates suggest that bHLH factors play a crucial role in neurogenesis also. For example, *Drosophila* AS-C bHLH factors are positive regulators and *hairy* and *Enhancer of split* encoded factors are negative regu-

lators for sensory organ formation (Ghysen and Dambly-Chaudiere, 1989; Cabrera, 1992; Campos-Ortega and Jan, 1991; Jan and Jan, 1993).

MASH1 transcription factor which is expressed in neuronal precursor cells and HES family transcription factors are mammalian homologues of *Drosophila* above mentioned factors. It has been suggested that HES1 prevents mammalian neural differentiation in the CNS by inhibiting bHLH transcription factors, such as MASH1, from binding to the E-box sequences of target genes. Thus, it is possible that an interplay of these transcription factors has an important role during mammalian neurogenesis. However, the possible bHLH partners of HES1 protein are not identified yet. (Ishibashi *et al.,* 1994 and references herein).

4.5. NUCLEAR HORMONE RECEPTORS

Many members of the nuclear hormone receptor superfamily mediate control of differentiation, development, and homeostasis in vertebrates by ligand dependent regulation of gene transcription. Among the best characterized members of the superfamily of nuclear hormone receptors are receptors for retinoids, thyroid hormones, steroid hormones, and glycocorticoids. Extensive studies have shown that the members of this superfamily activate and/or repress gene transcription through direct binding to discrete *cis*-acting elements known as hormone response elements. Beside the ligand dependent nuclear hormone receptors, a group of transcription factors with high homology to the nuclear hormone receptors have been isolated. These factors are known as orphan receptors which ligands are virtually unknown.

4.5.1. Structure and response elements

Nuclear hormone receptors have two specific common regions. DNA-binding domain (DBD) is a short, well conserved cysteine rich domain which forms two zinc finger structures. C-terminal part is known as ligand binding domain (LBD) (Fig. 3). This region is relatively well conserved between different members of the subfamilies. Based on the structure of zinc fingers and different functions, nuclear hormone receptors can be divided into subfamilies. First group consists of receptors for glucocorticoid, progesteron, androgen and mineralocorticoids (GR) and second for estrogen, thyroid hormone (TR), retinoids (RAR, RXR) and vitamin D3 (VDR) receptors (ER-TR) (Umesono *et al.,* 1989; Beato, 1989 and references herein).

DNA sequences responsive to nuclear hormone receptors have been found in many inducible genes. These hormone response elements contain two different palindrome motifs. The core consensus of GR response element half-site is GAACA. The DNA motif is recognized by first amino acid residues glycin-serin in the first zinc finger of these receptors. The ER-TR response element half-site is GGTCA with different spacing between the half-sites of each response element. The binding is possible with receptors which contain glutamic acid-glycin in the N-terminal zinc finger (Sthrale *et al.*, 1987). 1-3-4-5 rule of binding by RXR, VDR, TR, and RAR has been postulated. These receptors bind to A/GGGTCA direct repeats with a spacing of 1-3-4-5 bp-s respectively (Umesono *et al.*, 1991).

4.5.2. Retinoic acid receptors and receptors of retinoids

The vitamin A derivative retinoic acid (RA) and other retinoids regulate large spectrum of biological processes including differentiation and morphogenesis. RA and retinoid signals are mediated by nuclear receptors. Retinoic acid receptors (RAR) activate transcription in response to RA treatment by binding to the response element of the target genes (Petkovich *et al.*, 1987). RARs have a modular structure which comprises six different domains (Fig 3).

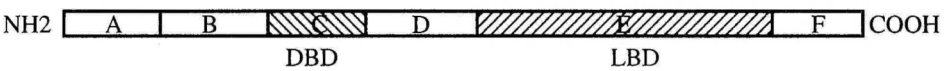


Figure 3. Schematic representation of nuclear hormone receptor transcription factors.

The N-terminal hypervariable region (A) and immediately adjacent, highly conserved region (B) carry cell and promoter specific activation functions. The DNA-binding domain (C) has two zinc finger structures which determine DNA binding specificity and are involved in receptor dimerization. This region is most highly conserved (93-95% identity between RARs). The hinge region (D) shows 61–74% of conservation. The C-terminal ligand binding domain (E) is the second most highly conserved (75–86%) and beside ligand binding it has a number of additional functions, including dimerization and transcriptional activation. The C-terminal region (F) mediates dimerization (Fig. 3). (De Luca, 1991; Luisi *et al.*, 1991; Zhang X.-K. *et al.*, 1991).

Receptors of retinoids known as retinoid X receptors (RXR) have been discovered. All three isolated receptors of this subfamily have low homology to RAR's. RXRs form active heterodimers with RAR s and increase DNA-binding specificity of RAR's. In addition, RXR's may function as auxiliary receptors for TRs and VDRs. RXRs bind DNA as homodimers in response to the 9-*cis*

retinoic acid treatment. These homodimers act in the regulation of transcription (Tran *et al.,* 1992). Analysis of retinoids and their corresponding receptors have revealed that both RAR and RXR may play an important role during development of vertebrates.

4.5.3. Orphan receptors

Orphan receptors share structural homology with nuclear hormone receptors described above and their ligands are virtually unknown.

4.5.3.1 Members of the subfamily and their structure

The chicken ovalbumin upstream promoter transcription factor (COUP TF) was first identified by O'Malley group from the HeLa and chick oviduct extracts. They have described low and high molecular weight forms (at least 8) of COUP TFs and identified two highly conserved proteins named COUP TF I and COUP TF II (Wang *et al.,* 1991). The COUP TFs homologues have been isolated from different organisms including *Drosophila* (Mlodzik *et al.,* 1990), zebrafish (Fjose *et al.,* 1993) and human (Miyajima *et al.,* 1988; Ladias and Karathanasis, 1991).

COUP TFs are classified on the basis of structure of DNA-binding domains as members of the ER-TR subfamily of nuclear hormone receptors. COUP TFs have relatively short N-terminal domain compared to other members of the superfamily. Probably, there might be less steric hindrance from the smaller domain allowing the freedom required for any structural changes in the flexible hinge region which is likely the reason of promiscuous DNA-binding of the orphan receptors. COUP TFs bind as functional homodimers to spatial variants of the GGTCA repeats with different spacing. In contrast, other nuclear hormone receptors from the ER-TR family require determined specific spatial organization of the binding sites. (1-2-3-4-5 rule of the binding for steroid hormone receptors) (Cooney *et al.,* 1992). It has been shown that COUP TFs lack dimerization domain in their DNA-binding domain which has been observed in other families of the superfamily (Ladias and Karathanasis, 1990).

4.5.3.2. Target genes and mechanism of action

Analysis of COUP TFs in a variety of systems demonstrate that these factors repress transcription mediated by thyroid and retinoid hormone receptors. Repression of the human transferrin gene (Sawaya and Schaeffer 1995), human acetytransferase chimeric promoter (Quirin-Stricer *et al.,* 1994) and peroxisome

proliferator-responsive element (Miyata *et al.,* 1993) transcription, have been demonstrated. As discussed above, RXR heterodimers with VDR, TR and RAR have higher DNA binding and transactivation activity than homodimers of these proteins. Important role of COUP TF I/RXR inactive heterodimers has been demonstrated in retinoid response pathway. These complexes have been suggested to restrict multitude of the retinoid responses in certain cell types (Tran *et al.,* 1992). COUP TF/RXR heterodimers repress whereas heterodimers of RARs/RXRs activate reporter gene transcription. It demonstrates competition for heterodimerization partners in retinoid response pathway (Cooney *et al.,* 1992). COUP TFs form active homodimers which may independently bind to variety of thyroid-steroid hormone receptor binding sites and repress hormonal induction of target gene. Thus, competition for the DNA binding sites is possible. In addition, COUP TF I has been shown to mediate an active silencing of reporter gene transcription. Transfer of putative ligand binding domain of COUP TF to GAL4 DBD resulted in repression of the reporter gene transcription. It confirmed that COUP TF possesses silencing function within its C-terminal domain (Cooney *et al.,* 1993). Thus, inhibition of transcription by COUP TFs can occur at three different levels: (1) by competition for DNA binding site occupancy; (2) the formation of nonfunctional heterodimers between COUP TF and RXR; (3) and by active silencing of transcription with the C-terminal domain of COUP TF s.

Besides inhibition of transcription, COUP TFs may also function as stimulatory transcription factors. Neurotransmitter dopamine may activate COUP TF I, which becomes converted to positive transcriptional regulator *in vitro* (Power *et al.,* 1991). COUP TF I stimulates transcription of arrestin gene by binding to a direct repeat with a 7-bp spacer located upstream of the transcription start site (Lu *et al.,* 1994). In combination with HNF4 transcription factor COUP TFs may stimulate activity of phosphoenole pyruvate carboxykinase gene (Hall *et al.,* 1995).

4.5.3.3. Role in differentiation

Complex expression patterns of COUP TF I and COUP TF II during development argue for their functional role in several developmental processes. In *Drosophila*, *seven-up* gene is required for the development of the embryonic central nervous system and specific photoreceptor cells of the eye (Mlodzik *et al.,* 1990). Detection of COUP TFs transcripts reveal discrete spatial and temporal expression pattern within special domains of the central and peripheral nervous system during zebrafish (Fjose *et al.,* 1993), chick (Lutz *et al.,* 1994) and mouse (Pereira *et al.,* 1995) embryogenesis. These data indicate that COUP TFs may play a crucial role in controlling a subset of neural-specific programs during development.

Very little is known about the functional role of COUP TFs during mammalian cell differentiation. Thyroid hormone and retinoids have been demonstrated to promote terminal muscle differentiation via activation of the muscle specific MyoD gene. Since the COUP TF II mRNA level decreases during the myoblast differentiation, it may be involved in the differentiation as a partner of these receptors. *In vitro* studies have revealed COUP TF II binding to thyroid hormone response elements of MyoD and myogenin genes. Thus, COUP TF II functions as an antagonistic regulator of myogenesis via direct effects on the tissue specific bHLH genes. These observations provide direct evidence for the developmental role of COUP TF II during mammalian cell differentiation (Muscat *et al.*, 1995).

5. AIMS OF THE PRESENT STUDY

A general objective of the present thesis was to describe the role of HLH and nuclear orphan receptor transcription factors during neurogenesis. The specific aims of the work were:

1. To characterize class A bHLH transcription factors ME1 and ME2 during neurogenesis:
   compare their expression patterns during mouse embryonic development and in adult brain;
   determine their dimerzation properties and specificity of binding to different E-boxes;
   characterize their interactions with a negative regulator (Id2);
   examine their activities in different neuronal cell lines.

2. To study blocking and reversion of neuronal differentiation using teratocarcinoma PCC7 cells as a model system:
   isolate genes which block retinoic acid induced neuronal differentiation and initiate proliferation;
   study orphan receptor COUP TF I induced block of neuronal differentiation;
   analyze effects of these regulators on the expression of neuron differentiation marker genes.

3. To characterize COUP TF II gene promoter; to analyze its cell type specific regulation by RA, dBcAMP and COUP TF transcription factors.

6. MATERIALS AND METHODS

Libraries

Postnatal day 1 mouse brain library in lambda ZAP II vector (Stratagene) (I, II), subtraction library of teratocarcinoma PCC7 (undifferentiated and neuronally differentiated) (Neuman *et al.,* 1995) (III),
mouse genomic library (Clontech) (V).

Plasmids

Bluescript SK(Stratagene),
Bluescript SK ME2 — mouse ME2 cDNA in EcoRV site (I),
Bluescript II KS DR1 — DR1 HRE in EcoRV site (Neuman *et al.,* 1995) (V),
Bluescript II KS β-RARE — β-RARE HRE in EcoRV site (V),
Bluescript II KS CRBP I — CRBP I HRE in EcoRV site (Neuman *et al.,* 1995).
pBLCAT2 (Luckow and Schütz, 1987),
pBLCAT2 (MEF)X4, TKCAT — four copies of MEF1 site carring E-box oligonucleotides (CACCTG) (II),
pBLCAT2 DR1 — DR1 HRE in HindIII-XbaI site (IV),
pBLCAT2 β-RARE — β-RARE HRE in HindIII-XbaI site (IV),
pBLCAT2 CRBP I — CRBP I HRE in HindIII-XbaI site (IV),
pCAT3N-4000/BglII, pCAT3N-1500/BglII, pCAT3N-621/BglII, pCAT3N -320/ApaI, pCAT3N-97/ApaI, pCAT3N-320/SacII, pCAT3N-40/SacII, pCAT3N-285/ApaI, pCAT3N-200/ApaI, pCAT3N-StyI/ApaI, — COUP TF II promoter fragments cloned into the unique BglII site (V).
pRcCMV (InVitrogen)
pRcCMV ME2 — full length ME2 cDNA subcloned into HindIII site (II),
pRcCMV(-216) — ME2 truncated expression vector (II),
pRcCMV ME1a — SpeI-BstEII full length was blunted and subcloned into HindIII site with HindIII linkers (II),
pRcCMV Id2 — contains Id2 full length cDNA XbaI-HindIII fragment (II),
pRcCMV E1A12S, pRcCMV E1A13S — adenovirus E1A12S cDNA and, E1A13S cDNA (gift from Nevins and Moran) (III),
pRcCMV E2F — E2F1 cDNA subcloned into HindIII and NotI site (Neuman *et al.,* 1995) (III),
pRcCMV RNP1 — RNP1 cDNA cDNA subcloned into HindIII and NotI site (III),
pRcCMV RNP2 — RNP2 cDNA subcloned into HindIII and NotI site (III),
pRcCMV ME1 — ME1 cDNA SpeI-BstEII full length was blunted and subcloned into HindIII site with HindIII linkers (Neuman *et al.,* 1993) (II, III),
pRcCMV COUP TF I — mouse COUP TF I cDNA from newborn library (IV),
pRcCMV COUP TF II — COUP TF II cDNA (V).

Cell Lines

cervical carcinoma C33A, human (American Type Culture Collection ATCC) (V),
glioblastoma C6, rat (ATCC) (V),
glioblastoma U373, human (ATCC) (V),
fibroblasts 3T3, mouse(ATCC) (V),
neuroblastoma N18 (II),
neuroblastoma-glioma NG108 (II)
teratocarcinoma PCC7, mouse (from S. E. Pfeiffer) (III, IV, V).

Antibody

anti-CAS linked to the carrier KLH, IgG fraction of the anti-rabbit polyclonal serum purified on Protein G-Sepharose Fast Flow affinity chromatography (II).

Methods

DNA cloning and sequence analysis (I, II, III, IV, V)
Screening of bacteriophage λ library (V)
Polymerase chain reaction (PCR) (II, III, IV)
Northern blotting (I, II, III, IV)
RNA *in situ* hybridization (I, II)
Electrophoretic mobility-shift assay (EMSA) (II, IV)
Primer extension (V)
β-galactosidase assay (V)
Chloroampenicol acetyl transferase (CAT) assay (II, IV, V)
RNase protection (V)
In vitro transcription site directed mutgenese (V)
Exonuclease III digestion (V)
Transfection of the cells (II, III, IV, V)
Cell culture (II, III, IV, V)

7. RESULTS AND DISCUSSION

7.1. CLASS A bHLH TRANSCRIPTION FACTORS ME1 AND ME2 DURING NEUROGENESIS (I, II)

The role of class A transcription factors during myogenesis and haematopoiesis as partners for tissue specific HLH transcription factors has been well characterized. The role of these factors during neurogenesis is still obscure. We isolated (ME2) and characterized (ME2 and ME1) two class A bHLH transcription factors which are expressed during mouse neurogenesis.

7.1.1. ME2 trascription factor — a member of class A bHLH transcription factor family (I)

The proneural genes were originally identified as essential regulators of *Drosophila* nervous system development. Many of these proneuronal genes were identified as helix-loop-helix transcription factors (Jan and Jan, 1990). Based on remarkable similarity between *Drosophila* proneuronal and mammalian bHLH genes it is likely that mammalian bHLH transcription factors are also involved in the neuronal development. In order to study the role of bHLH transcription factors during neuronal differentiation we cloned mouse ME2 cDNA which is related to *Drosophila* neurogenic gene *da*, from postnatal day 1 cDNA library. Sequence analysis revealed that ME2 is mouse homologue of human ITF2 bHLH transcription factor (Henthorn *et al.*, 1990).Homology of these factors is 98% at amino acid level and 94% at nucleotide level. ME2 has high homology to known class A bHLH transcription factors in the HLH region (80% to daughterless, 95% to ME1, 97% to E12 and 90% to E47) (Neuman *et al.*, 1993a)

7.1.2. Expression of ME1 and ME2 during mouse development (I, II)

Northern blot analyses demonstrated high levels of ME1 and ME2 mRNAs during embryonic development. During the early postnatal development ME1 is expressed at low level whereas ME2 expression remains high and gradually decreases as the mouse brain reaches maturity (Neuman *et al.*, 1993a).

At embryonic day 12, ME2 expression is detectable in the cerebral cortex, cerebellum, pons, medulla and spinal cord (I; Fig. 2A) as detected by *in situ* hybridization analysis. From the embryonic day 18 until adulthood, ME2 is expressed at high levels in the pyramidal cells of hippocampal layers CA1-CA4, and in the granular cell layer of the dentate gyrus(I; Fig. 2C). At postnatal day 7, expression of ME2 is remarkably high in the visual cortex which is undergoing a critical period of development (I; Fig. 2B) and in the subependymal region extending from the anterior lateral ventricle into the olfactory bulb (I; Fig. 2C, E18 and P7). Cerebellar granule and Purkinje neurons and the ventricular zone of the olfactory bulb express ME2 at all stages studied (Fig. 2C). In non-neuronal tissues, myotomes and developing limbs have the strongest hybridization signal at embryonic day 12.

We examined differences and similarities between ME1 and ME2 expression patterns in adult mouse brain. Parallel *in situ* hybridization analyses showed high levels of ME1 and ME2 expression in the internal granular cell layer of the cerebellum, and granular cells of the dentate gyrus of the hippocampus where also low level ME1 expression was detected (II; Fig. 2A, B). ME2 is expressed at high levels in pyramidal cells from CA1-CA3 whereas ME1 expression is barely detectable. ME1 expression was not detectable in the cerebral cortex where ME2 signals were observed.

Expression of ME1 and ME2 are generally distinct but show some spatial and temporal overlap in several regions. These data demonstrate that ME1 and ME2 are expressed in the regions of neuronal cell proliferation and initial differentiation. This expression pattern is similar to E2A expression with high level of mRNA in the ventricular zone (area of intensive cell proliferation) during embryonic development (Roberts *et al.,* 1993). Also G1, chick homologue of ME1, is expressed in regions of cell proliferation.

Our data demonstrate specific expression of ME2 during development and also distinct but overlapping expression patterns of ME1a and ME2 in the regions of neuronal plasticity in the adult brain. These observations suggest that ME1 and ME2 may have a regulatory function in developmental processes as well as during neuronal plasticity.

7.1.3. DNA binding properties of ME1 and ME2 *in vitro* (II)

Analyses of DNA binding and dimerization properties of ME1a and ME2 demonstrate that both factors form dimers and bind to the E-box sequences. Electrophoretic mobility shift assay was used to examine DNA binding of C-terminal fragment of the ME1a which contains bHLH motif and 24 amino acids. Truncated C-terminal fragment containing last 300 amino acids was used to analyze ME2 protein. ME1 and ME2 proteins were expressed in *E. coli* and

purified by metal affinity chromatography. An oligonucleotide containing the E-box found in the muscle creatine kinase enhancer (MEF1) was used to study ME1a and ME2 DNA binding properties. Our analyses demonstrate that ME1 and ME2 bind as homodimers to oligonucleotide carrying the MEF1 site. Specificity of ME1/DNA and ME2/DNA complexes was confirmed by supershift using polyclonal antibody raised against the CAS domain. Preimmune serum was used as a control (II; Fig. 3B, D).

MyoD protein was used to investigate whether ME1a or ME2 could form functional heterodimers with a class B bHLH protein. We observed formation of ME1a homodimers, ME1a/MyoD heterodimers, and MyoD homodimers. MyoD homodimer formation is likely a result of high concentration of the proteins used in this assay. Additionally we found that incubation of ME2 with ME1a or MyoD results in a formation of complex with an intermediate electrophoretic mobility suggesting existence of ME2/MEa heterodimers. These results demonstrate that ME1a, ME2 and MyoD form homo- and heterodimers *in vitro* and bind MEF1 DNA. DNA-binding specificity of ME1a and ME2 homodimers was analyzed using competition assays. 25-fold excess of each of several competitors was added to a reaction mix containing 40 ng of ME1a or ME2 protein and 40 fmoles of MEF1 probe. A strong competitor E-box element would decrease the signal from the labeled shifted DNA-protein complex. The specific sequences of the various E-boxes used for this assay are indicated in Table 1(II). The ME1a/DNA complex is competed successfully with a 25-fold excess unlabeled MEF1 oligonucleotide but not with non-specific DNA (II; Fig. 4A, lanes 3 and 5). DNA containing μE5 E-box competed equally well with the MEF1 probe whereas DNA containing μE2 E-box did not (II; Fig. 4A, lanes 6 and 7). The *c-fos* E-box located in the *c-fos* promoter reduced the specific binding by 50% (Fig. 4A, lane 9). A relatively weaker competition was obtained with the κ E2 E-box (II; Fig. 4A, lane 8). These data clearly demonstrate that ME1a homodimers bind different E-boxes with different affinities. The ME2/DNA complex was greatly reduced by the addition of 25-fold excess of unlabeled MEF1 oligonucleotide. A similar excess of nonspecific DNA did not affect the amount of specific binding (II; Fig. 4B, lane 3, 5). DNA containing μE5 E-box or μE2 binding site competed as well as MEF1 probe (II; Fig. 4B, lanes 6, 7). However, DNA containing the κ E2 site did not compete for binding with the MEF1 E-box (II; Fig. 4B, lane 8). The *c-fos* E-box slightly reduced the specific binding (II; Fig. 4B, lane 9). These results show that ME1a and ME2 homodimers recognize a variety of E-box sequences and also that they are characterized by significantly different DNA-binding specificities.

To elucidate specific functions of ME1a and ME2 in neurogenesis we examined their ability to activate transcription from a minimal promoter linked to several MEF1 E-boxes. We constructed two expression vectors pRcCMV ME1a and pRcCMV ME2, and a reporter plasmid TKCAT (MEF)X4 with four

MEF1 E-boxes upstream of the basic TK promoter. Cotransfection of ME1a or ME2 expression vector with reporter plasmid resulted in significant increase in CAT expression in N18 neuronal cells and in NG108 neuroblastoma-glioma cell line (II; Fig. 5). These data clearly indicate that both factors function as transcriptional activators in mammalian neuronal cells. In contrast, previous studies revealed transcriptional activation only with GAL4:ITF2 chimeric protein (ITF2 is human homologue of ME2) which activated transcription of a reporter plasmid with GAL4 binding sites (Henthorn *et al.*, 1990). In our experiments ME2 behaves as a strong activator on its own. Furthermore, truncated form of ME2, which lacks the first 216 N-terminal amino acids is still capable of stimulating the gene expression through the MEF1 E-box.

7.1.4. Interaction of ME1 and ME2 with the inhibitory factor Id2 *in vitro* (II)

Id -like proteins have been described to interfere differentiation by forming inactive heterodimers with bHLH proteins during embryonic development. We cotransfected expression plasmids pRcCMV Id2, pRcCMV ME1a or pRcCMV ME2 and CAT-reporter into neuroblastoma N18 cells to test effects of Id proteins on transcriptional activity of ME1a and ME2. A significant reduction in CAT activity was observed in the presence of the expression vector pRcCMV Id2. Thus, Id2 inhibits transcriptional activity of both ME1a and ME2 in neuronal cells. Since Id proteins are believed to prevent binding of bHLH proteins to DNA due to the formation of inactive heterodimers, we examined the protein-protein interactions of ME1a, ME2 and Id2 using EMSA and MEF-1 E-box DNA. We found that both ME1a and ME2 form not-DNA-binding complexes with Id2 protein.

7.2. BLOCK AND REVERSE OF DIFFERENTIATION (III, IV)

Neuroblasts exit cell cycle and become arrested in the G0 phase during neuronal differentiation. This transition is accompanied by switching off cell cycle specific genes. Our goal was to identify genes that are capable to dedifferentiate neuronally differentiated teratocarcinoma cells and initiate proliferation.

Teratocarcinoma PCC7 cells stop proliferation and differentiate into neuronal-like cells after treatment with retinoic acid (RA) alone or RA plus dibutyryl cyclic-AMP (dBcAMP). Differentiation is irreversible as removal of RA and dBcAMP does not cause dedifferentiation and reentry into the cell cycle. Thus, one could to study these cells as a model for nerve cell differentiation.

We used CMV promoter-based expression vectors in our experiments. CMV promoter activity is weak in proliferating PCC7 cells and is induced about 45 times after induction of differentiation by RA and dBcAMP in transient assays using bacterial CAT gene as a reporter. Neuronal differentiation of PCC7 cells is also inducible with RA alone and this treatment does not induce CMV promoter activity. By contrast, dBcAMP treatment alone stimulates CMV promoter activity but does not result in neuronal differentiation. pRcCMV eukaryotic expression vectors allow expression of introduced genes at high levels during neuronal differentiation of PCC7 cells treated with RA and dBcAMP, or at low levels during neuronal differentiation of PCC7 cells induced with RA alone. Thus, CMV promoter-based eukaryotic expression vectors allow expression of introduced genes at different levels during neuronal differentiation.

7.2.1. Blocking neuronal differentiation of the teratocarcinoma PCC7 cells (III)

We developed an expression cloning system for the detection and isolation of cDNAs which block differentiation and induce proliferation of neuronally differentiated teratocarcinoma PCC7 cells. As a first step, we generated subtraction cDNA library from undifferentiated versus differentiated PCC7cells. PCC7 cells stop proliferation and differentiate into neuron-like cells after treatment with RA and dBcAMP. Expression screening of the subtraction cDNA library was performed to isolate genes which block neuronal differentiation of PCC7 cells and induce proliferation. The cDNA library in pRcCMV expression vector was transfected into neuronally differentiated PCC7 cells. Cultures of neuronally differentiated cells did not contain proliferating cells as it was estimated using thymidine incorporation and cell cycle analysis. After 3 weeks of selection in the presence of G418, three proliferating clones were isolated. Cells of these clones continued proliferation in the presence of RA an dBcAMP and were morphologically identical to the original undifferentiated PCC7 cells. These clones were propagated in the presence of RA and dBcAMP and transfected cDNAs were isolated using PCR and retested for dedifferentiation and induction of proliferation. Sequence analysis revealed that one cDNA corresponds to mouse homologue of human E2F1; second cDNA, RNP1 (Regulator of Neuronal Proliferation), lacks significant homology to any GeneBank sequences: and third cDNA, RNP2, is 99,5% homologous to HLH transcriptional regulator Id4.

7.2.2. Expression of E2F, RNP1, and RNP2 in differentiating PCC7 cells (III)

Neuronal differentiation results in downregulation of E2F1, RPN1 and RPN2 expression in PCC7 cells. E2F1 mRNA level decreases slightly after 3 days of differentiation. The level of RNP1 mRNA decreases significantly during the first 24 h of differentiation and is undetectable by 48 h. Decrease of RNP2 mRNA levels occurs more gradually and mRNA disappears by the third day of differentiation (III; Fig. 2). E2F expression during initial step of differentiation is consistent with the data demonstrating that E2F expresses in the adult nervous system and may have a role during initial steps of differentiation (Zhao *et al.*, 1995).

7.2.3. Efficiency of isolated cDNAs to initiate proliferation (III)

Efficiency of isolated RNP1 and RNP2 cDNAs to dedifferentiate and initiate proliferation of neuronally differentiated PCC7 cells was tested by transfection of cDNAs into neuronally differentiated PCC7 cells (the same conditions used in screening the expression library) and by transfection into proliferating PCC7 cells (followed immediately by treatment with dBcAMP and RA to induce neuronal differentiation). Adenovirus oncogene E1A12S and E1A13S forms, bHLH transcription factor ME1 and the pRcCMV vector without insert were used as controls. ME1 is expressed in several proliferating cell types and is downregulated during differentiation. No proliferating clones were observed after transfection with ME1 cDNA or pRcCMV vector without insert. All three isolated cDNAs, E2F, RNP1 and RNP2 induced the formation of proliferating clones with the same efficiency under both experimental conditions. The efficiency of E1A to induce proliferation is approximately 10 times higher than for E2F, RNP1 and RNP2 cDNAs (III; Table 1).

7.2.4. Effect of overexpression of RNP1 and RNP2 on neuronal specific genes (III)

Expression of NF-L and GAP43 genes was studied to analyze the effect of RNP1 and RNP2 overexpression on neuronal differentiation. To normalize differences in the expression of RNP1 and RNP2 between individual clones we used randomly selected pools of RNP1 and RNP2 expressing clones. Northern blot analyses demonstrated expression of the RNP1 and RNP2 mRNAs after treatment with dBcAMP and RA as a result of CMV promoter induction by dBcAMP in transfected PCC7 cells. In non-transfected cells NF-L is undetect-

able in proliferating cells, and its level increases after differentiation begins. The induction of NF-L 3.5 kb mRNA occurs more rapidly than that of 2.5 kb mRNA. Overexpression of RNP1 results in the delay in the induction of NF-L gene, and this induction is transient. The increase of NF-L mRNA is detectable on the second day after induction with RA and dBcAMP, and the mRNA level decreases on the third day. No expression of NF-L was detected in RNP2 pools (III; Fig. 3).

GAP-43 is expressed at relatively low levels in undifferentiated PCC7 cells, and its mRNA levels rapidly increase after induction with RA and dBcAMP. No induction of GAP-43 was detected in RNP1 overexpressing pools, after treatment with RA and dBCAMP; moreover, its mRNA becomes undetectable after 1 day of treatment. No expression of GAP-43 was detected in undifferentiated RNP2 overexpressing pools or after induction with RA and dBcAMP (III; Fig. 3).

We isolated three different genes which overexpression blocks neuronal differentiation and induces proliferation of neuronally differentiated PCC7 cells. E2F family factors are known to be key regulators of cell cycle progression. Transcription factor E2F1 plays a central role in the cell cycle regulation through its ability to activate genes necessary for G1/S phase transition. Activity of E2F is regulated by formation of transcriptionaly inactive or inhibitory complexes with pRB family members. During the G1 phase of the cell cycle pRB becomes hyperphosphorylated by cyclin/cdk complexes which leads to the release of transcriptionally active E2F (Beijersbergen *et al.*, 1995). Overexpression of E2F may lead to a situation where pRb is limiting and thus results in the excess of active E2F which may trigger the initiation of the cell cycle. Alternatively, excess of E2F could sequester pRBs and block expression of the differentiation specific genes as it has been demonstrated for the muscle cell differentiation. Knock-out experiments demonstrate that pRB is necessary for normal neurogenesis. The RNP2 is highly homologous to Id4, a helix-loop-helix transcriptional regulator which may block neuronal differentiation by forming inactive heterodimers with bHLH transcription factors that are expressed in neuronally differentiating cells.

Our analysis of neuronal-specific genes demonstrate that overexpression of RNP1 and RNP2 interferes with the induction of NF-L and GAP43 gene expression. RNP2 suppresses expression of the neural marker genes NF-L and GAP43 after induction with RA and dBcAMP. In contrast overexpression of RNP1 does not suppress expression of NF-L and does suppress GAP43. These observations argue that isolated genes RNP1 and RNP2 have at least partially different mechanisms of action during blockage of neuronal differentiation. Delayed and transient induction of NF-L gene in RNP1 overexpressing cells after induction with RA and dBcAMP suggest that it does not completely block signal transduction at the initial stages of neuronal differentiation. However, overexpression of RNP1 suppresses expression of GAP43 which demonstrates

that RNP1 completely blocks expression of at least one neuronal specific gene in PCC7 cells. Our results support the hypothesis that different neuronal genes are regulated by a different mechanisms.

7.2.5. Orphan receptor COUP TF I arrests the morphological differentiation of PCC7 cells (IV)

Above we demonstrated that overexpression of E2F, RNP1 and RNP2 results in the suppression of neuronal differentiation of PCC7 cells. The neuronal differentiation of PCC7 cells is a result of RA and dBcAMP treatment. Reason of the differentiation blockade could be suppression of RA effects by elevated levels of these factors during RA induced differentiation. Nuclear hormone receptors (RARs and RXRs) mediate RA effects on gene expression. COUP TFs are expressed in the developing nervous system and interact with nuclear hormone receptors to regulate expression of different genes. Importance of ligand-activated nuclear hormone receptors during neurogenesis is well described. At the same time, role of orphan receptors is unknown. To study the possible function of COUP TF I during neuronal differentiation, we generated COUP TF I overexpressing PCC7 cell lines and analyzed RA-induced neuronal differentiation of these cells.

Using randomly selected clones we analyzed correlation between COUP TF I expression and morphological differentiation in response to RA and dBcAMP induction. Two of the tested clones demonstrated induced expression of COUP TF I mRNA and failed to differentiate morphologically (IV; Fig 2A, Fig. 3). To verify if the loss of morphological differentiation is a result of COUP TF I expression or selection of aberrant clones, we examined those clones during neuronal differentiation induced by RA alone. No inhibition of morphological differentiation of PCC7 clones were observed and no COUP TF I mRNA expression detected after treatment with RA alone. In contrast, expression of COUP TF I was induced after induction with dBcAMP plus RA as well as with dBcAMP alone (IV; Fig. 2B). These results demonstrate that overexpression of COUP TF I blocks neuronal differentiation of PCC7 cells.

Flow cytometric analyses were performed to characterize changes in the cell cycle during differentiation of control and COUP TF I overexpressing PCC7 cells. During normal growth of wild type PCC7 cells and COUP TF I overexpressing clones, the percentage of cells in G1 phase was 41–42%, in S phase 36–38%, and in G2 phase 20–24%. Differentiation of wild type PCC7 cells blocks cell cycle in G1 phase and percentage of cells in G1/G0 reaches 98% after three days of treatment. In contrast, treatment of COUP TF I overexpressing cells with RA and dBcAMP results in a significantly smaller reduction

of the proliferation rate: the portion of cells in G1/G0 phase increase from 41–42% in day 0 to 64–65% at day three (IV; Fig. 5, Table 1).

7. 2.6. Effect of COUP TF I on the expression of neuronal marker genes (IV)

We characterized expression of NF-L, GAP43 and MAP2 genes during neuronal differentiation of wild type and COUP TF I overexpressing PCC7 cells. Differentiation of PCC7 cells results in induction of NF-L and MAP2 genes 48 h and GAP43 gene 12 h after treatment with RA and dBcAMP. Overexpression of COUP TF I results in no changes of NF-L expression, delayed induction of GAP43 gene expression, and blocked expression of MAP2 gene after induction of neuronal differentiation with RA and dBcAMP. Induction of neuronal differentiation without stimulation of COUP TF I expression results in stimulation of all neuronal marker genes similar to control (IV; Fig 4).

7.2.7. Induction of RARE enhancers in COUP TF I-overexpressing cells (IV)

COUP TFs have demonstrated to form inactive heterodimers with RXRs and bind to retinoic acid response elements (RARE) from different genes as homodimers to repress the retinoic acid response. To investigate whether COUP TF I blocks RA signaling pathway in neuronally differentiating PCC7 cells, we analyzed the activities of CAT reporter constructs containing different RAREs in front of the thymidine kinase promoter in COUP TF I overexpressing cells. We analyzed three different RAREs: β-RARE, a direct repeat with a 5-bp spacer that is activated by RARα; the CRBP I-RARE, a direct repeat with a 2-bp spacer that is optimally activated by RAR/RXR heterodimers but not RXR homodimers and the DR1, a direct repeat that contains a 1-bp spacer and has a high affinity to COUP TF I and RXRs. All three reporter constructs are inducible in PCC7 cells with RA or RA and dBcAMP, and dBcAMP potentiates the effect of RA. In COUP TF I overexpressing clones treated only with RA, induction of β-RARE and CRBPI is reduced and no induction was detected from DR1. Induction of COUP TF I by adding dBcAMP to RA results in further inhibition of β-RARE- and CRBPI-mediated transcription (IV; Fig. 6). Interestingly, transcription activity of the β-RARE containing reporter is inhibited in COUP TF I overexpressing cells. This result contradicts to the data of the cotransfection experiments with COUP TF I and RARα which demonstrate that COUP TF I does not inhibit induction of β-RARE by RARα (Tran *et al.*, 1992). All three RARs and RXRα and RXRβ have been described in differentiating

PCC7 cells. In differentiating PCC7 cells, expression of all three RARs and RXRs is induced and it is unknown which of the RAR/RXR complexes are involved in the induction of β-RARE enhancer in our experiments. It is possible that β-RARE induction is mediated by RAR/RXR complexes other than RARα homodimers and that these complexes are differently affected by COUP TF I.

7.3. CHARACTERIZATION OF THE PROMOTER REGION OF COUP TF II GENE (V)

COUP TF II is expressed in a complex spatio-temporal pattern during development of several organ systems including nervous system. This complex expression pattern argues for a different mechanisms of COUP TF II gene regulation in different cell types.

7.3.1. Localization of the 5' regulatory region

We isolated and cloned 1.5 kb 5' end fragment of the COUP TF II gene. The transcription start site was mapped by primer extension and RNase protection analyses using RNA isolated from embryonic day 11, 13, and 15 mouse embryos. Both methods demonstrated the presence of several transcription start sites. The 5'-proximal region contains several consensus TATA box sequences in the location where all the transcription start sites are localized. To determine the sequences that are essential for transcription of mouse COUP TF II gene, various portions of the 5'-flanking region were fused to the bacterial CAT gene as a heterologous reporter gene. The reporter plasmids we transfected transiently into the mouse teratocarcinoma PCC7, mouse fibroblast 3T3, and human cervical carcinoma C33A cells. This deletion analysis demonstrated that the COUP TF II basal promoter is localized in the 200 bp region upstream from the major transcription start site (V; Fig. 1A, 1B).

7.3.2. The regions responsible of RA and dBcAMP effect

Since COUP TF II is involved in the modulation of retinoic acid responses and may function as a part of the regulatory loop, we analyzed the effect of all-*trans* RA on the activity of its promoter. We used CAT-reporter constructs to identify regions responsible for RA and cAMP. Expression of all three RAR and RXR genes and induced expression of RARs during RA treatment has been demonstrated in PCC7 cells. Transient transfection of COUP TF II promoter-CAT

plasmids containing different fragments of 5' regulatory region demonstrated that RA treatment induces promoter activity of all plasmids which contain more than 40 bp of the promoter region and localizes responsive region between nucleotides –97 to –40. The same promoter region is also responsive for dBcAMP treatment. Cotreatment with RA and dBcAMP stimulated COUP TF II promoter activity about 15–20-fold (V; Fig. 2). The additive effect of RA and dBcAMP suggest that RA and dBcAMP have different pathways to stimulate COUP TF II promoter activity. Sequence analyses of –97 to –40 bp COUP TF II promoter region revealed a cAMP response element (CRE) and a possible consensus sequence for hormone response element (HRE) localized next to each other. Mutations were introduced into these elements to assess their functionality. Mutations which destroyed proposed HRE did not affect inducibility of COUP TF II promoter activity, however mutations in CRE or in both CRE and HRE together completely blocked the effect of dBcAMP and cooperative induction by RA and dBcAMP (V, Fig. 3). These results demonstrate that RA has indirect effect on the COUP TF II promoter activity in PCC7 cells.

7.3.3. Effects of RA and dBcAMP on the COUP TF II promoter activity in different cell lines

CAT assays demonstrated different effects of RA and RA plus dBcAMP treatment to the COUP TF II promoter in different cell lines. In rat glioblastoma C6 and human glioblastoma U373 cells the activity of promoter-CAT constructs decreased but in mouse fibroblast 3T3 and human cervical carcinoma C33A cell lines activities did not changed after treatment (V; Fig. 4A and B). Thus, analyses of COUP TF II promoter activity in different cell lines revealed that all-*trans* retinoic acid either stimulates (PCC7), suppresses (C6, U373) or has no effect (3T3, C33A) on transcription. These results suggest that COUP TF II is involved at least in two different functions of retinoids. First, in cells where COUP TF II promoter activity is stimulated by retinoids, it may function as a part of negative feedback loop to suppress effects of retinoids. Second, in cells where COUP TF II promoter activity is suppressed by retinoids, the retinoid response may be longer lasting. COUP TF II may also be involved in timing the switches of retinoic acid mediated gene regulation.

7.3.4. Possible regulation of RA induced COUP TF II promoter activity by COUP TFs (V)

Transcription factors COUP TF I and COUP TF II block the stimulatory effect of RA on transcription of several genes. We demonstrated that transcription from COUP TF II promoter is induced by RA which likely suggests feedback mechanism between RA level and COUP TF II expression level in PCC7 cells. We examined the possibility that COUP TF I and COUP TF II block the stimulatory effect of RA on COUP TF II promoter. Promoter-CAT construct and COUP TF I or COUP TF II expression vectors were cotransfected into PCC7 cells. As a positive control, plasmid containing a RARβ2 promoter lacZ reporter construct that has be shown to be inducible with activated RARs and RXRs was used. CAT assays demonstrated that both COUP TFs slightly suppress RA plus dBcAMP induced COUP TF II promoter activity. In contrast, COUP TF I and COUP TF II completely inhibited RA plus dBcAMP induced RARβ2 promoter. (V; Fig. 4C). This is an additional indication that RA does not directly regulate COUP TF II promoter activity through activation RAR/RXR complexes.

8. SUMMARY AND CONCLUSIONS

It has been shown that helix-loop-helix transcription factors regulate expression of tissue specific genes during cellular differentiation. In particular, several bHLH transcription factors are regulators of cell fate. We isolated and characterized cDNA encoding mouse class A bHLH transcription factor ME2. Sequence analyzes of ME2 revealed high homology of bHLH domain with the other known class A bHLH transcription factors. ME2 is expressed in cerebral cortex, Purkinje and granular cell layers of the cerebellum, olfactory neuroephithelium, pyramidal cells of hippocampal layers CA1-CA4, and in the granular cells of dentate gyrus. To evaluate differences and similarities between two class A bHLH transcription factors ME1a and ME2, we studied expression patterns of ME1a and ME2 in the adult brain. *In situ* hybridization analyses revealed distinct but in some regions overlapping pattern of these two bHLH transcription factors in the areas where neuronal plasticity occurs.

DNA-binding assay results show that both proteins bind to E-boxes as homo- and heterodimers with remarkable differences in DNA-binding specificities. These differences in DNA-binding properties suggest that above mentioned factors regulate transcription through selective interactions with different binding sites of target gene. *In vitro* DNA-binding assay revealed ME1a and ME2 interaction with dominantly negative transcription factor Id2, also. Id2/ME1a and Id2/ME2 heterodimers do not bind to the E-box sequences because the DNA-binding ability of these class A transcription factors is abolished by inhibitory factor Id2.

We tested activities of these HLH transcription factors in neuronal cell lines. Class A bHLH transcription factors (ME1a or ME2) activate transcription of reporter gene in neuronal cells. and their coexpression of ME1a or ME2 with Id2, a HLH transcription factor, interferes capability of these bHLH transcription factors to enhance transcription of the reporter gene.

Based on our data, we hypothesize that ME1a and ME2 may activate gene expression of different target genes and therefore are likely to be differently involved during neurogenesis.

We studied retinoic acid (RA) induced neuronal differentiation of teratocarcinoma PCC7 cells. These cells differentiate into neuron-like cells after induction with RA or RA plus dibutyryl c-AMP (dBcAMP) and are used as a model system of neuronal differentiation. Our approach was to identify factors that induce proliferation of neuronally differentiated teratocarcinoma PCC7 cells. Mouse E2F1 and novel cDNAs RNP1 and RNP2 (Regulator of Neuronal Pro-

liferation) were isolated. We demonstrate that overexpression of E2F1, RNP1, and RNP2 cDNAs in neuronally differentiated PCC7 cells results in blocking differentiation and initiation of proliferation.

Role of COUP TF orphan receptors in neurogenesis is virtually unknown. COUP TF transcription factors regulate activity of ligand-activated nuclear hormone receptors or function independently in the regulation of gene expression. Expression of COUP TFs and interactions with nuclear hormone receptors have been observed in the developing nervous system. We generated COUP TF I overexpressing teratocarcinoma PCC7 cell lines to study possible function of COUP TF I during neuronal differentiation. COUP TF I overexpression results in the blockade of morphological differentiation after induction to differentiate. Also, cells overexpressing COUP TF I do not stop proliferating after RA and dBcAMP treatment and possess suppressed transcriptional activation from different RA response elements. Our observations suggest that COUP TF I plays an important role in the control of neurogenesis by modifying activities of RAR/RXRs which regulate expression of neuronal-specific genes as well as genes regulating cessation of proliferation and initiation migration and differentiation of neuroblasts.

We tested effects of RNP1, RNP2 and COUP TFI on the expression of differentiation specific genes. Expression of RNP1 and RNP2 blocks NF-L and GAP43 gene transcription in differentiating PCC7 cells. In contrast, COUP TF I expression does not affect NF-L gene, although delays expression of GAP43 gene and represses MAP2 gene expression completely. These observation indicate that initiation of differentiation specific genes is allowed during proliferation and is not necessarily downregulated by blockade of the neuronal differentiation. These data also support the hypothesis that different neuronal genes are regulated by different mechanisms during differentiation.

COUP TF II has a complex expression pattern during development suggesting that different mechanisms are involved in the regulation of its expression. The molecular mechanisms responsible for the spatial and temporal expression of COUP TFs are still poorly understood. We isolated and analyzed the 5' regulatory region of the mouse COUP TF II gene and demonstrated that the basal promoter is localized in a –200 bp region 5' from major transcription start sites.

We examined the cell type specific effects of RA and dBcAMP on COUP TF II promoter and revealed different cell type specific effects from repression to activation of the gene expression. We localized the cAMP response element to the region localized 74 nucleotide upstream from the major transcriptional start site. Also, *in vitro* promoter analysis demonstrate that the effect of RA is not directly mediated by the binding of RARs or RXRs to the studied promoter sequences. Weak inhibition of RA induced COUP TF II promoter activity was observed by coexpression of COUP TF I or COUP TF II transcription factors.

We conclude that HLH transcription factors and nuclear hormone receptor

transcription factors have an important role in the neurogenesis. Our data indicate regulatory role of ME1a, ME2 and Id2 in neurgenesis like it has been demonstrated for the related bHLH transcription factors during myogenesis and haematopoiesis. Studies on teratocarcinoma PCC7 cells demonstrated that E2F1, a HLH transcription factor and RNP2 a HLH transcription factor Id4 homologue are able to interfere neuronal differentiation and support proliferation in the presence of the inductor, RA. Retinoids regulate expression of cell-type specific genes through specific nuclear hormone receptors, RARs, RXRs. COUP TFs are known as inhibitory molecules of nuclear hormone receptor transcription factors and in our experiments COUP TF I arrested neuronal differentiation of PCC7 cells. We observed the role of HLH transcription factors during neuronal differentiation and neuronal development as well as role of COUP TF I during induced neuronal development.

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10. *HELIX-LOOP-HELIX*- JA TUUMASEOSELISTE HORMOONIRETSEPTOR-TRANSKRIPTSIOONI-FAKTORITE OSA NEUROGENEESIS

Kokkuvõte

Rakkude diferentseerumise mehhanismi spetsialiseerunud kudedeks pole veel ammendavalt kirjeldatud. Protsessi alguseks on prolifereeruvate rakkudega toimuvad muutused mis viivad rakutsükli katkemisele (G0-faas) ja sellele järgnevale suunatud geenide avaldumisele, kuni kasvuomaselt ekspresseerunud geenide kogum asendub spetsialiseerunud rakule omasega.

Rakutsükli peatumine G0-faasis on diferentseerumise sõlmpunktiks. Mõnede transkriptsioonifaktorite osa selles on teada. Näiteks *helix-loop-helix* (HLH) perekonna E2F-valgu ja retinoblastoomi valgu pRb vahekord määrab, kas rakud jätkavad jagunemist või diferentseeruvad. Teine HLH perekonna valk Id2 oletatavasti osaleb diferentseerumisel. Id2 võib seonduda pRb-le vahetult või toimib tsükliini kinaaside (p16, p21) vahendusel. Imetajate rakkude diferentseerumise spetsiifilisust tagavad aluselise domeeniga HLH (bHLH) valgud. Osa nendest valkudest ekspresseerub koeomaselt, teine osa koetüübist sõltumatuna. Transkriptsiooni reguleerivad nad kui homo- või heterodimeerid, seondudes reguleeritavate geenide promootorite kindlatele järjestustele (E-box). Samuti seonduvad nad eespoolmainitud Id-valkudega, kusjuures vastavad heterodimeerid ei aktiveeri transkriptsiooni. Nii lihasrakkude kui ka vereloomerakkude kujunemine toimub bHLH-faktorite kaasabil (MyoD/E2A). Hormoonide mõju diferentseerumisele vahendavad ka tuumaseoselised hormooniretseptorid.

Drosophila neurogeneesis on olulised HLH ja tuumaseoselised hormooniretseptor-transkriptsioonifaktorid. Vastavate faktorite homolooge on leitud ka selgroogsetel. Kõrgelt konserveerunud talitluslikult olulised piirkonnad viitavad nende faktorite talitluslikule sarnasusele nii selgrootutel kui ka selgroogsetel loomadel.

Käesolevas töös uurisime hiire bHLH-transkriptsioonifaktorite ME1 ja ME2 ekspressiooni areneval hiire lootel ja täiskasvanud looma ajus. Nimetatud faktorid ekspresseeruvad nii närvikoe arengu seisukohalt olulistes piirkondades kui ka täiskasvanud looma aju neuraalse plastilisuse piirkondades. *In vitro* analüüs näitab, et need faktorid võimendavad mRNA sünteesi neuraalsetes rakkudes ja Id2-faktor pärsib selle mõju. Erinevatele vaadeldud *E-box*-järjestustele seondu-

vad uuritud faktorid erinevalt. Viimatinimetatu viitab uuritud faktorite võimalikule talitluslikule eripärale ja nende regulatoorsele tähtsusele diferentseerumisel.

Neuraalse diferentseerumise mudelina võib vaadata teratokartsinoomi PCC7-rakuliini. Nimetatud rakuliin diferentseerub retinoolhappega (RA) mõjutamisel neuronitelaadseteks rakukogumikeks. Eraldasime PCC7-rakuliinist neuraalset diferentseerumist pärssivad geenid. Need olid hästituntud E2F-transkriptsioonifaktor ja seni iseloomustamata faktorid RNP-1 ja RNP-2 (viimatinimetatu on Id4 homoloog), mis peatasid diferentseerumise ja suunasid diferentseerunud rakud prolifereeruma. RA mõju rakus on vahendatud retinoolhappe- ja retinoidiretseptorite ja orfanretseptorite, näiteks COUP TF-ide (*chicken ovalbumine upstream promoter transcription factor*) koos- ja vastumõjudega. Analüüsisime COUP TF I transkriptsioonifaktori poolt indutseeritud diferentseerumiseblokki PCC7-rakuliinil. Osutus, et COUP TF I kõrge tase nendes rakkudes takistab morfoloogilist diferentseerumist. Uuritud diferentseerumist pärssivate faktorite (RNP1, RNP2 ja COUP TF I) mõju markergeenidele (NF-L, GAP43 ja MAP2) PCC7-rakuliinis on erisugune, alates täielikust ekspressiooni peatumisest (RNP2 mõju NF-L, GAL43 ja COUP TFI mõju MAP2 ekspressioonile), osalise peetuse (RNP1 mõju NF-L ekspressioonile) ja täieliku puudumiseni (COUP TF I mõju GAP43 ekspressioonile). Saadud tulemus viitab erinevale mehhanismile uuritud markergeenide ekspressiooni suunamisel ja sellele, et diferentseerumisel avalduvate geenide ekspressioon võib alata juba jagunevas rakus.

Retinoolhappe vallandatud diferentseerumisel on oluline osa orfanretseptoritel. Et uurida orfanretseptori COUP TF II geeni regulatsiooni, isoleerisime selle geeni promootori, lokaliseerisime minimaalse transkriptsiooni aktiveeriva piirkonna sellel ja oletatavad cAMP ja RA mõju vahendavad DNA järjestused. Osutus, et RA mõju vaadeldava geeni promootorile on kaudne. Promootori analüüs erinevatel rakuliinidel (teratokartsinoom PCC7, hiire fibroblastid 3T3, roti glioblastoom C6, inimese glioblastoom U373 ja emakakaelakartsinoom C33A) näitab RA transkriptsiooni võimendavat toimet PCC7-, pärssivat glioblastoomidel C6- ja U373- ja efekti puudumist 3T3- ja C33A-rakuliinidel. Orfanretseptorite COUP TF I ja COUP TF II mõju uuritud promootorile on nõrgalt pärssiv. Selles töös näitame, et HLH transkriptsioonifaktorid ja COUP TF osalevad närvisüsteemi arengus.

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PUBLICATIONS

I

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MOLECULAR BRAIN RESEARCH

Short Communication

Expression of basic-helix-loop-helix transcription factor ME2 during brain development and in the regions of neuronal plasticity in the adult brain

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Abstract

We report the isolation of a cDNA encoding the mouse class A bHLH transcription factor ME2 and the analysis of its expression. ME2 is expressed in the cerebral cortex, Purkinje and granule cell layers of the cerebellum, olfactory neuroepithelium, pyramidal cells of hippocampal layers CA1–CA4, and in the granular cells of the dentate gyrus. The specific expression of ME2 during development and in the regions of neuronal plasticity in the adult brain suggest that ME2 may have a regulatory function in developmental processes as well as during neuronal plasticity.

Key words: Brain development; Helix-loop-helix; Gene expression; Plasticity; Mouse development

Basic-helix-loop-helix (bHLH) transcription factors play important roles during development in many organisms [3]. These proteins have a conserved helix-loop-helix (HLH) domain essential for dimerization as well as a basic domain which mediates DNA binding [7] to a common hexanucleotide sequence known as the E-box (CANNTG) [8]. Class A bHLH proteins can form homo- and heterodimers [7] and contain an additional loop-helix (LH) domain used for transcriptional activation [11] and a class A specific (CAS) domain of unknown function [13]. Three class A bHLH genes homologous to *Drosophila* daughterless are expressed in the developing and adult nervous system of mammals. E2A gene is expressed at high levels in areas of cell proliferation whereas its expression is undetectable in nonproliferative regions of the brain and spinal cord [12]. ME1 gene [9], also known as HTF-4 [13], HEB [2], ALF1 [10], and REB [4], is expressed at relatively high levels in proliferating neuroblasts and decreases significantly when cells initiate differentiation [9]. In this paper, we describe the expression of ME2, the mouse homolog of human ITF-2 gene.

Cloning. ME2 cDNA [9] was used to screen a postnatal day 1 mouse brain cDNA library in lambda ZAPII vector (Stratagene). Filters were washed at high stringency conditions (0.2 × SSC, 65°C). Fifteen clones were isolated and characterized. For analyses and sequencing, the phage DNA with insert was converted to its plasmid form using an in vivo excision protocol (Stratagene).

In situ hybridization. In situ hybridization was performed as described elsewhere [9]. The hybridization buffer contained 5 × SSPE, 50% formamide, 12% dextran sulfate, yeast tRNA, and Denhardt's solution. The entire coding sequence of ME2 was subcloned into the Bluescript vector SK (Stratagene) and used to synthesize single-stranded antisense and sense RNA probes using T3 and T7 RNA polymerases, respectively. Probes were partially hydrolyzed with 0.2 M NaOH on ice for 30 min and neutralized with 1 M acetic acid. The hybridization was performed at 50°C for 15 h followed

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                                                                CAAAGTGCCTCCGATGGATGAACCCGGCAAACCCTG   36
AACTGTTCAGGCTTCAGATTGTAACTGGCGATCTGAGGGGGAAAATGAGGTGCTCGGTGAATTTTCCTTGGTTTGTGTGATTTTGCTAAA  126
ATGCATCACCAACAGCGAATGGCTGCCTTAGGGACGGACAAAGAGCTGAGTGATTTACTGGATTTCAGTGCGATGTTTTCGCCTCCTGTA  216
M  H  H  Q  Q  R  M  A  A  L  G  T  D  K  E  L  S  D  L  L  D  F  S  A  M  F  S  P  P  V    30

AGCAGTGGGAAAAATGGACCAACTTCTTTGGCGAGTGGACATTTCACTGGCTCAAATGTAGAAGACAGAAGTAGCTCAGGGTCCTGGGGA  306
S  S  G  K  N  G  P  T  S  L  A  S  G  H  F  T  G  S  N  V  E  D  R  S  S  S  G  S  W  G    60

ACTGGAGGCCATCCAAGCCCGTCCAGGAACTATGGAGATGGGACTCCCTATGACCACATGACTAGCAGGGATCTTGGGTCACACGACAAT  396
T  G  G  H  P  S  P  S  R  N  Y  G  D  G  T  P  Y  D  H  M  T  S  R  D  L  G  S  H  D  N    90

CTCTCTCCACCTTTTGTCAATTCCAGAATACAAAGTAAAACAGAAAGGGGCTCATACTCATCTTATGGGAGAGAAAACGTTCAGGGTTGC  486
L  S  P  P  F  V  N  S  R  I  Q  S  K  T  E  R  G  S  Y  S  S  Y  G  R  E  N  V  Q  G  C    120

CACCAGCAGAGTCTCCTCGGAGGGGACATGGATATGGGCAATCCAGGAACCCTTTCGCCCACCAAACCTGGCTCCCAGTACTATCAGTAT  576
H  Q  Q  S  L  L  G  G  D  M  D  M  G  N  P  G  T  L  S  P  T  K  P  G  S  Q  Y  Y  Q  Y    150

TCAAGCAATAATGCCCGCCGGAGGCCTCTTCACAGTAGTGCCATGGAGGTACAGACAAAGAAAGTCCGAAAAGTTCCTCCGGGTTTGCCG  666
S  S  N  N  A  R  R  R  P  L  H  S  S  A  M  E  V  Q  T  K  K  V  R  K  V  P  P  G  L  P    180

TCTTCAGTCTACGCTCCTTCAGCCAGCACTGCCGACTACAACAGGGACTCGCCAGGCTATCCTTCCTCCAAGCCAGCAGCCAGCACTTTC  756
S  S  V  Y  A  P  S  A  S  T  A  D  Y  N  R  D  S  P  G  Y  P  S  S  K  P  A  A  S  T  F    210

CCTAGCTCCTTCTTCATGCAAGATGGCCATCACAGCAGCGACCCTTGGAGCTCCTCCAGCGGGATGAATCAGCCCGGCTACGCAGGGATG  846
P  S  S  F  F  M  Q  D  G  H  H  S  S  D  P  W  S  S  S  S  G  M  N  Q  P  G  Y  A  G  M    240

CTGGGCAATTCTTCTCATATCCCACAGTCCAGCAGCTACTGTAGCCTGCATCCACATGAACGTTTGAGCTATCCATCCCACTCCTCGGCA  936
L  G  N  S  S  H  I  P  Q  S  S  S  Y  C  S  L  H  P  H  E  R  L  S  Y  P  S  H  S  S  A    270

GACATCAACTCCAGTCTTCCTCCGATGTCCACGTTCCATCGTAGTGGCACAAACCATTACAGCACCTCTTCCTGCACACCCCCTGCCAAC 1026
D  I  N  S  S  L  P  P  M  S  T  F  H  R  S  G  T  N  H  Y  S  T  S  S  C  T  P  P  A  N    300

GGAACAGACAGTATAATGGCAAACAGAGGAACTGGGGCAGCAGGCAGCTCGCAGACTGGAGACGCTCTGGGGAAAGCCCTAGCTTCGATC 1116
G  T  D  S  I  M  A  N  R  G  T  G  A  A  G  S  S  Q  T  G  D  A  L  G  K  A  L  A  S  I    330

TATTCTCCTGACCACACGAACAACAGCTTTTCCTCCAATCCTTCAACTCCTGTGGGCTCCCCTCCTTCACTCTCAGCAGGCACAGCTGTT 1206
Y  S  P  D  H  T  N  N  S  P  S  S  N  P  S  T  P  V  G  S  P  P  S  L  S  A  G  T  A  V    360

TGGTCTAGAAATGGAGGACAGGCCTCGTCATCTCCCAATTATGAAGGACCCTTGCACTCACTGCAAAGCCGAATCGAAGACCGTTTGGAA 1296
W  S  R  N  G  G  Q  A  S  S  S  P  N  Y  E  G  P  L  H  S  L  Q  S  R  I  E  D  R  L  E    390
                                  LOOP-HELIX
AGACTGGACGATGCGATTCATGTTCTCCGGAACCACGCAGTGGGCCCGTCCACAGCTGTGCCTGGTGGCCATGGGGACATGCATGGGATC 1386
R  L  D  D  A  I  H  V  L  R  N  H  A  V  G  P  S  T  A  V  P  G  G  H  G  D  M  H  G  I    420

ATGGGACCCTCCCACAACGGAGCGATGGGTAGCCTGGGCTCAGGGTACGGAACTAGTCTTCTCTCAGCCAACAGACACTCGCTCATGGTT 1476
M  G  P  S  H  N  G  A  M  G  S  L  G  S  G  Y  G  T  S  L  L  S  A  N  R  H  S  L  M  V    450

GGGGCCCACCGTGAAGATGGCGTGGCTCTGAGAGGCAGCCATTCTCTCCTGCCAAACCAGGTTCCGGTCCCACAACTTCCGGTCCAGTCT 1566
G  A  H  R  E  D  G  V  A  L  R  G  S  H  S  L  L  P  N  Q  V  P  V  P  Q  L  P  V  Q  S    480

GCAACTTCCCCTGACTTGAACCCACCCCAAGACCCTTACAGAGGGATGCCACCAGGCCTCCAGGGCCAGAGCGTGTCTTCTGGTAGCTCT 1656
A  T  S  P  D  L  N  P  P  Q  D  P  Y  R  G  M  P  P  G  L  Q  G  Q  S  V  S  S  G  S  S    510

GAGATCAAATCCGACGACGAGGGCGATGAGAACCTGCAAGACACAAAATCTTCTGAGGACAAGAAATTAGATGACGACAAGAAGGATATC 1746
E  I  K  S  D  D  E  G  D  E  N  L  Q  D  T  K  S  S  E  D  K  K  L  D  D  D  K  K  D  I    540

AAATCAATTACTAGCAATAACGATGATGAGGACCTGACCCCAGAGCAGAAGGCTGAGCGCGAGAAGGAACGGAGGATGGCCAATAATGCC 1836
K  S  I  T  S  N  N  D  D  E  D  L  T  P  E  Q  K  A  E  R  E  K  E  R  R  M  A  N  N  A    570

CGTGAGCGCCTGAGGGTCCGAGATATCAACGAGGCTTTCAAGGAGCTTGGCCGTATGGTGCAGCTCCACCTGAAGAGCGACAAGCCCCAG 1926
R  E  R  L  R  V  R  D  I  N  E  A  F  K  E  L  G  R  M  V  Q  L  H  L  K  S  D  K  P  Q    600
                                  basic HELIX-LOOP-HELIX
ACCAAGCTCCTGATTCTCCACCAGGCCGTGGCTGTCATCCTCAGCCTGGAGCAGCAAGTTCGAGAAAGGAATCTGAACCCGAAAGCTGCC 2016
T  K  L  L  I  L  H  Q  A  V  A  V  I  L  S  L  E  Q  Q  V  R  E  R  N  L  N  P  K  A  A    630
                                                                  CAS
TGTCTGAAAAGAAGGGAGGAAGAGAAGGTGTCCTCAGAGCCTCCCCCACTCTCCTTGGCTGGCCCACACCCTGGGATGGGAGACGCAGCG 2106
C  L  K  R  R  E  E  E  K  V  S  S  E  P  P  P  L  S  L  A  G  P  H  P  G  M  G  D  A  A    660

AATCACATGGGACAGATGTGAAAAGGTCCAAGTTGCTACCTTGCTTCATTAAACAAGAGACCACTTCCTTAACAGCTGTATTACCCTAAA 2296
N  H  M  G  Q  M

CCCACATAAACACTGCTCCTTAACCCCGTTTTTTTTTGTAAATAAGACAAGTCTGAGTAGTTATGAATCGCAGACGCAAGAGGTTTCAGC 2386
ATTCCCAATTATCCTCGTGCCGGACTCAACTAACACATGTACAGATGTGAAAAGGTCCAAGTTGTCACCTTGCTTCAT              2464
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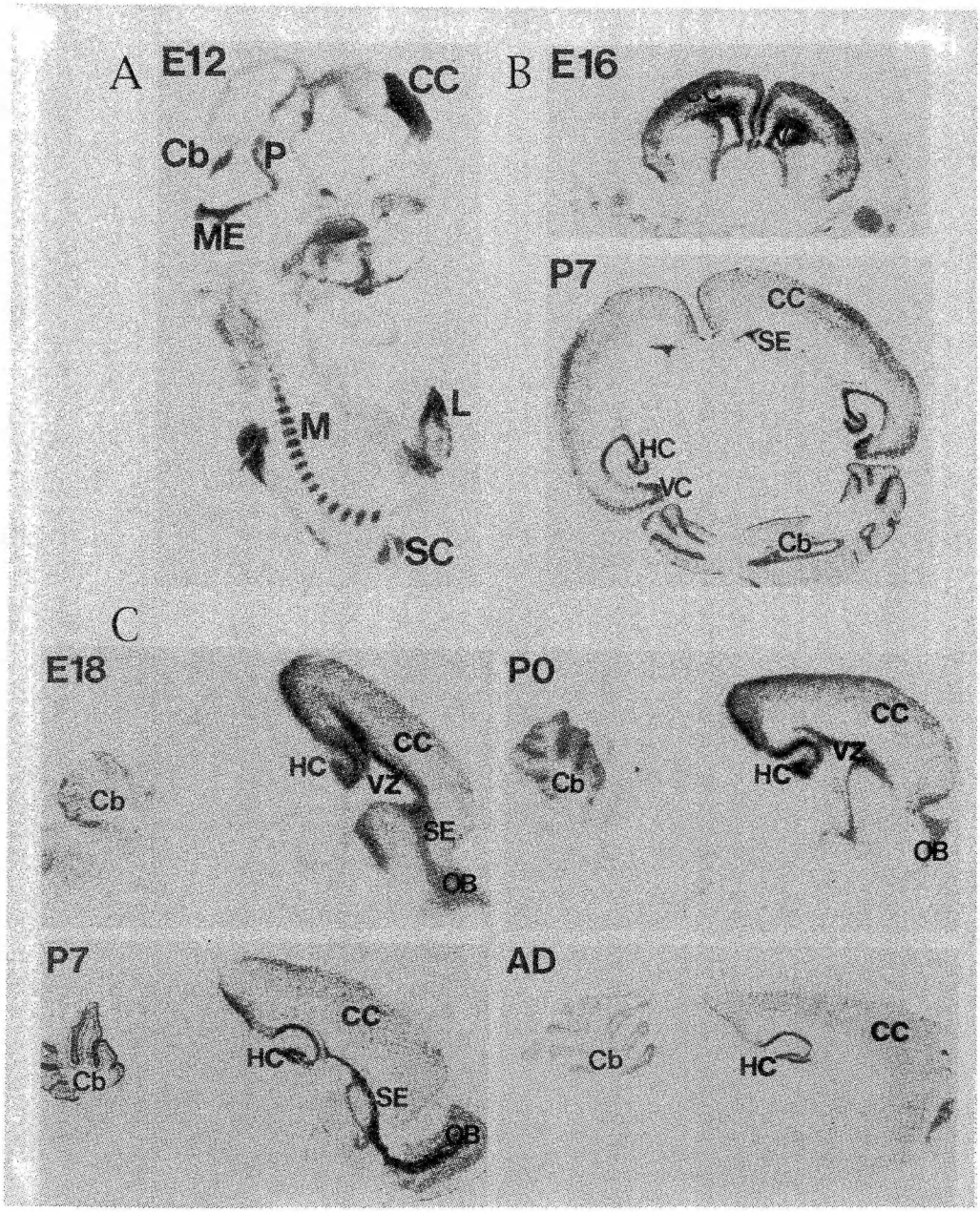
Fig. 1. Nucleotide and deduced amino acid sequences of ME2. The underlined amino acid sequences correspond to loop-helix, basic-helix-loop-helix, and CAS domains.


A
E12
CC
Cb
P
ME
M
L
SC
B
E16
P7
CC
SE
HC
VC
Cb
C
E18
Cb
HC
CC
VZ
SE
OB
P0
Cb
CC
VZ
HC
OB
P7
Cb
CC
HC
SE
OB
AD
Cb
HC
CC

by RNase treatment at 37°C. The sections were washed twice in 2 × SSC at 50°C, once in 0.2 × SSC 55°C, and finally in 50% formamide/2 × SSC at 50°C for 1–2 h. The slides were stained with Giemsa.

Characterization of ME2 cDNA. To obtain cDNAs encoding full length ME2, a mouse newborn brain cDNA library was screened with the PCR derived ME2 cDNA [9]. The longest cDNA obtained was approximately 2.4 kb with an open reading frame encoding 666 amino acids (Fig. 1) and a predicted molecular weight of 71,147 Da. ME2 is 98% identical to the human ITF2 at the amino acid level and 94% identical at the nucleotide level.

Expression of ME2 in the nervous system. Northern blot analysis revealed the presence of a single 6 kb mRNA expressed maximaly during embryonic development (data not shown). By in situ hybridization analysis, at embryonic day 12 (E12) ME2 expression is detectable in the cortex, cerebellum, pons, medulla and spinal cord (Fig. 2A). In non-neuronal tissues, myotomes and developing limbs have the strongest hybridization signal. From embryonic day 18 (E18) until adulthood, ME2 is expressed at high levels in the pyramidal cells of hippocampal layers CA1–CA4, and in the granular cells of the dentate gyrus (Fig. 2C) and at lower levels in the neocortex. At postnatal day 7, expression of ME2 is remarkably high in the visual cortex which is undergoing a critical period of development (Fig. 2B) and in the subependymal region extending from the anterior lateral ventricle into the olfactory bulb (Fig. 2C, E18 and P7). Cerebellar granule and Purkinje neurons and the ventricular zone of the olfactory bulb express ME2 at all stages studied (Fig. 2C).

In this paper, we demonstrate that ME2 is expressed in proliferative zones during development and in the adult in areas of neuronal plasticity (hippocampus, cerebellum, olfactory neuroepithelium and neocortex). The developmental profile of ME2 expression is different from the one of E12 [12] or ME1 [9] in that the later ones decrease to almost undetectable levels in the adult. Whether the function of ME2 is the same during development and during synaptic plasticity is unknown.

Of particular interest is the expression of ME2 in areas of neuronal plasticity. Induction of long-term potentiation (LTP) and kindling cause upregulation of several early response genes including the proto-oncogenes c-*fos* and c-*jun* [1,6]. The effect of transcriptional activation of c-*fos* depends on the promoter elements one of which is an E-box sequence which may have a critical role during transcriptional induction [5]. ME2, like other genes expressed in areas of neuronal plasticity (such as *fos*, *jun*, GAP-43 etc.) may increase during regeneration and LTP. It will be of interesting to investigate whether the levels of ME2 vary during these experimental conditions.

This work was supported by funds from the Colorado State University (BRSG), National Science Foundation and the March of Dimes Birth defects foundation.

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Fig. 2. In situ hybridization analysis of ME2 expression in mouse. A: saggital section of embryonic day 12 (E12). B: cross-section of the brain at E16 and horizontal section at postnatal day 7 (P7). C: saggital sections of brain at E18, P0 and P7, respectively), and adult (AD). During the early stages of embryogenesis (E12), ME2 is expressed at high levels in cerebral cortex (CC), cerebellar primordia (Cb), pons (P), medulla (ME), spinal cord (SC), and also in the developing limb (L) and in the myotomes (M). In later stages of brain development, strong hybridization is detectable in the hippocampus (HC), cerebellum (Cb), cerebral cortex (CC), olfactory bulb (OB), visual cortex (VC), subependymal region (SE), and ventricular zone (VZ). The expression of ME2 disappears from the ventricular zone (VZ) and subependymal region (SE) as proliferation decreases (AD).

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of ME1 and ME2 suggest a unique role during differentiation and
neuronal plasticity.
Mol. Brain Res. 29, 107–118.

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Molecular Brain Research 29 (1995) 107–118

MOLECULAR BRAIN RESEARCH

Research report

Differential expression and distinct DNA-binding specificity of ME1a and ME2 suggest a unique role during differentiation and neuronal plasticity

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Abstract

Class A basic-helix-loop-helix (bHLH) proteins have been referred to as ubiquitous and are believed to have redundant functions. They are involved in the control of several developmental pathways, such as neurogenesis and myogenesis. To rationalize the existence of multiple class A bHLH proteins, we evaluated the differences and similarities between ME1a and ME2, two class A bHLH proteins, highly expressed in differentiating neuronal cells. In situ hybridization analyses reveal that ME1a and ME2 are characterized by distinguishable patterns of expression in areas of the adult mouse brain where neuronal plasticity occurs. Also, DNA-binding assays show that both proteins bind to E-boxes as homodimers and heterodimers, and show differences in their DNA-binding specificities, which suggest selective interactions with different binding sites of target genes. In addition, in vitro DNA-binding assays demonstrate that Id2 forms heterodimers with ME1a and ME2. As a result of these interactions, their DNA-binding activity is abolished. Furthermore, overexpression of Id2 in neuronal cells suppresses ME1a and ME2 transcriptional activity. Based on our data, we hypothesize that ME1a and ME2 may activate gene expression of different target genes and therefore are likely to be differently involved during neurogenesis.

Keywords: Brain development; Neuronal plasticity; Helix-loop-helix; Gene expression

1. Introduction

Basic-helix-loop-helix (bHLH) transcription factors play important and specific roles in various developmental pathways of many organisms. In Drosophila, the requirement of bHLH transcription factors in determining cell fate during development of the nervous system has been well established [6,7,20]. Expression of proneural bHLH genes including daughterless, atonal and the Achaete–Scute Complex (AS-C) is a prerequisite for neuronal differentiation. For example, the achaete/daughterless heterodimer confers upon cells the potential of developing into neural precursors [14]. Based on a remarkable degree of similarity between several mammalian bHLH genes and Drosophila proneuronal genes, it seems very likely that mammalian bHLH factors also regulate neuronal development. Recently, the molecular mechanisms controlling the early steps of vertebrate neurogenesis have emerged. The rat homologues of Drosophila achaete-scute, Mash-1 and Mash-2 are expressed in the developing nervous system at a time when neurons are being generated [21]. In the mouse, the expression of Mash-1 is restricted to cells in the developing central and peripheral nervous systems between embryonic days 8.5 and 12.5, suggesting it plays a critical role during early stages of neurogenesis [15]. Recently, this has been confirmed by studies on mice carrying homozygous null mutations in the Mash-1 gene. Mutant mice died shortly after birth and showed defects in a number of olfactory and peripheral autonomic neurons [16].

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Taken together, these data clearly indicate the importance of bHLH proteins in neuronal differentiation.

During myogenesis, MyoD and Myf-5 bHLH transcription factors play pivotal rôles and are postulated to be responsible for myoblast determination [39]. MyoD acts as a regulator of muscle specific gene expression and when expresssed from a constitutive promoter, it converts different cell types into muscle cells [33,43,44]. Recently, it has been demonstrated that these myogenic regulatory factors are required for the determination and propagation of skeletal myoblasts, using mice carrying null mutations in Myf-5 and MyoD [35].

Basic-helix-loop-helix transcription factors contain at least four functional domains including a helix-loop-helix domain utilized for dimer formation [24,27,28], a basic domain required for DNA binding [10,42], a nuclear localization region [9,22], and a transcriptional activation domain [10,18,34]. The basic and the helix-loop-helix domains are conserved between many of the bHLH proteins [28,30] and because of this conservation they all bind the consensus DNA sequence CANNTG known as the E-box. Specific homodimers and heterodimers of bHLH proteins have different binding affinities depending upon the particular nucleotides within the internal and flanking sequences of the E-box [5,12].

Helix-loop-helix transcription factors have been grouped into different classes depending on their expression patterns and the homology of their functional domains with other bHLH proteins [27]. Class A bHLH transcription factors, such as E12, E47, da, ME1 and ME2, are expressed in more than one tissue and are characterized by their ability to readily form homodimers and heterodimers. They contain a conserved domain adjacent to the HLH motif, termed the Class A Specific (CAS) domain [46] and a newly identified loop-helix (LH) motif involved in transcriptional activation [34]. Class B transcription factors, like MyoD, myogenin, and members of the AS-C complex, have a more temporal and specific distribution and are believed to bind DNA as heterodimers with a class A bHLH factor. Both class A and class B bHLH proteins can interact with Id-like proteins (i.e. extramacrochaete, Id1, Id2, HLH462) which contain the HLH motif but lack the basic domain [3,11,38]. Heterodimers of a bHLH protein and an Id-like protein are devoid of DNA-binding activity.

As a first step in elucidating the role of bHLH proteins during neuronal development, we cloned several murine cDNAs encoding class A bHLH transcription factors, named ME1 through ME4 [30]. Protein sequence analysis between ME1 and ME2 reveals three highly conserved domains, LH [34], bHLH [21] and CAS [46]. ME1 is abundantly expressed during neuronal development and is down-regulated once cellular differentiation is completed [30]. As a result of alternative splicing. the ME1 gene encodes two proteins, ME1a and ME1b. ME1a is enriched in neuronal cells and the ratio between ME1a and ME1b increases considerably during mouse neuronal development and neuronal differentiation of PCC7 cells in culture [30]. ME1a is characterized by a 24 amino acid insertion that disrupts a leucine heptad repeat which shows a high degree of similarity with ankyrin-like domains [22]. A series of bHLH proteins closely related to the two forms of ME1, including the human E-box-binding protein HEB [19], the human transcription factor HTF4 [46], the rat E-box-binding proteins REBα and REBβ [22], the Akv murine leukemia virus E-box-binding proteins, ALF1A and ALF1B [32] and the chicken E-box-binding protein CTF4 [41], have been cloned from cDNA libraries by screening for binding of labeled E-box sequences. We believe ME1a (ALF1B) is the mouse counterpart of the rat REBβ while ME1b (ALF1A) is the mouse counterpart of rat REBα, human HEB, human HTF4, and chick CTF4.

It has been shown that HEB, the human homologue of ME1b, binds to several E-box sequences as a homodimer or a heterodimer with E12, ITF-2 or myogenic factors and functions as a transcritional activator [18]. REBα, the rat counterpart of HEB and ME1b binds as a homodimer or heterodimer to the E-box CE-2 site located in the control region of the calcitonin/CGRP gene [22]. However, REBβ (rat ME1a) poorly dimerizes or binds to DNA [22]. ME2, another abundant class A bHLH transcription factor, is expressed in the mouse brain from as early as embryonic day 12 and throughout the development [36] and is highly homologous to human ITF2 [18]. ITF-2 has been shown to behave as a transcriptional activator only as a chimeric GAL4 : ITF-2 protein using a reporter plasmid with several GAL4 DNA-binding sites [18,34].

As an attempt to rationalize the existence of multiple class A bHLH transcription factors, we have sought to analyze the differences and similarities between the ME1a and ME2 proteins. In situ hybridization analyses indicate overlapping as well as unique expression patterns between these two class A bHLH proteins in the adult mouse brain. We demonstrate that ME1a and ME2 proteins bind specific E-boxes as homodimers as well as heterodimers with MyoD. The assessment of their binding specificity reveals that ME1a preferentially binds to a different subset of E-box elements than does ME2. We demonstrate that both class A bHLH proteins function as transcriptional activators in neuronal cells, and that Id2 negatively regulates the transcriptional activities of ME1a and ME2 by forming inactive heterodimers. Taken together, these results suggest that although ME1a and ME2 are both class A bHLH proteins, they participate differently during the modulation of neurogenesis and neuronal plasticity.

2. Materials and methods

2.1. Constructions of eukaryotic expression plasmids

Full length ME2 cDNA was cloned into Bluescript SK vector (Stratagene) as described [36]. A truncated ME2 cDNA which lacks the first 216 N-terminal amino acids was also isolated and cloned into Bluescript SK vector (Fig. 1). Both ME2 forms were subcloned into the *Hin*dIII site of the eukaryotic expression vector pRcCMV (InVitrogen) to give CMV ME2 and CMV (−216)ME2 for full length and truncated ME2, respectively. The *Spe*I–*Bst*EII fragment containing the entire coding sequence of ME1a [30] was blunt ended and *Hin*dIII linkers were added in order to subclone into the *Hin*dIII site of pRcCMV. This construct is referred to as CMV ME1a. The *Xba*I–*Hin*dIII fragment carrying the complete cDNA sequence for Id2 was introduced into pRcCMV to give rise to the CMV Id2 plasmid. For in vivo studies, a reporter plasmid (MEF)X4 TKCAT carrying four copies of the MEF1 site (CACCTG) was constructed by inserting the oligonucleotides containing the MEF-1 E-box into the pBLCAT2 plasmid.

2.2. Construction of bacterial expression plasmids

The *Kpn*I–*Hin*dIII fragment which encodes the last 333 carboxy-terminal amino acids of ME1a, including the bHLH motif and the 24 amino acids specific to the neuronal form of ME1 (ME1a) was subcloned into the pRSET vector and this plasmid is referred to as pME1a/*Kpn*I. A *Nco*I–*Hin*dIII fragment carrying the last 238 C-terminal amino acids of ME2 was subcloned into the pRSET vector and this construct is called pME2/*Nco*I. A *Xmn*I–*Eco*RI fragment carrying the first 117 amino terminal amino acids was subcloned into pRSET B vector and this construct is referred as pRSET/Id2. All plasmid sequences were confirmed using the USB Sequenase kit version 2.0.

2.3. Cell culture

N18 (33) and NG108-15 (34) cell lines were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (Gibco).

2.4. Antibody production

A polyclonal antibody was raised in New Zealand white rabbits against a peptide carrying the sequence of the helix of the CAS domain (CLKRRREEEKVSAASAE) linked to the carrier KLH (Keyhole Limpet Hemocyanin). Protein G-Sepharose Fast Flow affinity chromatography (Pharmacia) was used to purify the IgG fraction.

2.5. Protein purification

The appropriate expression plasmid was transformed into the *E. coli* strain, BL21 DE3 pLys-S, a T7 lysozyme producing strain which contains the T7 RNA polymerase gene under the control of the inducible lac-UV5 promoter [37]. Cells were grown at 37°C to an $O.D_{600}$ of 0.6 and then induced with 1 mM IPTG (Sigma). The polyhistidine-containing recombinant proteins were purified by Ni^{2+}-NTA chromatography (Qiagen) under denaturing conditions. The cells were lysed in 6 M guanidine.HCl pH 8 (Sigma) at room temperature for 30 min. The lysate was cleared by centrifugation and the supernatant was incubated with the Ni^{2+}-NTA resin and loaded onto a column according to the manufacturers instructions (Qiagen). The bound recombinant proteins were eluted by lowering the pH. After purification, the proteins were allowed to refold by gradual dilution of the denaturing agents through dialysis. The recombinant protein was suspended in a buffer containing 10% glycerol, 20 mM HEPES pH 7.2, 1 mM EDTA, 0.1 M NaCl, 1 mM DTT, and 1 mM PMSF.

2.6. Preparation of DNA probe and specific competitors

The following DNA oligonucleotide probes were synthesized by Macromolecular Resources (Colorado State University). The sequences of the top strands of MEF1, μE5, μE2, κE2 oligonucleotides [19] and c-*fos* oligonucleotide [26] with the E-box sequence underlined are as follows: MEF1, 5'-CTAGATCTCC-AACACCTGCTGCGGAT-3'; Non-specific, 5'-TAAGGCTCTGAC-GTCTCCCCC-3'; μE5, 5'-TGCAAGAACACCTGCAAACA-3'; μE2, 5'-AGCTGGCAGCAGCTGGCAGCA-3'; κE2, 5'-GGGTCG-AAAGGCAGGTGGCCCAAGCT-3'; c-*fos*, 5'-GATGTCCATTAG-

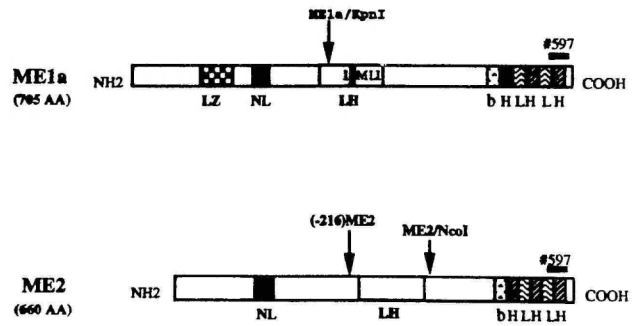

Fig. 1. Linear representation of ME1a and ME2 proteins. The major structural-functional regions are indicated; leucine zipper (LZ), nuclear localization domain (NL), loop-helix transcriptional activation motif (LH), leucine heptad repeat (LMLL), basic region (b), and helix-loop-helix-loop-helix (HLHLH). The second loop and third helix form the CAS domain. The ME1a specific mini-exon is represented by a black box. The polyclonal antibody #597, raised against the helix motif of the CAS domain, is indicated by a thick black line. The truncated proteins used for EMSA are indicated at the top of each linear representation by an arrow and their names indicate which restriction site was used for their cloning. The construct (−216)ME2 refers to the truncated form of ME2 cloned in the eukaryotic expression vector pRcCMV.

GACATCTGCGTCA-3'. The complementary oligonucleotides of the MEF1 site were annealed and the double-stranded MEF1 probe was labeled at the recessed 3' ends using [α^{32}P]dCTP (Amersham) and Klenow polymerase (Boeringer). Unlabeled competitor DNAs (non-specific, μE5, μE2, κE2 and c-*fos*) were prepared by annealing complementary oligonucleotides.

2.7. Electrophoretic mobility shift assays

DNA binding reactions consisted of 40 ng of purified protein incubated with 40 fmol of labeled oligonucleotide MEF1 and 100 ng of poly(dI.dC) (Sigma) for 20 min at room temperature in presence of binding buffer (10 mM Tris-HCl pH 7.5, 50 mM KCl, 0.1 mM EDTA, 1 mM DTT, 1 mM $MgCl_2$, 5% glycerol). When heterodimer formation was studied, 40 ng of purified ME1 or ME2 protein and 200 ng of MyoD were incubated for 20 min at room temperature. The probe (40 fmol) was added to the mixture and incubated for an additional 20 min at room temperature. For supershift assays, 40 ng of purified recombinant protein ME1a or ME2 was incubated with 1 μg of polyclonal antibody or rabbit preimmune serum prior to the incubation with the probe. For competition experiments, purified protein was incubated for 20 min with the MEF1 probe (40 fmol) in

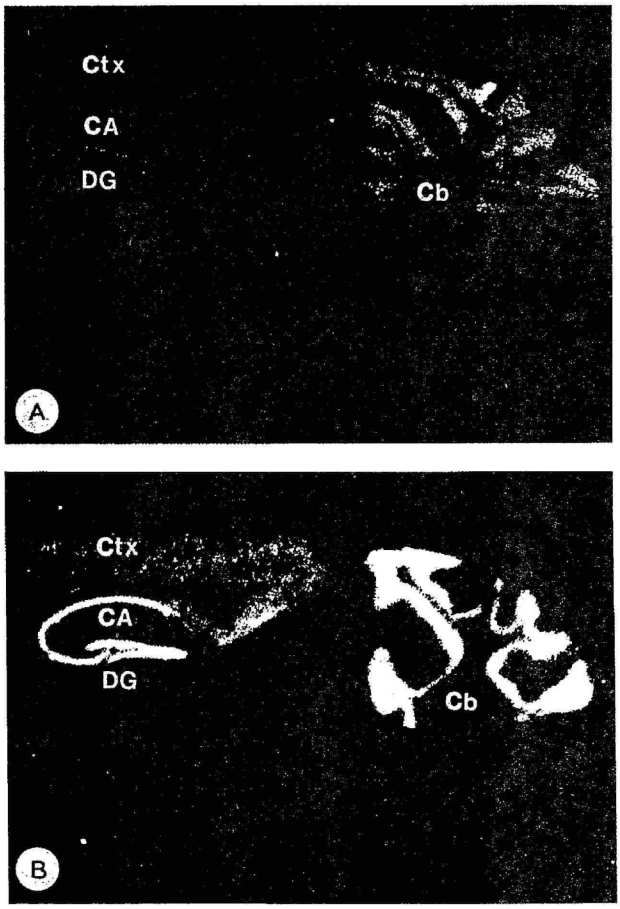

Fig. 2. ME1a and ME2 mRNA expression in adult mouse brain. A: in situ hybridization analysis of ME1a on a cross section of adult brain. Hybridization signals are strongly detected in the internal granular cell layer of the cerebellum (IGL) and the dentate gyrus of the hippocampus (DG) and poorly detected in the pyramidal cells from CA1 through CA3 (CA). B: in situ hybridization analysis of ME2 on a cross section of adult brain. Hybridization signals are strongly detected in the internal granular cell layer of the cerebellum (Cb), the dentate gyrus of the hippocampus (DG), the pyramidal cells from CA1 through CA3 (CA), and the cerebral cortex (Ctx). A similar picture for ME2 has been described [36] and is included here for comparison purposes.

presence of 25 fold excess of cold competitor. The products of the DNA binding reactions were electrophoresed on a 5% native gel, 0.5×TBE (45 mM Tris, 45 mM boric acid, and 1 mM EDTA) for 1.45 h at 150 V. Gels were dried and exposed on hyperfilm MP X-ray film (Amersham) at −70°C.

2.8. Transfections and CAT assays

Cells at a density of $0.5–1 \times 10^6$ in 60 mm dishes were transfected using a calcium phosphate coprecipitation method. The total amount of transfected DNA was kept constant by the addition of an appropriate amount of carrier DNA (empty expression vector). Twenty four hours later, the media was changed and cells were harvested after 48 h. Cells were washed in PBS, lysed in 250 mM Tris-HCl (pH 7.6) by three freeze/thaw cycles. A 150 μl mixture containing 0.4 mM acetyl coenzyme A (Sigma), 0.1 μCi of [dichloroacetyl-1,2-14C]chloramphenicol (Amersham), and 50–100 μg of total protein was incubated at 37°C for 1.5 h. After extraction with ethyl acetate, the radioactive forms of chloramphenicol were resolved by thin-layer chromatography, localized by exposing to X-ray film, and quantitated by PhosphorImager analysis. All experiments were done in triplicate.

2.9. In situ hybridization

In situ hybridization was performed as described [30]. The ME1a probe was transcribed from the ME1a vector pBSME1a which is a full length ME1a cDNA cloned into pBluescript SK [30]. The ME2 probe was synthesized by T3 RNA polymerase from the ME2 vector pBSME2 containing the full length ME2 cDNA [36]. The probes were partially hydrolyzed with 0.2 M NaOH on ice for 30 min and neutralized with 1 M acetic acid. All in situ experiments were repeated at least three times using sense riboprobes to determine the background level.

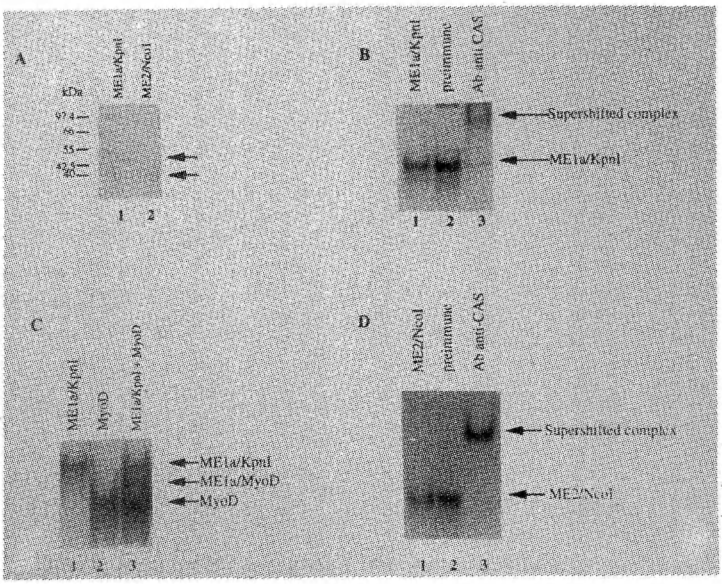

Fig. 3. ME1a and ME2 bind to the MEF1 E-box as homodimers and heterodimers. A: recombinant ME1a/*Kpn*I and ME2/*Nco*I proteins were expressed in *E. coli* and purified by Ni^{2+} chelate chromatography. The purified proteins were assayed by SDS/PAGE and visualized by Coomassie staining. The size of the protein molecular weight standards is shown on the left and the position of each purified protein is indicated on the right. B: ME1a/*Kpn*I protein is supershifted by a polyclonal antibody raised against the helix of the CAS domain. Forty ng of purified ME1a/*Kpn*I was incubated with 40 fmol of labeled oligonucleotide MEF1 in absence (lane 1) or presence of 1 μg of affinity-purified polyclonal antibody anti-CAS (lane 3) for 20 min at room temperature. Preimmune serum was added as a negative control (lane 2). The protein–DNA complexes were resolved by electrophoresis on a native 5% polyacrylamide gel, 0.5 × TBE. The gel was truncated to show only the protein-DNA complexes. C: ME1a/*Kpn*I forms heterodimers with MyoD. Forty ng of ME1a/*Kpn*I (lane 1) or 100 ng of purified MyoD (lane 2) was incubated with 40 fmol of labeled oligonucleotide MEF1 for 20 min at room temperature. When heterodimer formation was studied, the two purified proteins, ME1a and MyoD, were incubated together for 20 min prior to the addition of probe (lane 3). The different protein–DNA complexes are indicated by arrows. D: ME2/*Nco*I binds to the MEF1 E-box as homodimers and is supershifted by an antibody against the helix of the CAS domain. Forty ng of purified ME2/*Nco*I was incubated with 40 fmol of labeled oligonucleotide MEF1 in absence (lane 1) or presence of 1 μg of affinity purified polyclonal antibody anti-CAS (lane 3) for 20 min at room temperature. As a negative control, preimmune serum was also added (lane 2).

3. Results

3.1. ME1a and ME2 are expressed in overlapping and different areas of the adult brain

The level of expression of ME1 and ME2 is regulated during brain development [30,36]. Soon after birth, ME1 is expressed at low level whereas ME2 expression remains high and gradually decreases as the mouse brain reaches maturity. As a further means to dissect the differences and similarities between the two class A proteins, ME1a and ME2, parallel in situ hybridization analyses were performed on mouse adult brain using ME1 and ME2 antisense probes. High levels of ME1 and ME2 expression were detected at the internal granular cell layer (IGL) of the cerebellum (Fig. 2A,B). Other areas of common expression included the granular cells of the dentate gyrus of the hippocampus albeit at a lower level for ME1 (Fig. 2A,B). In the adult brain, ME2 expression was very abundant in the pyramidal cells from CA1 through CA3 whereas ME1 expression was barely detectable. Relatively strong ME2 signals were also observed throughout the cortex whereas ME1 expression was not detectable beyond background in this area. These data clearly indicate several spatial similarities and differences between ME1 and ME2 in areas of the adult brain where neuronal plasticity occurs.

3.2. ME1a and ME2 form dimers and bind to E-box elements

As an attempt to elucidate the molecular mechanisms of action of the two class A bHLH proteins, ME1a and ME2, we determined their dimerization and DNA-binding properties. We first examined, using an electrophoretic mobility shift assay (EMSA), whether ME1a could form homodimers even with the disruption of the leucine heptad repeat by the 24 amino acids specific to the neuronal form of ME1 (Fig. 1). For all EMSA analyses described in this report, we used bacterially purified proteins to avoid interferences with products of alternative translation initiation codons and contaminating proteins present in the rabbit reticulocyte lysate that can interact with the protein of interest [40]. Due to the large molecular weight of the full length ME1a protein, we expressed the C-terminal fragment of ME1a (ME1a/*Kpn*1). This truncated form carries the bHLH motif as well as the 24 amino acids specific to the neuronal form ME1a (Fig. 1). ME1a was expressed in *E. coli* and purified to near-homogeneity by metal affinity chromatography. An SDS/polyacrylamide gel of the purified ME1a protein is shown in Fig. 3A. Fig. 3B demonstrates that ME1a binds as a homodimer to oligonucleotides containing the E-box found in the muscle creatine kinase enhancer MEF1 site. To ascertain whether this protein–DNA complex is specific, we added to the reaction a polyclonal antibody raised against the helix of the CAS peptide. In the gel shift assay, this antibody supershifted the ME1a–DNA complex whereas the preimmune serum did not alter its mobility (Fig. 3B, lanes 2 and 3).

To analyze ME2 DNA-binding abilities by EMSA, a truncated ME2 protein containing the last 300 carboxy-terminal amino acids (ME2/*Nco*1; Fig. 1) was expressed in *E. coli*. ME2/*Nco*1 was then purified by nickel affinity chromatography to near homogeneity as shown on a coomassie stained SDS/polyacrylamide gel (Fig. 3A, lane 2). Binding of ME2 to an oligonucleotide carrying the MEF1 site produced a shifted ME2–DNA complex (Fig. 3D, lane 1). The specificity of this complex was confirmed by supershift using the polyclonal antibody raised against the CAS domain and the preimmune serum as a control (Fig. 3D, lanes 3 and 2).

Current models propose that class A bHLH factors modulate transcriptional activation differently depending on whether they are bound as homodimers or heterodimers [2,28]. MyoD was chosen to investigate whether ME1a or ME2 could form functional heterodimers with a class B bHLH protein. ME1a and ME2 are expressed in somites and myotomes at high levels [30] and are likely to be endogenous heterodimer partners of MyoD. Full length MyoD protein was expressed in bacteria using the bacterial T7 expression vector system [37] and purified to near-homogeneity. Heterodimer formation was studied by EMSA using the MEF1 site of the muscle creatine kinase enhancer. First, the heterodimers were allowed to form at room temperature in the absence of E-box which was then added to the reaction. Both ME1a homodimers and ME1a/MyoD heterodimers were detected (Fig. 3C, lanes 1 and 3). MyoD homodimers were also observed by EMSA because a high concentration (200 ng) of bacterially expressed MyoD was used in this assay (Fig. 3C, lane 2). Additionally, we observed that ME2, when incubated with ME1a or MyoD, produced an intermediate mobility complex suggesting that ME2 can form heterodimers with another class A bHLH as well as a class B bHLH (data not shown). Therefore, these results demonstrate that ME1a, ME2 and MyoD can associate in vitro and bind to the MEF1 site.

3.3. ME1a and ME2 homodimers have distinct DNA-binding specificities

Because it is not clear whether class A bHLH proteins have different functional properties during development, it is important to assess their differences in DNA-binding specificities in order to unravel their specific roles in gene expression. Therefore, the DNA-binding specificities of ME1a and ME2 homodimers were analysed and compared by a competition analysis

Table 1
Summary of ME1a and ME2 DNA-binding specificities

Wild type E-box		Binding activity [a]	
Name	Sequence [b]	ME1a	ME2
MEF-1	CAACACCTGCTG	++++	++++
μE5	GAACACCTGCAA	++++	++++
μE2	CAGCAGCTGGCA	+	++++
κE2	AGGCAGGTGGCC	++	–
c-*fos*	GGACATCTGCGT	++	+++

[a], The relative binding activities were determined from data in Fig. 4A,B.
[b], The E-box consensus sequences are underlined.

experiment. The design of this experiment was such that a 25-fold excess of each of several competitors was added to a reaction containing 40 ng of ME1a or ME2 protein and 40 fmoles of MEF1 probe (Fig. 4A). A strong competitor E-box element would decrease the signal from the labeled shifted DNA–protein complex. The specific sequences of the various E-boxes used for this assay are indicated in Table 1.

The ME1a–DNA complex could be competed successfully with a 25-fold excess unlabeled MEF1 oligonucleotide (CAACACCTGCTG) but not with non-specific DNA (Fig. 4A, lanes 3 and 5). DNA containing μE5 E-box (GAACACCTGCAA) competed equally well with the MEF1 probe whereas DNA containing μE2 E-box (CAGCAGCTGGCA) did not (Fig. 4A, lanes 6 and 7). The c-*fos* E-box (GGACATCTGCGT) located in the c-*fos* promoter (39) reduced the specific binding by 50% (Fig. 4A, lane 9). A relatively weaker competition was obtained with the κE2 site (AGGCAGGTGGCC) as shown in Fig. 4A, lane 8. The relative levels of ME1a binding affinity to different E-boxes are summarized in Table 1. Clearly, not all E-boxes are capable of binding ME1a homodimers with the same affinity.

The ME2–DNA complex was greatly reduced by the addition of a 25-fold excess unlabeled MEF1 oligonucleotide (Fig. 4B, lane 3). A similar excess of non-specific DNA did not affect the amount of specific binding (Fig. 4B, lane 5). DNA containing μE5 E-box (GAACACCTGCAA) or μE2 binding site (CAGCAGCTGGCA) competed as well as the MEF1 probe (Fig. 4B, lanes 6 and 7). However, DNA containing the κE2 site (AGGCAGGTGGCC) did not compete for binding with the MEF1 E-box (Fig. 4B, lane 8). The c-*fos* E-box (GGACATCTGCGT) slightly reduced the specific binding (Fig. 4B, lane 9). The relative levels of ME2 binding activity are summarized in Table 1. These results show that not only ME1a and ME2 homodimers recognize a variety of E-box sequences but also that they are characterized by significantly different DNA-binding specificities.

3.4. ME1a and ME2 activate gene expression in neuronal cells

To elucidate the manner by which ME1a and ME2 proteins may be involved in neuronal development, it is important to determine their specific functions. Thus, we examined their ability to activate transcription from a minimal promoter linked to several MEF-1 E-boxes. The in vivo studies were performed using the N18 neuroblastoma cell line [1]. We constructed two expression vectors, CMV ME1a and CMV ME2, and a re-

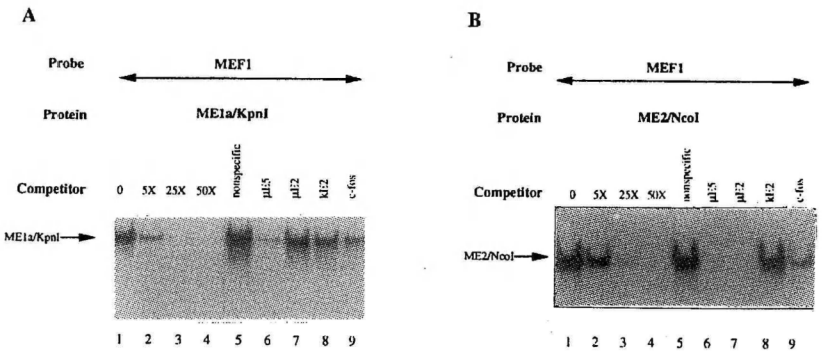

Fig. 4. ME1a and ME2 are characterized by different DNA-binding specificities. A: 40 ng of ME1a/*Kpn*I was incubated for 20 min with the MEF1 probe in absence (lane 1) or in presence of increasing amounts of cold MEF1 oligonucleotide as indicated on top of the figure (lane 2–4). The DNA-binding specificity of ME1a was determined using a 25-fold excess of non-labeled competitor, such as non-specific (lane 5), μE5 E-box (lane 6), μE2 E-box (lane 7), κE2 E-box (lane 8), and c-*fos* E-box (lane 9). The sequence of each cold competitor is indicated in Table 1. The reactions were run on a 5% native polyacrylamide gel in 0.5 × TBE. B: 40 ng of ME12/*Nco*I was incubated for 20 min with the MEF1 probe in absence (lane 1) or in presence of increasing amounts of cold MEF1 oligonucleotide as indicated on top of the figure (lane 2–4). The DNA-binding specificity of ME1a was determined using a 25 fold excess of cold competitor (lane 5–9), as described for panel A.

porter plasmid containing either four MEF1 E-boxes (MEF)X4 TKCAT upstream of the thymidine kinase TATA box.

Cotransfection of ME1a or ME2 expression vector with a reporter plasmid containing MEF1 E-boxes resulted in significant increases in CAT expression in N18 neuronal cells (Fig. 5, lanes 3, 4, 7 and 8). This transactivation activity was also observed in a different neuronal cell line such as the neuroblastoma–glioma NG108 cell line [17] (data not shown). The transient

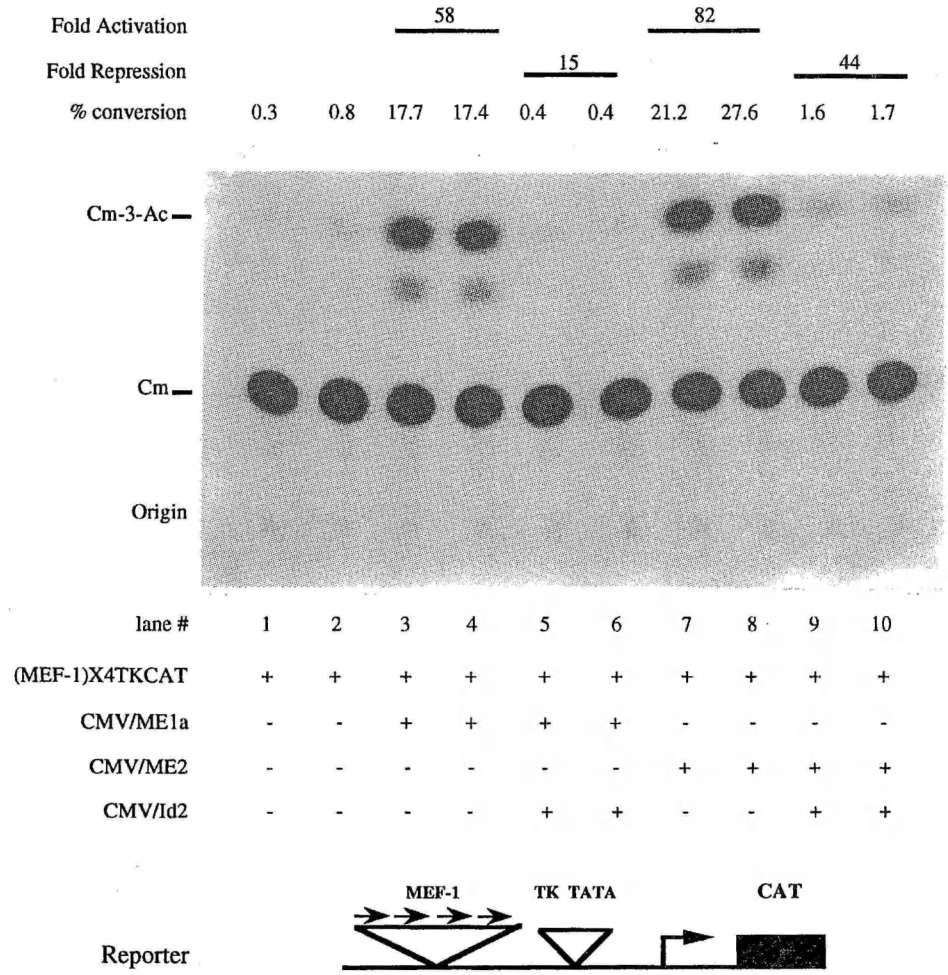

Fig. 5. ME1a and ME2 activates transcription through MEF1 E-boxes in neuronal cells. Neuronal cells N18 were transfected with 5 μg of expression plasmid as indicated at the bottom of the figure and 5 μg of CAT reporter plasmid (MEF1)X4 TKCAT by calcium phosphate precipitation. A schematic representation of the CAT reporter plasmid is shown at the bottom of the figure. Cells were harvested and CAT activities were determined. Protein concentration of cell extract was measured by Bradford assay and used for standardization of CAT activities which were determined by PhosphorImager analysis. The percent of acetylated ^{14}C-labeled chloramphenicol and the fold of activation and repression are indicated at the top of the figure.

CAT assay data clearly indicate that in mammalian neuronal cells, ME1a and ME2 behave as transcriptional activators. It is worth noting that ME2 behaves as a strong activator on its own, since previous transfection studies showed that only a GAL4:ME2 fusion protein was capable of weakly activating transcription [18] (see Discussion). Furthermore, a truncated form of ME2, which lacks the first 216 N-terminal amino acids (Fig. 1) is still capable of stimulating gene expression through the MEF1 E-box at levels comparable to that of full length ME2 (data not shown).

3.5. Overexpression of Id2 in neuronal cells interferes with transcriptional activity of ME1a and ME2

Id-like proteins have been shown to negatively regulate the DNA binding ability of bHLH proteins in vitro through formation of inactive heterodimers [3,38]. Their level of expression is usually down-regulated upon cellular differentiation and their relative concentration varies in different cell lineages [3,23,31]. We addressed the question of whether Id2, an Id-like protein expressed during mouse neurogenesis [31], would impair the transcriptional activity of ME1a and ME2 in neuronal cells. The expression plasmid CMV Id2 was cotransfected with CMV ME1a or CMV ME2 along with a reporter plasmid in the N18 neuronal cell line. A significant reduction in CAT activity was observed in the presence of the expression vector CMV Id2 (Fig. 5, lanes 5, 6, 9, and 10). However, a difference in the sensitivities of ME1a and ME2 to Id2 inhibition was observed; a maximal repression of 44 fold and 15 fold was obtained when Id2 was cotransfected with ME2 and ME1a, respectively (Fig. 5, lanes 5 and 6, 9 and 10). Thus, Id2 inhibits the transcriptional activity of both ME1a and ME2 in neuronal cells.

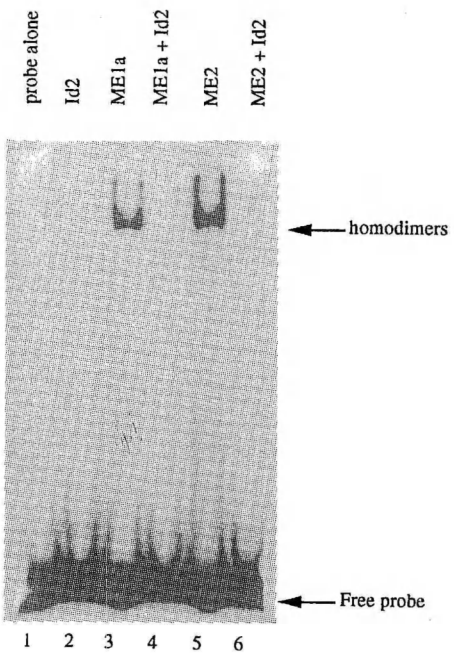

Fig. 6. Id2 interacts with ME1a and ME2 bHLH proteins. Two hundred ng of Id2 (lane 2) or 40 ng of ME1a/*Kpn*I (lane 3) or ME2/*Nco*I (lane 5) was incubated with 40 fmol of labeled oligonucleotide MEF1 for 20 min at room temperature. When heterodimer formation was studied between Id2 and the two class A bHLH proteins ME1a and ME2, purified Id2 was incubated for 20 min to form heterodimer with ME1a/*Kpn*I (lane 4) or ME2/*Nco*I (lane 6) prior to the addition of probe. As a control, lane 1 shows the probe alone.

Since Id proteins are believed to prevent the binding of bHLH proteins to DNA due to the formation of inactive heterodimers, we examined whether Id2 inhibits the binding activities of ME1a and ME2 through protein–protein interactions. We first subcloned the Id2 coding sequence into the bacterial expression vector pRSET and then purified recombinant Id2 protein to homogeneity by nickel affinity chromatography. To detect heterodimer formation with ME1a or ME2, we incubated excess of Id2 (5 fold) with either protein for 20 min before adding the MEF1 probe. These protein complexes were analysed for their DNA-binding ability by EMSA (Fig. 6). We found that both ME1a and ME2 formed inactive complexes with Id2 (lanes 4 and 6, respectively) and therefore lost their DNA-binding function. These results strengthen the functional data obtained by CAT assay on the transcriptional inactivation of ME1a and ME2 by Id2.

4. Discussion

Cellular differentiation is a multistep process involving activation and repression of many genes. Transcription factors containing the basic-helix-loop-helix (bHLH) motif are one set of regulatory proteins controlling this process. A comprehensive analysis of binding properties of class A bHLH homodimers is likely to provide insights as to whether these proteins interact selectively with specific target genes. This knowledge is particularly relevant in the case of class A bHLH proteins because it has been postulated only recently that class A homodimers might play an important role in establishing the differentiated state in different cell types [2,29].

Class A bHLH proteins can readily form homodimers or heterodimers with a tissue-specific class B bHLH protein and thereby stimulate gene expression.

It is generally assumed that the E2A gene products (E12 and E47) are the heterodimeric partners of MyoD during muscle development. Because, ME1a and ME2 are expressed at high levels in the somites early in development [30,36] and readily form heterodimers with MyoD in vitro as shown in this report, they might dimerize with MyoD in vivo. However, it has been shown using in vitro translated proteins that REBβ, the rat homologue of ME1a, poorly forms homodimers or heterodimers with MyoD [22]. This may be due to the slight divergence in sequence, but is more likely due to the use of reticulocyte lysate-translated proteins, since Klein et al. [22] report that bacterially purified REBβ can form homodimers. We and others [22,32] have shown by CAT assays that ME1a and REBβ are able to activate transcription, which further supports the fact they have similar dimerization/DNA-binding properties.

Until recently, it has been emphasized that class A proteins might function primarily as heterodimers [25]. However, examples of class A homodimers controlling gene expression during differentiation have been described. For example, E47 homodimers bind to regulatory sequences present in the immunoglobulin heavy- and light-chain gene enhancers, and induce transcription activation resulting in B-cell differentiation [29]. Therefore, a detailed analysis of the DNA-binding specificity of ME1a and ME2 homodimers is likely to yield significant insight into processes involved in neuronal differentiation. The precise nature of ME1a and ME2 binding specificities is a key issue given the absence of information concerning potential targets for transcriptional regulation by these two proteins. Our EMSA analyses have shown that ME1a homodimers bind strongly to MEF1 and μE5 E-box sequences, moderately to κE2 and c-*fos* E-box elements, and poorly to the μE2 E-box element. By contrast, ME2 homodimers bind strongly to MEF1, μE2, and μE5 E-box sequences, less to the c-*fos* E-box, and not at all to the κE2 E-box. These results demonstrate that the class A bHLH ME1a and ME2 homodimers exhibit significant differences in DNA-binding specificities, which may contribute to selective interactions of these proteins to the E-boxes of target genes. The specificities of class A homodimer-DNA interactions are likely to provide additional mechanisms of gene regulation without the participation of class B bHLH proteins. We can also predict that gene regulation by heterodimers between two class A bHLH proteins might play important roles during differentiation. And that, in vivo formation of class A homodimers might be regulated by events such as posttranslational modification which has been reported for the class C bHLH Max protein [4], and/or the relative concentrations of class A and B bHLH proteins and Id-like proteins which are cell-stage specific and cell-type dependent.

Although expression of ME1a and ME2 is different, they overlap in their spatial and temporal expression patterns during mouse brain development. Most of ME1a expression is down-regulated shortly after birth whereas ME2 expression persists much longer [30,36]. In the adult mouse brain, ME1a and ME2 expression patterns are restricted to areas such as the internal granular-cell layer of the cerebellum and the dentate gyrus of the hippocampus albeit at a lower level for ME1a in the latter tissue. In addition, ME2 expression is also present in the cortex and the pyramidal-cell layer of the hippocampus. These results show that class A bHLH proteins are not ubiquitous as has been suggested [28], but are highly regulated in their temporal and spatial distribution. Furthermore, by using a polyclonal antibody raised against the CAS domain of all class A bHLH proteins, we have detected brain specific class A bHLH proteins (unpublished results). In the adult brain, the functional significance for the expression of ME1a and ME2 is still unknown. However, since their expression is high in areas of neuronal plasticity, this suggests a potential gene regulation involved in the remodeling of neuronal connections.

In neuronal cells, both ME1a and ME2 behave as transcriptional factors. Previous transfection studies showed transcriptional activation only with GAL4:ME2 chimeric protein (ITF-2, E2–2) from a reporter plasmid carrying GAL4 binding sites [18,34]. The data presented here clearly demonstrate that ME2 is able to function as a transcription factor. Therefore, it contains distinct elements that dictate both efficient DNA binding activity, transcriptional activation and nuclear localization. In vitro, Id2 selectively binds to ME1a and ME2 to form inactive heterodimers, and consequently inhibits their DNA-binding activity. Furthermore, in vivo results support these protein-protein interactions which result in a loss of transcriptional activity of both ME1a and ME2 bHLH proteins. Therefore, it appears that the decrease in the level of Id2 in some neuronal cells [36] allows ME1a and ME2 to form active homodimers and/or heterodimers and thereby bind to E-box sequences inducing neuronal gene expression. Our results indicate that Id2 may suppress mammalian neuronal differentiation in a similar manner to extramachrochaetae, a negative regulator of differentiation during Drosophila neurogenesis [11,13]. A similar mechanism has been proposed for B-cell differentiation during which Id inhibits the homodimer formation of E47 in pro-B cells [29,45].

Clearly, it is essential to determine the heterodimer partners for ME1a and ME2 as well as their target genes in order to elucidate their specific cellular function during mammalian neuronal differentiation. It is possible that like daughterless, which controls sex determination and neuronal differentiation [8], ME1a and ME2 may have pleiotropic functions during devel-

opment. These proteins have different DNA-binding specificities as well as differential expression during neurogenesis and in the adult brain. In the future, it should be possible to analyze the in vivo concentrations of bHLH homodimers and heterodimers present in a given tissue at a given time and to comprehend how the interplay between the different types of bHLH proteins results in the appropriate expression of tissue specific genes.

Acknowledgements

This manuscript is dedicated to Mauricio Zuber who was recently killed in an accident. He will greatly be missed by his collaborators and the members of his laboratory: his technician Anne Keen, his undergraduate students Mike Ross, Dawit Haile and Dena Peavy, his graduate student Todd Verrastro, his recently graduated Ph.D. student and presently post-doctoral fellow Dan Shain and his post-doctoral associate Anne Chiaramello. He will dearly be remembered for his enthusiasm in science and his mentorship. We thank his colleague and friend Jim Bamburg for unwavering support. We also thank James Hoeffler and Harold Weintraub for gifts of plasmids. This work was supported by grants from the National Science Foundation (BNS-91558411), National Institute of Health (NS33804), Cancer League of Colorado and the March of Dimes to MXZ and the Spinal Cord Society to TN.

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III

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Isolation of Genes Which Block Neuronal Differentiation of Teratocarcinoma PCC7 Cells

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Since several viral oncoproteins block differentiation and induce proliferation of differentiated cells, we developed an expression screening method to isolate cDNAs which block neuronal differentiation and induce proliferation of teratocarcinoma cells. Mouse E2F1, RNP-1, and RNP-2 (Regulator of Neuronal Proliferation) were isolated using the developed screening method. Overexpression of E2F1, RNP-1, and RNP-2 cDNAs in neuronally differentiated teratocarcinoma PCC7 cells results in blocking differentiation and initiation of proliferation. Also, expression of RNP-1 and RNP-2 blocks the expression of neurofilament-L and GAP-43 genes in PCC7 cells. © 1995 Academic Press, Inc.

INTRODUCTION

The regulation of differentiation and dedifferentiation is a fundamental issue for understanding both development and maintenance of tissues. The molecular mechanisms which regulate these processes are essentially unknown, especially for neuronal cells.

Several data demonstrate reversibility of differentiation by overexpression of specific proteins in differentiated cells. Expression of adenovirus E1A proteins disrupts neuronal differentiation and growth factor responsiveness of PC12 cells [2]. In E1A-expressing cells, nerve growth factor receptors, $p140^{trk}$ and $p75^{LNGFR}$, epidermal growth factor receptor, tyrosine hydroxylase, and peripherin genes are repressed, while more ubiquitously expressed genes remain unaffected [2]. The E1A conserved region 1 which binds cell cycle regulators $p105^{Rb}$ and p300 is necessary for the above described effects. Expression of E1A also inhibits myogenic differentiation [4]. This inhibition may be based on the interaction of E1A with basic helix-loop-helix transcription factors which leads to the suppression of cell-specific gene transcription [23]. The 12S form of E1A has also been shown to dedifferentiate F9 teratocarcinoma cells [24]. Recently, it has been demonstrated that terminally differentiated myotubes can be induced to reenter the S phase by expressing large T antigen [5].

We developed an expression cloning system for the detection and isolation of cDNAs which block differentiation and induce proliferation of neuronally differentiated teratocarcinoma PCC7 cells. Three cDNAs were isolated and the effect of their overexpression in neuronally differentiated PCC7 cells was characterized.

MATERIALS AND METHODS

Cell culture. Mouse teratocarcinoma cell line PCC7 [17] was grown in Dulbecco's modified Eagle's medium containing 10% fetal calf serum (Sigma). Neuronal differentiation of PCC7 cells was induced with dibutyryl cyclic AMP (Bt_2cAMP, 1 m*M*) and all-*trans* retinoic acid (RA, 0.5 μM).

Generation of subtraction cDNA library. Undifferentiated and neuronally differentiated teratocarcinoma PCC7 cells were used to isolate poly(A)$^+$ RNA (FastTrack, Invitrogen). Twenty micrograms of poly(A)$^+$ RNA from undifferentiated cells was used to synthesize first-strand cDNA. Oligo(dt) primer with a *Not*I restriction site at the 5′ end (CTAGATCGCGAGCGGCCGCCCTTTTTTTTTTTTTTT-TTT) was used to synthesize first-strand cDNA using SuperScript RNase H reverse transcriptase (200 units/μg of poly(A)$^+$ RNA, Gibco). [^{32}P]dCTP (50 μCi, >3000 Ci/mmol, Amersham) was added to the first-strand synthesis reaction to trace DNA. First-strand cDNA was hybridized to poly(A)$^+$ RNA (200 μg) isolated from differentiated cells in sealed ampules (total volume 100 μl, buffer 0.5 *M* sodium phosphate, pH 6.8, 300 m*M* NaCl, 2 m*M* EDTA, and 0.2% SDS) for 18 h at 70°C. The hybridization mix was diluted to a final molarity of 0.08 *M* sodium phosphate, loaded onto a DNA grade hydroxylapatite column (4 ml vol, Bio-Rad), and washed extensively with 0.08 *M* sodium phosphate buffer (pH 6.8). Single-stranded cDNAs were eluted in 10 ml of 0.15 *M* sodium phosphate buffer. Column fractions (0.5 ml) exhibiting radioactivity above background were pooled, and the cDNAs were concentrated by butanol extraction followed by chromatography in STE buffer (100 m*M* NaCl, 10 m*M* Tris–HCl, pH 7.4, 1 m*M* EDTA) on a Sephadex G-25 column (Pharmacia). Single-stranded cDNAs were mixed with 40 μg of poly(A)$^+$ RNA from differentiated cells for the second cycle of hybridization and this yielded 0.6 μg of first-strand cDNA. First-strand cDNAs were hybridized with 5 μg of the original poly(A)$^+$ RNA isolated from undifferentiated cells, and the resulting DNA/RNA hybrids were used as template for second-strand synthesis with RNase H and *Escherichia coli* DNA polymerase I (Gibco). Blunt ends were created with T4 DNA polymerase, and the *Hin*dIII adapter, 5′ AGCTTGGCACGAG 3′, 3′ ACCGTGCTC 5′ was ligated to the cDNA. Preparation of cDNAs longer than 700 bp for ligation into the expression vector (pRc/CMV, Invitrogen) was performed by digestion with *Not*I followed by size selection on a Sephacryl S-400 column (Pharmacia). cDNAs were cloned into the expression vector, pRcCMV (Invitrogen), between the *Hin*dIII and *Not*I

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restriction sites. The library was divided into 20 aliquots and used to transform *E. coli* DH5 cells (MAX Efficiency, Gibco). Each aliquot yielded 5–8 × 10^3 colonies which were combined and grown for large-scale plasmid isolation (> plasmid < maxi kit, Qiagen). The cDNA expression library has 2 × 10^5 independent clones with an average insert size of 1.5 kb (range: 0.6–3.7 kb). To characterize the efficiency of subtraction cloning, we selected 10 clones and performed Northern blot analyses using total RNAs from undifferentiated and neuronally differentiated PCC7 cells. Five cDNAs hybridized exclusively with RNA from undifferentiated cells, three cDNAs hybridized strongly with RNA from undifferentiated cells and weakly with RNA from differentiated cells, and two cDNAs hybridized equally with RNA from undifferentiated and differentiated cells. This analysis demonstrated that subtraction cloning was successful and the expression library is enriched for sequences expressed in undifferentiated cells.

Transfection of cells. PCC7 cells were differentiated 3 days before transfection. Transfection of the cDNA library was performed by the calcium phosphate coprecipitation technique using 20 μg of DNA per 100-mm tissue culture plate (Falcon) at a cell density of 2 × 10^6 cells per plate with an incubation time of 15–16 h. This treatment results in 10–15% transfection efficiency (estimated using transient β-galactosidase assay). Each aliquot of the cDNA library (n = 20) was used to transfect cells in 20 plates. Transfectants which were stably proliferating were identified following culture of cells in the presence of 400 μg/ml G418 (Gibco) for 18–21 days. Proliferating clones were isolated and subcloned.

Isolation of transfected cDNAs. Genomic DNA was prepared from isolated clones (TurboGen, Invitrogen) and used as a template for amplification of cDNAs by polymerase chain reaction (PCR). Primers for cDNA amplification corresponding to flanking sequences in the pRcCMV vector (5′ primer 5′-AGCTCTCTCTGGCTAACTAGAGAAC and 3′ primer 5′-AGCGAGCTCTAGCATTTAGGTGA) were prepared, and 35 cycles of PCR were performed using the following conditions: 92°C 1.2 min, 58°C 2 min, and 72°C 4 min. Amplified DNAs were cloned into the *Eco*RV site of a Bluescript plasmid (Stratagene) for sequencing. Isolated cDNAs with vector (pRcCMV) sequences were subcloned into the pRcCMV expression vector between the *Hin*dIII and *Not*I sites and retested on differentiated PCC7 cells. Adenovirus E1A 12S and 13S forms (Generous gifts from Dr. J. Nevins and Dr. E. Moran) and mouse ME1 [15] cDNAs were cloned into pRcCMV vector using *Hin*dIII linkers and used as controls.

Northern blot analyses. The RNA was fractionated on a 1.2% agarose-formaldehyde gel and transferred to a nylon membrane (Hybond N, Amersham). Twenty-five micrograms of total RNA was run in each lane. RNA was isolated using the acid guanidinium/phenol/chloroform extraction procedure [7]. Full-length mE2F, RNP-1 (regulator of neuronal proliferation), RNP-2, GAP-43, and a mouse NF-L *Sma*I/*Bgl*II fragment were radiolabeled (^{32}P) using the Multiprime DNA labeling system (Amersham) and used as probes. The blots were washed at high stringency (0.2× SSC, 65°C) and exposed to X-ray film for 3 days. The amount and quality of transferred RNA were monitored by methylene blue staining of the filters before hybridization.

RESULTS

Molecular Cloning of Cellular Genes Which Block Differentiation and Initiate Proliferation of Neuronally Differentiated Teratocarcinoma PCC7 Cells

Teratocarcinoma PCC7 cells stop proliferation and differentiate into neuronal-like cells after treatment with Bt_2cAMP and RA [17]. Differentiation is irreversible as removal of Bt_2cAMP and RA does not cause dedifferentiation and reentry into the cell cycle. Expression screening of the subtraction cDNA library was performed to isolate genes which block neuronal differentiation of PCC7 cells and induce proliferation. The cDNA library in pRcCMV expression vector was transfected into neuronally differentiated PCC7 cells. The cultures of neuronally differentiated cells did not contained proliferating cells as it was estimated using thymidine incorporation and cell cycle analysis (data not shown). After 3 weeks of selection in the presence of G418, three proliferating clones were isolated (Fig. 1a). All three clones proliferated in the presence of RA and Bt_2cAMP and were morphologically identical to the original undifferentiated PCC7 cells. These clones were propagated in the presence of RA and Bt_2cAMP, and transfected cDNAs were isolated using PCR and retested for dedifferentiation and induction of proliferation. Sequence analysis reveals that one cDNA is the mouse homolog of human E2F1; the second cDNA, RNP-1, lacks significant homology to any GeneBank sequences; and RNP-2 is 99.5% homologous to helix-loop-helix transcriptional regulator Id4 [18]. RNP-1 cDNA has a nuclear translocation sequence which suggest that it is a nuclear protein.

Efficiency of Isolated cDNAs to Initiate Proliferation

The efficiency of isolated RNP-1 and RNP-2 cDNAs to dedifferentiate and initiate proliferation of neuronally differentiated PCC7 cells was tested under two conditions: (1) transfection of cDNAs into neuronally differentiated PCC7 cells (the same conditions used in screening the expression library) and (2) transfection of cDNAs into proliferating PCC7 cells followed immediately by treatment with RA and Bt_2cAMP to induce neuronal differentiation. Adenovirus oncogene $E1A^{12S}$ and $E1A^{13S}$ forms and helix-loop-helix transcription factor ME1 were used as controls. ME1 is expressed in several proliferating cell types, and its expression is downregulated during differentiation [15]. Both the 12S and 13S forms of E1A block neuronal differentiation and initiate proliferation of neuronally differentiated PCC7 cells (Table 1). No proliferating clones were observed after transfection with ME1 cDNA or the pRcCMV vector without insert. All three isolated cDNAs, mE2F, RNP-1, and RNP-2, induced the formation of proliferating clones with the same efficiency under both experimental conditions (Table 1). The efficiency of E1A to induce proliferation is approximately 10 times higher than that for E2F1, RNP-1, and RNP-2 cDNAs.

Expression of E2F, RNP-1, and RNP-2 in Differentiating PCC7 Cells

Neuronal differentiation results in downregulation of E2F1, RNP-1, and RNP-2 expression in PCC7 cells. E2F1 mRNA level decreases slightly after 3 days of differentiation (Fig. 2). This result is consistent with the data that E2F1 is expressed in the adult nervous system [10, our unpublished data]. Since E2F1 mRNA level is

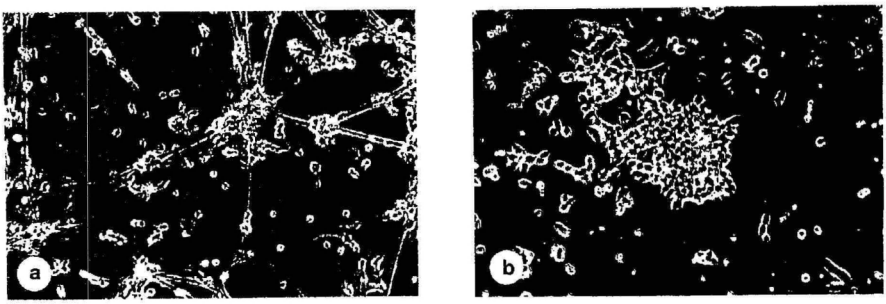

FIG. 1. Induction of proliferation of neuronally differentiated PCC7 cells. (a) Differentiated PCC7 cells after 3 days of treatment with RA and Bt_2cAMP which represents the cells used for transfection of cDNAs. (b) A proliferating clone 21 days after transfection of neuronally differentiated PCC7 cells (as in a) with E2F cDNA growing in the presence of RA and Bt_2cAMP. Arrow indicates the clone of proliferating cells.

only slightly downregulated during differentiation, it may raise the question of how it was possible to isolate its cDNA from the subtraction library. Our analyses demonstrated that the subtraction was not complete (see Materials and Methods) and using functional screening allowed us to isolate even very rare cDNAs. The level of RNP-1 mRNA (3.1 kb) decreases significantly during the first 24 h of differentiation and is undetectable by 48 h. Decrease of RNP-2 mRNA levels occurs more gradually, and its 2.1-kb mRNA disappears by the third day of differentiation (Fig. 3).

Expression of Neuronal-Specific Genes in Differentiating PCC7 Cells That Overexpress RNP-1 and RNP-2

Induction of neuronal differentiation of PCC7 cells results in the activation of neuronal-specific genes such as neurofilament L (NF-L) and GAP-43 (Fig. 3). Expression of NF-L and GAP-43 genes was studied to analyze the effect of RNP-1 and RNP-2 overexpression on neuronal differentiation. The expression of RNP-1 and RNP-2 varies from almost undetectable to very high levels, comparable to the actin mRNA level (data not shown) in individual clones. To normalize these differences, we used randomly selected pools of RNP-1 (RNP-1 pool)- and RNP-2 (RNP-2 pool)-expressing clones (20–25 clones). RNP-1 and RNP-2 pools were cultured in regular media (DMEM + 10% FCS) in the presence of G418 to avoid the loss of transfected cDNAs for 7 days followed by treatment with RA and Bt_2cAMP to induce differentiation. Northern blot analyses demonstrate expression of transfected RNP-1 (1.9 kb) and RNP-2 cDNAs (2.1 kb) in RNP-1 and RNP-2 pools, respectively. The RNP-1 and RNP-2 mRNA levels in-

TABLE 1

Efficiencies of Different cDNAs to Induce Formation of Proliferating Clones from Neuronally Differentiated PCC7 Cells

	Number of clones					
	Transfection into undifferentiated cells followed by differentiation Experiments			Transfection into differentiated cells Experiments		
Construct	1	2	3	1	2	3
pRcCMV	0	0	0	0	0	0
pRcCMV-E1A12S	150	231	112	146	219	110
pRcCMV-E1A13S	184	191	132	172	180	109
pRcCMV-E2F	25	19	14	15	19	12
pRcCMV-RNP-1	9	11	15	12	12	15
pRcCMV-RNP-2	12	9	17	9	11	16
pRcCMV-ME1	0	0	0	0	0	0

Note. Results of three separate experiments are presented. All transfections were done in triplicate and each number represents the average number of clones from three culture dishes. The results are normalized to the transfection efficiency measured by β-galactosidase (β-gal) activity after cotransfection of β-gal driven by CMV promoter.

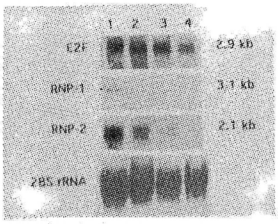

FIG. 2. Northern blot analyses of E2F, RNP-1, and RNP-2 expression during differentiation of teratocarcinoma PCC7 cells. RNA was isolated from untreated cells (lane 1) and cells were treated for 1, 2, and 3 days with RA and Bt_2cAMP (lanes 2, 3, and 4). The same membranes were hybridized with ^{32}P-radiolabeled E2F, RNP-1, and RNP-2 cDNA probes. Methylene blue-stained 28S ribosomal RNA demonstrates the amount of RNA in each lane.

creased after treatment with RA and Bt_2cAMP (Fig. 3). This increase is a result of CMV promoter induction by Bt_2cAMP (Neuman *et al.*, unpublished data). In nontransfected PCC7 cells, NF-L mRNA is undetectable in proliferating cells, and its levels increase after induction with RA and Bt_2cAMP (Fig. 3). The induction of the 3.5-kb mRNA occurs more rapidly than that of 2.5-kb mRNA. Overexpression of RNP-1 results in the delay in the induction of NF-L gene, and this induction is transient. The increase of NF-L mRNA levels is detectable on the second day after induction with RA and Bt_2cAMP, and the mRNA levels decrease on the third day (Fig. 3). No expression of NF-L was detected in RNP-2 pools.

GAP-43 is expressed at relatively low levels in undifferentiated PCC7 cells, and its mRNA levels increase rapidly after induction with RA and Bt_2cAMP (Fig. 3). No induction of GAP-43 was detected in RNP-1 pools after treatment with RA and Bt_2cAMP; moreover, its mRNA becomes undetectable after 1 day of treatment (Fig. 3). No expression of GAP-43 was detected in undifferentiated RNP-2 pools or after treatment with RA and Bt_2cAMP.

Analyses of several individual RNP-1 and RNP-2 overexpressing clones result in the same changes of neuronal marker genes as was described for pooled clones (data not shown).

DISCUSSION

During neuronal differentiation, neuroblasts exit the cell cycle and become arrested in the G_0 phase. This transition is accompanied by switching off of the cell cycle regulatory genes. The aim of this work was to identify genes which reverse this process in neuronally differentiated teratocarcinoma cells. Our approach was to develop a protocol to screen for cDNAs which induce proliferation of neuronally differentiated teratocarcinoma PCC7 cells. This approach enabled us to isolate three cDNAs (mE2F, RNP-1, and RNP-2). Overexpression of these cDNAs blocks neuronal differentiation and induces proliferation of PCC7 cells.

How the isolated genes function to induce proliferation is a subject of speculation. Cell cycle-dependent transcription factor E2F regulates several genes which are necessary for the S phase of the cell cycle [16]. The activity of E2F is regulated by formation of transcriptionally inactive or inhibitory complexes with $p105^{Rb}$ [1, 6, 21, 22, 25]. During the G1 phase of the cell cycle, $p105^{Rb}$ becomes hyperphosphorylated and leads to the release of transcriptionally active E2F [16]. Overexpression of E2F may lead to a situation where $p105^{Rb}$ is limiting and thus result in the excess of active E2F which may trigger the initiation of the cell cycle. This explanation is supported by the data demonstrating that overexpression of E2F1 induces quiescent cells to enter S phase [12]. Alternatively, excess of E2F could sequester $p105^{Rb}$ and block differentiation. The evidence for this is that the expression of $p105^{Rb}$ is necessary for the first stages of muscle differentiation when it forms complexes with bHLH transcription factors to regulate muscle-specific genes [9]. The same may be true in neuronal differentiation, as knock-out experiments demonstrate that $p105^{Rb}$ is necessary for normal neurogenesis [8, 11, 14]. The RNP-1 lacks remarkable homology to gene sequences contained in the Genbank; thus, it is premature to speculate on its function. The RNP-2, which is highly homologous to Id4, a helix-loop-

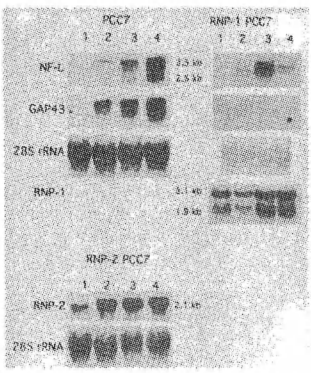

FIG. 3. Northern blot analyses of NF-L, GAP43, RNP-1, and RNP-2 expression in differentiating PCC7 cells and in pools of PCC7 cells overexpressing RNP-1 and RNP-2 (RNP-1 PCC7 and RNP-2 PCC7). RNA was isolated from untreated cells (lane 1) and cells were treated for 1, 2, and 3 days with RA and Bt_2cAMP (lanes 2, 3, and 4). The same membranes were hybridized with ^{32}P-radiolabeled NF-L, GAP43, RNP-1, and RNP-2 probes. Methylene blue-stained 28 rRNA demonstrates the amount of RNA in each lane.

helix transcriptional regulator, may block the neuronal differentiation by forming inactive heterodimers with basic helix-loop-helix transcription factors which are expressed in neuronally differentiating cells [3, 13, 15, 19, 20].

Analyses of neuronal-specific genes demonstrate that overexpression of RNP-1 and RNP-2 interferes with the induction of NF-L and GAP-43 gene expression. Delayed and transient induction of NF-L in RNP-1-overexpressing cells after induction with RA and Bt_2cAMP suggest that RNP-1 does not completely block signal transduction at the initial stages of neuronal differentiation. By contrast, the lack of induction of GAP-43 and even its repression demonstrate that RNP-1 completely blocks the expression of at least one neuronal-specific gene in PCC7 cells. These data support the hypothesis that different neuronal genes are regulated by different mechanisms. No expression of NF-L and GAP-43 in RNP-2-overexpressing PCC7 cells argues that the two isolated genes, RNP-1 and RNP-2, have at least partially different mechanisms of action during blockage of neuronal differentiation.

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Orphan Receptor Coup-TF I Antagonizes Retinoic Acid-Induced Neuronal Differentiation

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Chicken ovalbumin upstream promoter-transcription factors (COUP-TF) are expressed in the developing nervous system and interact with nuclear hormone receptors to regulate expression of different genes. The role of COUP-TF orphan receptors in neurogenesis is virtually unknown. To study the possible function of COUP-TF I during neuronal differentiation, we generated COUP-TF I overexpressing teratocarcinoma PCC7 cell lines and analyzed retinoic acid (RA)-induced neuronal differentiation of these cells. COUP-TF I overexpression results in the blockade of morphological differentiation after induction to differentiate. COUP-TF I represses expression of microtubule-associated protein 2 (MAP2) gene and delays induction of growth-associated protein 43 (GAP43) gene expression. In contrast, expression of the neurofilament light subunit (NF-L) gene is not affected by COUP-TF I overexpression during neuronal differentiation. Also, cells overexpressing COUP-TF I do not stop proliferating after RA and dBcAMP treatment and possess suppressed transcriptional activation from different RA response elements. These results suggest that COUP-TF I plays an important role in regulating RA-induced neuronal differentiation.

Key words: teratocarcinoma, nuclear hormone receptors, neurofilament, GAP43, MAP2, gene expression

INTRODUCTION

A variety of regulatory mechanisms at different levels are involved in neuronal development. Transcription factors belonging to the superfamily of nuclear hormone receptors have a crucial role in establishing initial cellular diversity in the nervous system. Among the best characterized members of the superfamily are the receptors for retinoids, thyroid hormones, steroid hormones and glucocorticoids whose importance during neurogenesis is well-described (for review see Beato 1989; Evans and Arriza, 1989; McEwen et al., 1991; Linney, 1992). The nuclear hormone receptors are characterized by a highly conserved DNA binding domain and a less conserved ligand binding domain (for review see Green and Chambon, 1988; Beato, 1989; Fuller, 1991). The DNA binding domain has two zinc finger structures which determine DNA binding specificity and are involved in receptor dimerization (Luisi et al., 1991). The ligand binding domain is localized in the carboxy-terminal region of the molecule and besides ligand binding has a number of additional functions, including dimerization (Zhang et al., 1991, 1992) and transcriptional activation (Zenke et al., 1990, Zhang et al., 1991, 1992). Hormone receptors activate or repress gene transcription through binding to cis-acting hormone response elements (HRE; Green and Chambon, 1988, Beato, 1989). Besides the ligand-activated transcription factors, the hormone receptor superfamily comprises several orphan receptors for which ligands are not known (for review see Evans, 1988; Green and Chambon, 1988). One function of the orphan receptors is to regulate the activity of ligand-activated hormone receptors through heterodimer formation or competition for binding to specific response elements (Tran et al., 1992).

Orphan receptors, chicken ovalbumin upstream promoter-transcription factor (COUP-TF) homologs, have been isolated from Drosophila (Mlodzik et al., 1990), sea urchin (Chan et al., 1992), zebrafish (Fjose et al., 1993), Xenopus (Matharu and Sweeney, 1992), chick (Lutz et al., 1994) and mammals (Miyajima et al., 1988; Wang et al., 1989, 1991; Ritchie et al., 1990; Ladias and Karathanasis, 1991). COUP-TFs have high binding activity to hormone response elements and can repress hormonal induction of target genes (Cooney et al., 1992; Kliewer et al., 1992; Tran et al., 1992; Widom

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et al., 1992). Several different mechanisms may contribute to the repression of induction including heterodimerization with retinoid X receptors (RXRs), direct competition of COUP-TFs for the hormone response elements, and suppression of transcription by COUP-TF homodimers (Cooney et al., 1992, 1993; Kliewer et al., 1992; Tran et al., 1992; Widom et al., 1992; Segars et al., 1993). Analyses of nuclear hormone receptors in a variety of systems clearly demonstrate that regulation of gene expression by these transcription factors depends on the presence of different ligands and also on the interactions with activators and repressors.

Retinoic acid (RA) affects several processes in neurogenesis. High doses of RA alter the development of nervous system, including development of rhombomers in the hindbrain (Morris-Kay et al., 1991; Marshall et al., 1992) and morphogenesis of the brain (Durston et al., 1989; Ruiz i Altaba and Jessell, 1991; Agarwal and Sato, 1993). The effect of RA on gene expression is mediated via retinoic acid receptors (RAR) and (RXR; for review see Linney, 1992). RARs and RXRs are expressed in a complex pattern during the nervous system development (Mangelsdorf et al., 1992; Rowe et al., 1991; Ruberte et al., 1991, 1993; Smith and Eichele, 1991). At the cellular level, RA induces neurite outgrowth and survival of different neuronal types (Berrard et al., 1993; Wuarin and Sidell, 1991). It also induces nerve growth factor responsiveness of sympathetic neurons (Rodriguez-Tebar and Rohrer, 1991) and $p75^{NGFR}$ (Scheibe and Wagner, 1992; Metsis et al., 1992) and TrkB expression (Kaplan et al., 1993). RA induces neuronal differentiation in several teratocarcinoma and neuroblastoma cell lines (Jetten, 1990; Slack et al., 1992; Kaplan et al., 1993). In teratocarcinoma cells, RA treatment stimulates expression and activity of RARs (Wu et al., 1992) which results in induction of downstream genes including Hox genes (Murphy et al., 1988; Simeone et al., 1990). The importance of RA during differentiation is supported by the observation that cell lines expressing mutated RARs do not differentiate properly (Boylan et al., 1993; Espeseth et al., 1989; Pratt et al., 1993). The complexity of RA responses in different systems is displayed by six receptor genes, encoding three RA receptors, RARα, RARβ, and RARγ and three retinoid X receptors, RXRα, RXRβ, and RXRγ (reviewed by Linney, 1992). In addition to six genes, multiple isoforms of these receptors are generated by an alternative splicing and multiple promoter usage.

The role of COUP-TF orphan receptors in neurogenesis is virtually unknown. In Drosophila, the expression of COUP-TF homolog *seven-up* is required for development of specific subset of photoreceptor neurons during eye development (Mlodzik et al., 1990). COUP-TF I and COUP-TF II (Lutz et al., 1994; Qiu et al., 1994; our unpublished data) are expressed in a complex spatial and temporal pattern during development of the nervous system. To begin to investigate the function of COUP-TF I during neuronal differentiation, we have analyzed the effects of overexpression of COUP-TF I in neuronally differentiating teratocarcinoma PCC7 cells. Elevated levels of COUP-TF I block morphological differentiation and expression of the microtubule-associated protein 2 (MAP2) gene, whereas expression of the neurofilament light subunit (NF-L) gene is unchanged and the growth-associated protein 43 (GAP43) gene expression is altered. Also, cells overexpressing COUP-TF I do not stop proliferation after induction to differentiate. Transcriptional activation from different RA response elements (RARE) is inhibited in COUP-TF1-expressing PCC7 cells.

MATERIALS AND METHODS

Cell Culture and Establishing COUP-TF I Overexpressing Cell Lines

Mouse teratocarcinoma PCC7 cells (generous gift from Dr. S.E. Pfeiffer) were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (Gibco, Burlington, ON). Differentiation of PCC7 cells was induced with all-trans retinoic acid (0.5 μM, Sigma, St. Louis, MO) in the presence or absence of dibutyryl cyclic AMP (1 mM). Mouse COUP-TF I cDNA (2.3 kb) in pRcCMV expression vector (Invitrogen, San Diego, CA) was transfected into PCC7 cells followed by selection with G418 (400 μg/ml, Gibco) for 18–21 days to establish COUP-TF I-overexpressing cell lines. Mouse COUP-TF I cDNA was isolated from the newborn mouse brain cDNA library using rat COUP-TF I cDNA which was cloned in our laboratory (GeneBank accession number U10995, Connor et al., manuscript submitted). For growth analyses, cells were plated onto 100 mm tissue culture dishes (Falcon, Becton Dickinson, Franklin Lakes, NJ) at an initial density of 1×10^6 cells per plate. The cell number was counted using a hemocytometer (VWR) in triplicate samples from each time point.

Northern Blot Analyses

The RNA was fractionated on 1.2% agarose-formaldehyde gel and transferred to a nylon membrane (Hybond N, Amersham, Arlington Heights, IL). Twenty-five micrograms of total RNA were run in each lane. RNA was isolated using acid guanidinium/phenol/chloroform extraction procedure (Chomczynski and Sacchi, 1987). Full-length mouse COUP-TF I, rat GAP43 (gift from M.X. Zuber), mouse NF-L SmaI/BglII fragment, and mouse MAP2 clone 56 (gift from N.J. Cowan; Lewis et al., 1986) were radiolabeled (^{32}P) using Multiprime DNA labeling system (Amersham) and used as

probes. The blots were washed at high stringency (0.2 X SSC, 65°C) and exposed to X-ray film for 1 to 10 days. The amount and quality of transferred RNA were monitored by methylene blue staining of the filters before hybridization.

Transfections and CAT Assays

Cells were transfected using the calcium phosphate coprecipitation method (Okayama and Chen, 1991). Cells (4×10^5) in 60 mm dishes were transfected with 10 μg of plasmid DNA. Twenty-four hours later, the media were changed to regular media or differentiation media. For the expression assays, cells were harvested 48 hr later. Cells were washed and harvested in phosphate-buffered saline (PBS), lysed in 150 μl of 0.25 M Tris-HCl (pH 7.6) by freeze/thawing three times, and incubated at 65°C for 10 min to minimize deacylation activity. Protein concentration in the lysates was determined by a protein assay reagent (Pierce Chemical Co., Rockford, IL) with bovine serum albumin (BSA) used as a standard. A 150 μl mixture containing 0.4 mM acetyl coenzyme A, 0.1 μCi of [dichloroacetyl-1,2-^{14}C]chloramphenicol, and 10–25 μg of protein was incubated at 37°C for 0.5 to 1.5 hr. After extraction with ethyl acetate, the radioactive forms of chloramphenicol were resolved by thin-layer chromatography, localized by exposing to X-ray film, and quantitated by liquid scintillation counting. All experiments were done in triplicate.

For reporter plasmid construction, oligonucleotides containing corresponding HRE were synthesized, trimerized and cloned into the EcoRV site of Bluescript II KS (Stratagene, San Diego, CA). The orientation of the oligonucleotides was determined by sequencing. To insert oligos into HindIII/XbaI digested pBLCAT2 (Luckow and Schütz, 1987), the Bluescript vectors containing oligos were digested with HindIII/XbaI and the fragments were separated by polyacrylamide gel electrophoresis using a 10% gel in TBE buffer. The oligos were electroeluted and ligated to the HindIII/XbaI site of pBLCAT2. Oligonucleotides based on DR-1 (Kadowaki et al., 1992), β-RARE (Tran et al., 1992), and CRBP I (Tran et al., 1992) were synthesized.

DR-1:
5′-GGCTTCAGGTCAGAGGTCAGAGA
5′-GGTCTCTGACCTCTGACCTGAAG
β-RARE:
5′-GGTGTAGGGTTCACCGAAAGTTCACTCA
5′-GGTGAGTGAACTTTCGGTGAACCCTACA
CRBP I:
5′-CCATCCAGGTCAAAAAGTCAGGA
5′-GGTCCTGACTTTTTGACCTGGAT
HRE sequences are underlined.

Flow Cytometry

Cells were fixed in 70% ethanol/PBS and stained with propidium iodine (50 μg/ml) for 30 minutes at +4°C. DNA content of individual cells was measured using flow cytometer COULTER EPICS 5, and cell cycle analyses was performed using Multicycle analysis software (Phoenix Flow Cytometry System).

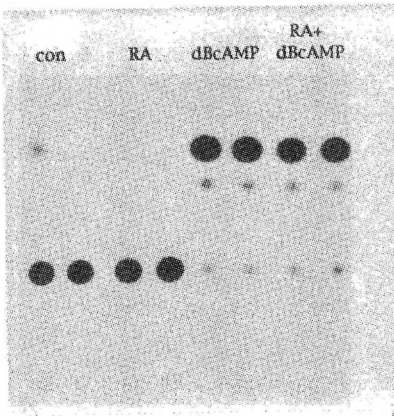
RESULTS

Overexpression of COUP-TF1 in Neuronally Differentiating Teratocarcinoma PCC7 Cells Results in Blocking Morphological Differentiation

Cytomegalovirus (CMV) promoter-based eukaryotic expression vectors allow expression of introduced genes at different levels during neuronal differentiation of PCC7 teratocarcinoma cells. CMV promoter activity is weak in proliferating PCC7 cells and is induced about 45 times after induction of differentiation by RA and dibutyryl cyclic AMP (dBcAMP) in transient assays using bacterial chloramphenicol acetyl transferase (CAT) as a reporter gene (Fig. 1). Similar activation of CMV promoter occurs in established cell lines with integrated pRcCMV-CAT vector (data not shown). Neuronal differentiation of PCC7 cells is also inducible by RA alone; however, it does not increase the CMV promoter activity (Fig. 1). By contrast, dBcAMP treatment alone stimulates CMV promoter activity but does not result in neuronal differentiation. Thus, these described characteristics of the CMV promoter activity during differentiation of PCC7 cells makes it possible to analyze neuronal differentiation in conditions where the introduced gene is expressed at low or high levels.

Teratocarcinoma PCC7 cells were transfected with COUP-TF I cDNA cloned into pRcCMVneo expression vector in order to analyze the effect of its overexpression during neuronal differentiation. Several clones were randomly selected, and COUP-TF I expression was correlated with their ability to differentiate. Northern blot analyses demonstrate that two clones express introduced COUP-TF I RNA at high levels after induction with dBcAMP (Fig. 2A). The same clones, 1 and 3, do not differentiate morphologically after treatment with RA and dBcAMP (Fig. 3).

Is the failure of isolated clones to differentiate a result of overexpression of COUP-TF I, or selection of aberrant clones? To answer this question, the expression of transfected COUP-TF I and morphological differentiation of clones 1 and 3 were analyzed after treatment with RA alone (weak expression of COUP-TF I) or with RA plus dBcAMP (induced expression of COUP-TF I). COUP-TF I mRNA was undetectable in control PCC7 cells during differentiation. Expression of transfected



CAT activity 1 1 45 43

Fig. 1. Effect of dBcAMP on induction of CMV promoter activity in neuronally differentiating teratocarcinoma PCC7 cells using transient CAT assay. After transfection of cells with the pRcCMV-CAT vector, neuronal differentiation was induced with RA or RA plus dBcAMP. CMV promoter activity is induced 45- and 43-fold after dBcAMP treatment compared to control or RA-treated cells.

COUP-TF I cDNA was induced in clones 1 and 3 after treatment with dBcAMP + RA as well as with dBcAMP alone but not in RA-treated cells (Fig. 2B). Morphological differentiation of clones 1 and 3 was blocked after treatment with RA + dBcAMP (Fig. 3), i.e., in conditions which induce overexpression of transfected COUP-TF I. No changes in morphological differentiation of clones 1 and 3 were observed after treatment with RA (Fig. 3). These results demonstrate that clones 1 and 3 differentiate normally in the conditions where introduced COUP-TF I is expressed at low levels and confirm the hypothesis that overexpression of COUP-TF1 blocks neuronal differentiation of teratocarcinoma PCC7 cells.

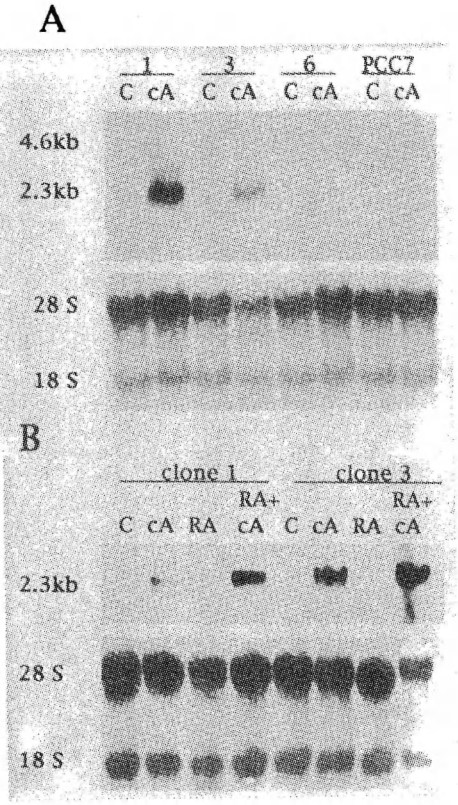


Fig. 2. Northern blot analysis of COUP-TF I expression in teratocarcinoma PCC7 cells. **A:** Total RNA was isolated from randomly selected clones 1, 3, and 6 of PCC7 cells after transfection with COUP-TF I cDNA cloned into eukaryotic expression vector pRcCMV and from control PCC7 cells. Clones 1 and 3 express introduced COUP-TF I mRNA (2.3 kb) at high levels after treatment of cells with dBcAMP (cA) compared to untreated cells (C). Overexpression of COUP-TF I also induces expression of endogenous COUP-TF I (4.6 kb). **B:** Induction of COUP-TF I mRNA expression in clones 1 and 3 after treatment with dBcAMP (cA), retinoic acid (RA), and RA plus dBcAMP (RA + cA) compared to untreated controls (C). Amount of RNA ran in each lane is demonstrated by methylene blue staining of filters before hybridization. Positions of 28S and 18S ribosomal RNAs are indicated (28 S and 18 S, respectively).

Effect of COUP-TF1 on the Expression of Neuronal Marker Genes

Expressions of NF-L, GAP-43, and MAP2 mRNAs in overexpressing COUP-TF I PCC7 cells were analyzed using Northern blot. Differentiation of PCC7 cells results in induction of NF-L and MAP2 genes 48 hours, and GAP-43 gene 12 hours after treatment with RA + dBcAMP (Fig. 4). Difference in the time course of induction indicates that neuronal specific genes are regulated by different mechanisms during neuronal differentiation of PCC7 cells. Overexpression of COUP-TF I results in no changes of NF-L expression, delayed induction of GAP-43 gene expression, and blocked expression of MAP2 gene (Fig. 4) after induction of neuronal differentiation with RA and dBcAMP. Induction of neuronal differentiation without stimulation of COUP-TF I expression (RA treatment) results in stimulation of the

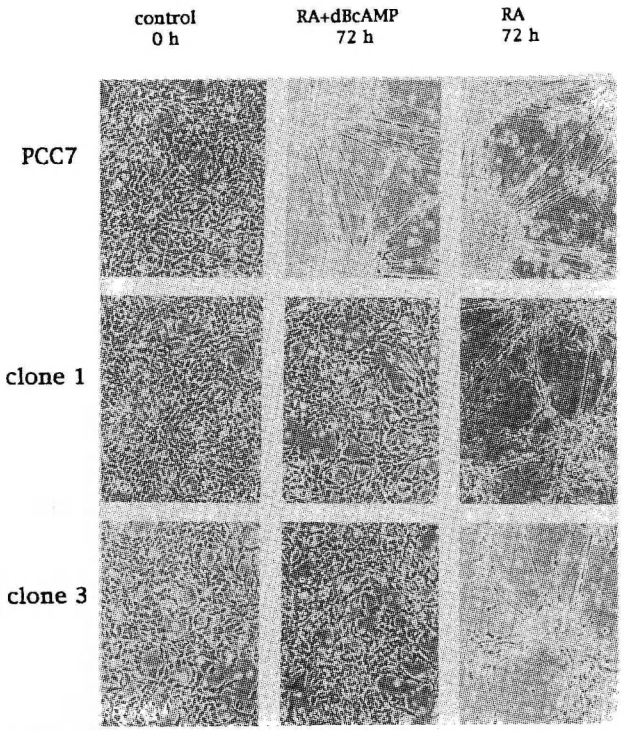


Fig. 3. Effect of COUP-TF I expression on neuronal differentiation of PCC7 cells. Control PCC7 cells and COUP-TF I-expressing clones 1 and 3 (0 h) were treated for 72 hours (72 h) with RA plus dBcAMP to induce neuronal differentiation and high level expression of COUP-TF I or RA alone which induces neuronal differentiation but does not result in high level expression of COUP-TF I.

neuronal marker genes similar to control. These data argue for the hypothesis that separate pathways of neuronal development are affected differently by COUP-TF I.

Overexpression of COUP-TF1 Blocks Exit of PCC7 Cells From the Cell Cycle After Treatment With RA and dBcAMP

Neuronal differentiation of PCC7 cells is accompanied by cessation of cell proliferation (Pfeiffer et al., 1981). Flow cytometric analyses were performed to characterize changes in cell cycle during differentiation of control PCC7 cells and COUP-TF I overexpressing clones. No changes in the cell cycle were detected in clones 1 and 3 during normal growth (Table I). For all analyzed untreated clones, wild type PCC7 and COUP-TF I-expressing clones 1 and 3, the percentage of cells in the G1 phase was 41–42%, in the S phase 36–38%, and in the G2 phase 20–24%. Differentiation of PCC7 cells blocks the cell cycle in G1 phase. During differentiation, the percentage of cells in G1/G0 phase increases constantly and reaches 98% after three days of treatment (Table I). Contrary to wild type PCC7 cells, the cell cycle is not blocked in COUP-TF I-overexpressing clones 1 and 3. Treatment of COUP-TF I-overexpressing cells with RA and dBcAMP results in an increase in the portion of cells in G1/G0 from 41–42% in day 0 to 64–65% at day three (Table I). To quantitate the proliferation of COUP-TF I-expressing clones, cell counts were made at 12 hour intervals after RA and

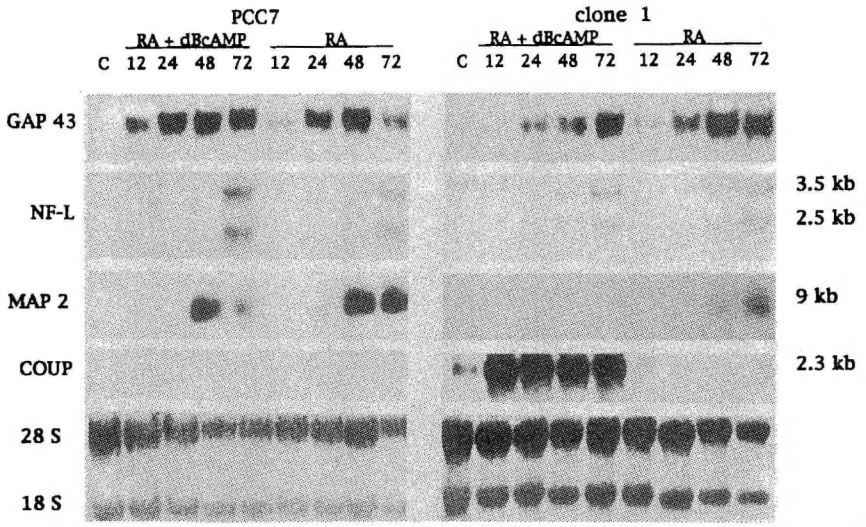


Fig. 4. Northern blot analyses of COUP-TF I effect on induction of GAP43, NF-L, and MAP2 gene expression during neuronal differentiation of PCC7 cells and COUP-TF I-expressing clone 1. Neuronal differentiation was induced with RA plus dBcAMP (RA + dBcAMP) or with RA alone (RA) and total RNA was isolated before treatment (C) and after 12, 24, 48, and 72 hours (12, 24, 48, and 72, respectively). COUP-TF I (COUP) expression is detectable only in clone 1 but not in control PCC7 cells. Amount of RNA ran in each lane is demonstrated by methylene blue staining of filters before hybridization. Positions of 28S and 18S ribosomal RNAs are indicated (28 S and 18 S, respectively).

dBcAMP induction (Fig. 5). Control PCC7 cells stop proliferation after 36–48 hours of treatment. On the contrary, COUP-TF I-overexpressing cells continue proliferation; however, the proliferation rate becomes reduced after 36 hours.

Induction of RARE Enhancers in COUP-TF1 Overexpressing Cells

COUP-TF I forms inactive heterodimers with RXRs and binds to retinoic acid response elements (RARE) from different genes as a homodimer to repress the retinoic acid response. To investigate whether COUP-TF I blocks RA signalling pathway in neuronally differentiating PCC7 cells, we analyzed the activities of CAT reporter constructs containing different RAREs in the front of a thymidine kinase promoter in COUP-TF I-overexpressing cells. Since the effects of COUP-TFs depend on the RARE sequences and promoter context, we analyzed two natural and one synthetic RAREs: the βRARE, a direct repeat with a 5-bp spacer that is activated by RARα (Tran et al., 1992); the CRBP I-RARE, a direct repeat with a 2-bp spacer that is optimally activated by RAR/RXR heterodimers but not by RXR homodimers (Hermann et al., 1992; Zhang et al., 1992), and the DR-1, a direct repeat that contains a 1-bp spacer and has a high affinity to COUP-TF I (Kadowaki et al., 1992) and RXRs (Mangelsdorf et al., 1990, 1991). The activity of all three reporter constructs is inducible in PCC7 cells with RA alone or with RA plus dBcAMP, and the effect of RA is potentiated by dBcAMP (Fig. 6). In COUP-TF I-overexpressing cells treated only with RA, the induction of βRARE and CRBPI enhancers is reduced (2.5 and 1.5 times, respectively) compared to control cells, and no induction was detected from DR-1 reporter construct. The induction of COUP-TF I expression in clones 1 and 3 by dBcAMP in the presence of RA results in further inhibition of βRARE- and CRBPI-mediated transcription (Fig. 6). Inhibition of βRARE-driven reporter gene activity in COUP-TF I-overexpressing cells contradicts data obtained from RARα and COUP-TF I cotransfection experiments which demonstrate that COUP-TF I does not inhibit induction of βRARE by RARα (Tran et al., 1992). In differentiating PCC7 cells, expression of all three RARs and RXRα and RXRβ is induced (our unpublished data) and it is unknown which of the RAR/RXR complexes are involved

TABLE I. Effect of COUP-TF1 Expression on Cell Cycle Changes During Neuronal Differentiation of PCC7 Cells*

	% Cells in G1, S, and G2 phases of the cell cycle											
	Differentiation with RA and dBcAMP											
	Untreated			1 day			2 days			3 days		
Clone	G1/G0	S	G2	G1/G0	S	G2	G1/G0	S	G2	G1/G0	S	G2
PCC7	42	38	20	58	24	18	75	13	12	98	0	2
Clone 1	41	38	21	50	25	25	58	23	19	65	18	17
Clone 3	42	36	24	47	30	23	55	26	19	64	19	17

*Numbers represent means from three independent experiments. Standard deviations were less than 5% from the average values in all experiments.

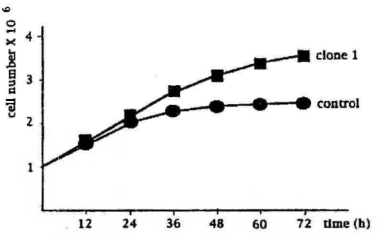


Fig. 5. Growth of COUP-TF-I-expressing (clone 1) and control PCC7 cells after RA + dBcAMP induction of neuronal differentiation. Cells were treated with RA + dBcAMP for 72 hours, and cell counts were made at 12 hour intervals using hemocytometer. All experiments were done in triplicate. Standard deviations were less than 7% from the average values in all experiments.

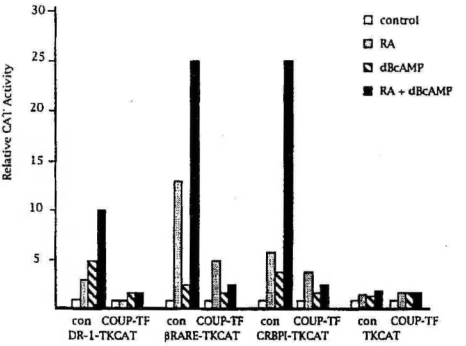


Fig. 6. The effect of COUP-TF I expression on activity of different RAREs in PCC7 cells treated with RA, dBcAMP, and RA plus dBcAMP. The transient CAT activity with DR-1-TKCAT, βRARE-TKCAT, CRBP I-TKCAT, and TKCAT constructs was measured in control (con) and COUP-TF I-expressing clone 1 (COUP-TF) PCC7 cells. The activities represent averages of three experiments and are expressed relative to the value obtained by transfection of corresponding construct into untreated cells, for which activity was set at 1. All activities are normalized to total cellular protein. Standard deviations were less that 10% from the average values in all experiments.

in the induction of βRARE enhancer. It is possible that βRARE induction is mediated by RAR/RXR complexes other than RARα homodimers and that these complexes are differentially affected by COUP-TF I.

DISCUSSION

During the development of the nervous system, different hormone receptors including COUP-TF I and II have a complex spatiotemporal expression pattern. Several in vitro studies demonstrate that COUP-TFs act as repressors of various steroid/thyroid hormone pathways in a dose-dependent manner. Analyses of apolipoprotein AI (Ladias and Karathanasis, 1991; Widom et al., 1992), apolipoproteins B, CIII, and AII (Ladias et al., 1992), and ornithine transcarbamylase (Kimura et al., 1993) promoters show that COUP-TFs bind to direct or inverted repeats of GGTCA sequences with variable nucleotide spacings and block the transcriptional activation by other hormone receptors. Repression by COUP-TFs may also sensitize promoters of different genes to positively acting hormone receptors (Widom et al., 1992). Apolipoprotein AI promoter site A is refractory to transactivation by RXRα and RA, but prior repression of this promoter by ARP-1 (COUP-TF II) results in full transactivation by RXRα and RA. It is proposed that ARP-1 repression uncouples the endogenous regulatory proteins bound to site A and thus converts site A into a form accessible to RXRα (Widom et al., 1992). This example brings up the possibility that COUP-TFs may play a fundamental role in switching transcription activation pathways from hormone-independent to hormone-dependent. Alternatively, COUP-TFs may be converted to positive transcriptional regulators by unknown ligands or neurotransmitters as it has been shown in the latter case for dopamine which activates COUP-TF I in transient transfection assays (Power et al., 1991a,b).

Despite the numerous data on molecular interactions of COUP-TFs, the role of these orphan receptors in neurogenesis remains unknown. We analyzed the effect of COUP-TF I on neuronal differentiation of PCC7 cells in conditions where the COUP-TF I was expressed either at low or highly induced levels. The inducible system has certain advantages in comparison to constitute expression systems. First, it allowed us to avoid potential artifacts associated with chronic high level COUP-TF I expression during weeks of selection. Second, it allowed us to compare the behavior of the same genetically homogeneous clones under inducing and noninducing conditions, factoring out clonal variations. Our data demonstrate that overexpression of COUP-TF I in PCC7 cells results in the block of morphological differentiation and induction of MAP2 gene. Further, COUP-TF I overexpression results in blocking cessation of cell proliferation, which normally occurs after treatment of PCC7 cells with RA and dBcAMP. Disturbances in morphological differentiation and withdrawal from the cell cycle may be related to the suppression of MAP2 induction by COUP-TF I. It has been shown that inhibition of MAP2 expression by antisense oligonucleotides in RA-induced P19 teratocarcinoma cells results in blockade of normal morphological differentiation and changes in the cell cycle (Dinsmore and Solomon, 1991).

Our results also demonstrate that induction of NF-L is not affected and induction of GAP43 is delayed in COUP-TF I-overexpressing PCC7 cells after treatment with RA and dBcAMP. Treatment with RA alone, which results in differentiation but not high level expression of introduced COUP-TF I, does not affect induction of any of the analyzed neuronal genes. However, transient CAT assays with different RARE sequences demonstrate the inhibition of transcriptional induction in COUP-TF I-overexpressing cells compared to control PCC7 cells after RA treatment. Induction of COUP-TF I expression by dBcAMP further inhibits RARE-mediated transcriptional activation but does not block it completely. Considering that NF-L, GAP43, and MAP2 genes have several RARE sequences in the 5′ regulatory regions (our observation) it is possible that induction of NF-L gene by RA requires much lower levels of activated RARs or RXRs than activation of GAP43 and MAP2 genes. If this is the case, the ratio of COUP-TF I and RAR/RXRs may regulate the timing of switching on different neural-specific genes when neuroblasts become committed to differentiate. This hypothesis is supported by the observations that expression of NF-L gene starts before the final mitoses (Bennett and DiLullo, 1985) when the neuroblasts are in the ventricular zone and express detectable levels of COUP-TF I (our unpublished data). Initiation of neuronal differentiation and migration out from the ventricular zone correlates with the reduction of COUP-TF I expression. Also, withdrawal of neuroblasts from the cell cycle may be related to the reduced levels of COUP-TF I expression during the final cell cycle.

We conclude that COUP-TF I may be involved in control of neurogenesis by modifying activity of RAR/RXRs (and other steroid hormone receptors) which regulate the expression of neuronal marker genes as well as genes regulating cessation of proliferation and initiation of migration and differentiation of neuroblasts.

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Cell type specific regulation of COUP-TF II promoter activity.

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Abstract COUP-TF family orphan receptors regulate activity of ligand-activated nuclear hormone receptors or function independently in the regulation of gene expression. COUP-TF II has a complex expression pattern suggesting that different mechanisms are involved in the regulation of its expression. We isolated the 5′ regulatory region of the mouse COUP-TF II gene and demonstrated that the basal promoter is localized in a −200 bp region 5′ from the transcription start site. All-*trans* retinoic acid and dibutyryl cyclic AMP have cell type specific effects on COUP-TF II promoter activity. The effect of cyclic AMP is mediated by the cyclic AMP response element that is localized 74 nucleotides upstream from the major transcriptional start. In vitro promoter analyses also demonstrated that the effect of all-*trans* RA is not directly mediated by the binding of RARs or RXRs to the promoter sequence.

Key words: COUP-TF II; Gene regulation; Promoter; Retinoic acid; Dibutyryl cyclic AMP

1. Introduction

Nuclear hormone receptors play an important role during development of different cell types and organ systems [1,2]. Retinoic acid (RA) receptors, retinoid X receptors, thyroid hormone receptors, steroid hormone receptors, and several other members of the superfamily of nuclear hormone receptors regulate expression of a variety of target genes [3,4]. These ligand dependent transcription factors bind as homo- or heterodimers to hormone response elements (HRE) located in the regulatory regions of target genes and regulate transcription [3]. Recently, it has been shown that nuclear hormone receptors can also suppress transcriptional activity by complexing with non DNA binding co-repressors [5–8]. Beside the ligand activated transcription factors, the nuclear hormone receptor superfamily comprises orphan receptors for which ligands are not known [9,10]. Orphan receptors, such as chicken ovalbumin upstream promoter transcription factor (COUP-TF) homologs, have been isolated from several species including rodents and human [11–21]. COUP-TF orphan receptors function as transcriptional activators or suppressors depending on the complexes they form and DNA sequences on which they bind. One function of the COUP-TFs is to regulate the activity of ligand activated nuclear hormone receptors [22,23]. Several different mechanisms may contribute to the repression of induction including direct competition of COUP-TFs for the hormone response elements, heterodimerization with retinoid X receptors (RXR) and suppression of transcription by COUP-TF homodimers [22,24–27]. Repression by COUP-TFs may also sensitize promoters of different genes to positively acting nuclear hormone receptors [26]. COUP-TFs may also function as a stimulatory transcription factors. COUP-TFs stimulate transcription of arrestin gene by binding to a direct repeat with a 7 bp spacer located upstream of the transcription start site [28]. In combination with HNF-4 transcription factor, COUP-TF I and COUP-TF II may also stimulate transcription of the phosphoenolpyruvate carboxykinase gene [29].

Complex expression patterns of COUP-TF I (Ear-3) and COUP-TF II (ARP-1) during development argue for their functional roles in several developmental processes. While the potential functions of COUP-TFs have been extensively investigated, the molecular mechanisms responsible for the spatial and temporal expression of COUP-TFs are still poorly understood. Expression of COUP-TFs in different regions of developing and adult organisms indicates that different mechanisms are involved in the regulation of their spatial and temporal expression.

Here we demonstrate that COUP-TF II basal promoter is localized in the 200 bp region 5′ from the transcription start site, and that all-*trans* retinoic acid and dibutyryl cyclic AMP have cell type specific effects on COUP-TF II promoter activity. Also, COUP-TF I and COUP-TF II weakly inhibit RA induced COUP-TF II promoter activity in PCC7 cells.

2. Materials and methods

2.1. Plasmid construction

A mouse genomic DNA library (Clontech) was screened with mouse COUP-TF II cDNA probe (isolated in our laboratory) using high stringency conditions (0.1×SSC, 65°C; 1×SSC = 150 mM NaCl, 15 mM Na-citrate). Fragments of isolated clones were subcloned into Bluescript II KS plasmid (Stratagene) for sequencing and generation of promoter constructs. All the COUP-TF II promoter fragments were cloned into the unique *Bgl*II site of the vector pCAT3N using *Bgl*II linkers. COUP-TF II promoter fragments of various lengths were generated by digestion of 5′ regulatory region DNA with endonucleases listed below: for plasmid −4000/*Bgl*II with *Bam*HI and *Bgl*II, for −1500/*Bgl*II with *Hin*dIII and *Bgl*II, for −621/*Bgl*II with *Bst*XI and *Bgl*II, for −320/*Apa*I with *Apa*I, for −97/*Apa*I with *Sty*I and *Apa*I, for −320/*Sac*II with *Apa*I and *Sac*II, for −40/*Sac*II with *Sac*I and *Sac*II, for −40/*Apa*I with *Sac*I and *Apa*I, and for *Sac*II/*Apa*I with *Sac*II and *Apa*I. Constructs −258/*Apa*I and −200/*Apa*I were generated by digestion of *Apa*I fragment with Exonuclease III. Construct Δ*Sty*I/*Apa*I was generated by deletion of *Sty*I fragment from the −320/*Apa*I plasmid.

Site directed mutagenesis was performed using plasmid −320/*Apa*I and oligonucleotides 5′-GTTGCAGCAGTCGTGATGCATTTCAC-TATATAGAGAG (mut 1) and 5′-ACGTGCGCTAAGTTGCA-TATGTCGTGTCAAAGTTCACT (mut 3). Erase-a-Base System (Promega) was used to generate mutations mut 2 and mut 4 by digesting plasmids mut 1 and mut 3 with *Nsi*I and *Nde*I, respectively.

Mouse COUP-TF I and COUP-TF II (cloned in our laboratory) cDNAs were cloned into eukaryotic expression vector pRcCMV-neo (Invitrogen) using appropriate restriction endonucleases.

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A

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BstXI
TGGTTTGCAAGCCCAGATCCCGGCACCTTGCCACCTCCGCTGCGTACCCCTCTACAAAGA -562
AGGGGAGAGCATTATTCAGTCTTTTGGAATAGTGGGTTCTACATAATGCGCCGGGAGTCC -502
CGGTGGACCGCGAGCTCGCATTTAGAGGCATCGCAAGTTGCGAGAGACTAAGTCTTTGGC -442
TCCATCCTCAACCCCTTGCGGACGCTTTAAGAGTCGTAGTGTGTGCGCCGCACTAGTCTT -382
GCGCAGGGCGGAAACCACTGTGCCGATGCGGCGGCGGGGTCCGCGCTCGGCTGCCACCGG -322
ApaI
GCCCCCATCCCCCTCTCTGTGGCCAGGACCGCGCGGCCACCCGTCCCCGCCCCCCTCCGT -262
CCCCGCGCGCCCCGTGCGCTTGTCTCGACGCCGCTCGCGCTAGGACCGGGCTGCTCCCGC -202
    StyI
TGCCGCCATGGCGGGCCGCTGCGCTCCGGCCAATGACGACGAGGGGGCGGCGCGCGCGCG -142
                                          StyI
CGGCCGGGCCAACCCCGGCGGCTGCCTTATAAGGCGGCCCGTCGCCATGGCAACGTGCGC  -82
                        * *          SacI        * *
TAAGTTGCAGCAGTCGTGTCAAAGTTCACTATATAGAGAGCTCAGTGAGTGATCGAGGAG  -22
                      * *1*                       * *
AAGCAACTTCTGCCAGCCCGGAGCCTATAAATCGCATTCCCTCCCGAGCCCCCCTTTTTA   37
                                  SacII
GCATATTTGATCACTTTGATTCTGCTCTTTTCTCTCCGCGGTGTGTGCGTCGGTGCGCGC   97
GTGTGTGTTTTCTTCTCCTCCTCCTCCTCTGCGAGTTGCCTCCTTTCTCCGGGTGCGGCT  157
GCTCTTCCCTCTTTCATTCTTTCTCTCCGTTTCTCCCCCCTCTGCGCACGAAGGATGCGC  217
TTCTAGGTGCTGATCTGCCCTCCTTTCTCTCTTATCATCTCTCCCGCCGCCGGCGAGTTG  277
ACTCTTTCCCTATTGCTGCTTGAGGTGTGCGGCAGCAGCAGCAGCAGCAGCAGCAGCAGC  337
GGCTCCACCGGCGGCGGCAGCAGCGGCAGCAGCAGCAGCGACTCCACCGGGGACGGCGGG  397
                ApaI
TAGACGCAGCGGCTCCGGGCCC
```

B

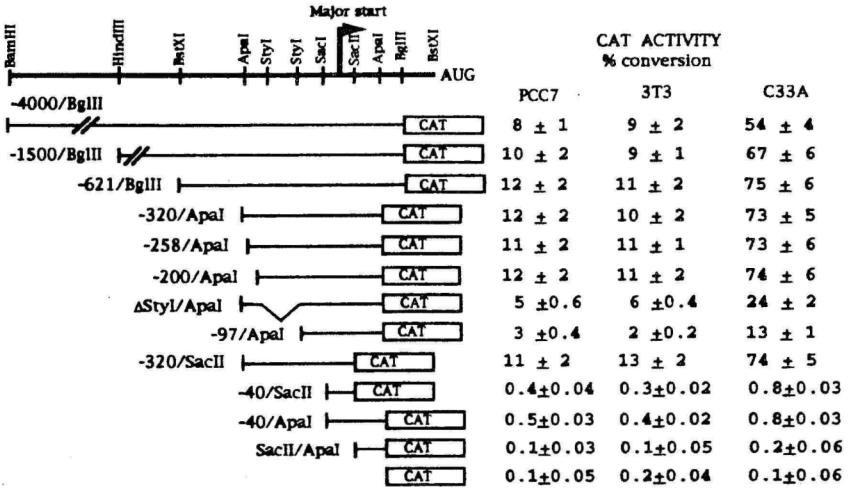
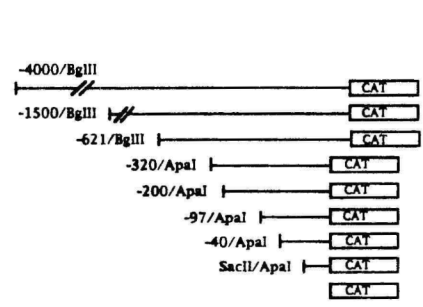


Fig. 1. Nucleotide sequence of the mouse COUP-TF II 5' regulatory region (A) and its promoter activity in mouse embryo carcinoma PCC7, mouse fibroblast 3T3, and human cervical carcinoma C33A cells (B). (A) Nucleotide numbering starts at the major transcriptional start site (1). upstream nucleotides have negative numbers. Minor start sites are indicated by * (above the corresponding nucleotides). The TATA-box core motifs are indicated by boldface letters, and restriction sites used in generation of CAT constructs are indicated. (B) In the schematic representation of mCOUP-TF II promoter region restriction sites and major start site (arrow) are indicated. The data shown are representative of at least three independent CAT assays.

2.2. Cell culture

Embryonic carcinoma cell line PCC7 was obtained from S. Pfeiffer and grown in Dulbecco's modified Eagle's medium (Gibco) containing 10% fetal bovine serum (Summit Biotechnology). PCC7 cells were



	CAT ACTIVITY % conversion			
	CONTROL	RA	dBcAMP	dBcAMP+RA
-4000/BglII	2.5 ± 0.5	11 ± 2	10 ± 1	38 ± 3
-1500/BglII	2.8 ± 0.3	9 ± 1	11 ± 1	41 ± 3
-621/BglII	2.3 ± 0.2	11 ± 2	10 ± 1	39 ± 2
-320/ApaI	2.0 ± 0.1	10 ± 1	9 ± 1	42 ± 2
-200/ApaI	2.1 ± 0.3	11 ± 1	10 ± 1	42 ± 3
-97/ApaI	0.4 ± 0.05	3 ± 0.3	4 ± 0.5	9 ± 1
-40/ApaI	0.3 ± 0.05	0.3 ± 0.04	0.3 ± 0.05	0.4 ± 0.06
SacII/ApaI	0.2 ± 0.06	0.1 ± 0.05	0.2 ± 0.06	0.1 ± 0.05
CAT	0.1 ± 0.06	0.2 ± 0.05	0.2 ± 0.05	0.1 ± 0.06

Fig. 2. Effects of all-*trans* retinoic acid and dibutyryl cAMP on COUP-TF II promoter activity in CAT assay using mouse embryo carcinoma PCC7 cells. Cells were treated with all-*trans* RA (0.5 μM), dBcAMP (1 mM) or combination of all-*trans* RA and dBcAMP. The data shown are representative of at least three independent experiments.

differentiated into neuronal-like cells with all-*trans* RA (0.5 μM) and dibutyryl cyclic AMP (dBcAMP; 1 mM) treatment. Mouse fibroblast 3T3, rat glioblastoma C6, human glioblastoma U373, and human cervical carcinoma C33A cells were obtained from the American Type Culture Collection and were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum.

2.3. DNA transfection and CAT assays

Cells were transfected with 15 μg of total plasmid DNA, using the calcium phosphate precipitation method [30] in 60 mm diameter dishes (1×10^5 to 3×10^5 cells per dish). The medium was changed 12–18 h after transfection to normal growth medium or medium containing all-*trans* RA (5×10^{-7} M) or dBcAMP (1 mM) as indicated for each experiment. Cells were harvested 48 h later. Cells were washed and harvested in PBS, lysed in 150 μl of 0.25 M Tris-HCl (pH 7.6) by freeze/thawing three times, and incubated at 65°C for 10 min to minimize deacylation activity. Protein concentration in the lysates was determined by a protein assay reagent (Pierce Chemical Co.) with BSA used as a standard. A 150 μl mixture containing 0.4 mM acetyl coenzyme A, 0.1 μCi of [dichloroacetyl-1,2-^{14}C]chloramphenicol, and 10–25 μg of protein was incubated at 37°C for 0.5–1.5 h. After extraction with ethyl acetate, the radioactive forms of chloramphenicol were resolved by thin-layer chromatography. Quantitation of acetylation ratios was obtained by PhosphorImager (Molecular Dynamics) analysis. To normalize transfection efficiencies, cells were cotransfected with 1 μg of plasmid pRcRSVlacZ. All the reported CAT activities were normalized to total protein and lacZ activity. The CAT assay values represent the means of at least three independent transfections.

3. Results

3.1. Analysis of COUP-TF II gene promoter region

We isolated a phage clone containing the 5′ end of the COUP-TF II gene and sequenced a 1.5 kb fragment that extended for 622 nucleotides upstream of the major transcription initiation site (Fig. 1A). The transcription initiation sites were mapped by primer extension and RNase protection analyses (data not shown) using RNA isolated from embryonic day 11, 13, and 15 mouse embryos. Both methods demonstrated the presence of several transcription start sites (Fig. 1). The sequence of the 5′-proximal region contains several consensus TATA box sequences in the region where all the transcription start sites are localized (Fig. 1A). TATA box sequences in COUP-TF II promoter do not lie at typical distances (20–30 nucleotides) from start sites which is not an unusual situation.

To determine the sequences that are essential for transcription of mouse COUP-TF II gene, various portions of the 5′-flanking region were fused to the bacterial chloramphenicol acetyltransferase (CAT) gene as a heterologous reporter gene. Reporter plasmids containing different fragments of the COUP-TF II gene 5′ region (Fig. 1B) were transiently transfected into the mouse teratocarcinoma PCC7, mouse fibroblast 3T3, and human cervical carcinoma C33A cells. CAT assays demonstrated that COUP-TF II promoter CAT plasmids −4000/*Bgl*II, −1500/*Bgl*II, −621/*Bgl*II, −320/*Apa*I, −258/*Apa*I, −200/*Apa*I, and −320/*Sac*II have similar promoter activity in all studied cell lines (Fig. 1B). Deletion of additional 103 nucleotides (construct −97/*Apa*I) resulted in 4–6-fold reduction of promoter activity. Also, deletion of nucleotides −196 to −97 from −320/*Apa*I construct (plasmid Δ*Sty*I/*Apa*I) resulted in 2–3-fold reduction of promoter activity. CAT constructs containing 40 bp promoter sequences (constructs −40/*Sac*II and −40/*Apa*I) and first exon sequences (construct *Sac*II/*Apa*I) showed almost no CAT activity. This deletion analysis demonstrated that the COUP-TF II basal promoter is localized in the 200 bp region 5′ from the transcription start site.

3.2. All-trans retinoic acid and cAMP induce COUP-TF II promoter activity in teratocarcinoma PCC7 cells and suppress it in C6 and U373 glioma cells

Since COUP-TF II is involved in the modulation of retinoic acid responses and may function as a part of the regulatory loop, we analyzed the effect of all-*trans* RA on the activity of its promoter. Initially, we used teratocarcinoma PCC7 cells which have been shown to express all three RAR and RXR genes [31]. Also, RA treatment induces expression of all three RAR genes in these cells ([31] and our unpublished data). CAT assays following transient transfection by COUP-TF II promoter-CAT plasmids containing different fragments of 5′ regulatory region (−4000/*Bgl*II, −1500/*Bgl*II, −621/*Bgl*II, −320/*Apa*I, −200/*Apa*I, −97/*Apa*I, −40/*Apa*I, and *Sac*II/*Apa*I) demonstrated that RA treatment induces promoter activity of all plasmids which contain more than 40 bp of the promoter region (Fig. 2). These CAT assay data suggest that RA response element(s) are localized in the −97 to −40 nucleotide promoter region. Increased levels of cAMP have been shown to potentiate the effect of RA on neuronal differentiation in teratocarcinoma cells (our unpublished data). Based on

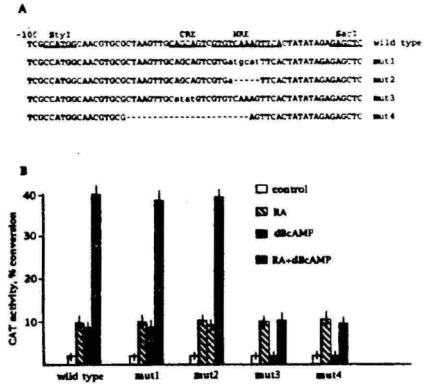


Fig. 3. Effects of mutations in CRE and HRE on mCOUP-TF II promoter activity. (A) Nucleotide sequences of wild type and mutated (mut 1, mut 2, mut 3, mut 4; changed nucleotides are in lower case) promoter regions. (B) Effect of all-*trans* retinoic acid and dibutyryl cAMP on wild type and mutated COUP-TF II promoter activity in transient CAT assay using mouse embryo carcinoma PCC7 cells. The wild type −320/*Apa*I CAT plasmid and mutated reporter plasmids, mut 1, mut 2, mut 3, and mut 4 were transfected into PCC7 cells and cells were treated with all-*trans* RA (0.5 μM), dBcAMP (1 mM) or combination of all-*trans* RA and dBcAMP. The data shown are representative of at least three independent experiments.

these observations, we also analyzed the effect of dibutyryl cAMP (dBcAMP) on COUP-TF II promoter activity in teratocarcinoma PCC7 cells. The same promoter CAT constructs that responded to the RA treatment were also responsive to dBcAMP treatment (Fig. 2). This argues that cAMP response element(s) are localized in −97 to −40 bp region of COUP-TF II promoter. Cotreatment of PCC7 cells with RA and dBcAMP stimulated COUP-TF II promoter activity about 15–20-fold.

Sequence analyses of −97 to −40 bp COUP-TF II promoter region revealed a cAMP response element (CRE) and a possible consensus sequence for hormone response element (HRE) localized next to each other (Fig. 3). Mutations were introduced into these elements to assess their functionality (Fig. 3). COUP-TF II 320 bp promoter CAT plasmids (−320/*Apa*I CAT) containing mutated CRE and HRE were transfected into PCC7 cells, and effects of RA and dBcAMP on promoter activity were analyzed. Surprisingly, mutations which destroyed HRE (mut 1 and mut 2) did not affect inducibility of COUP-TF II promoter activity (Fig. 3). However, mutations in CRE (mut 3) or in both CRE and HRE together (mut 4) completely blocked the effect of dBcAMP on the inducibility of the promoter. These results argue that RA has an indirect effect on COUP-TF II promoter activity in PCC7 cells. Also, the additive effect of RA and dBcAMP suggests that RA and dBcAMP have different pathways to stimulate COUP-TF II promoter activity.

To further explore the role of RA on COUP-TF II promoter activity, we performed CAT assays using mouse fibroblast 3T3, rat glioblastoma C6, human glioblastoma U373, and human cervical carcinoma C33A cells. Treatment of 3T3 and C33A cells with RA and dBcAMP did not affect the activity of COUP-TF II promoter constructs −320/*Apa*I and −4000/*Bgl*II (Fig. 4A,B). On the other hand, both RA and dBcAMP suppressed activity of COUP-TF II promoter constructs in C6 and U373 cells.

3.3. COUP-TF I and COUP-TF II weakly inhibit RA induced COUP-TF II promoter activity in PCC7 cells

Transcription factors COUP-TF I and COUP-TF II block the stimulatory effect of RA on transcription of several genes [24–27]. Our data demonstrate that RA induces COUP-TF II promoter activity in PCC7 cells. We then asked whether COUP-TF I and COUP-TF II block the stimulatory effect of RA on COUP-TF II promoter activity. We cotransfected the −320/*Apa*I COUP-TF II CAT construct with COUP-TF I and COUP-TF II expression plasmids into PCC7 cells and analyzed CAT activities. As a positive control, we used a plasmid containing an RARβ2 promoter *lac*Z construct (from A. Zimmer, NIH) that has been shown to be inducible with activated RARs and RXRs. CAT assays demonstrated that both COUP-TF I and COUP-TF II slightly suppress RA and dBcAMP induced COUP-TF II promoter activity (Fig. 4C). By contrast, COUP-TF I and COUP-TF II completely inhibit the RA and dBcAMP induced RARβ2 promoter activity (Fig. 4C). This is an additional indication that RA does not directly regulate COUP-TF II promoter activity through activation of RAR/RXR complexes.

4. Discussion

The central issue addressed in this study is the transcriptional regulation of the COUP-TF II gene. COUP-TF II gene is expressed in a complex spatio-temporal pattern during development of several organ systems including nervous system [19–21,32]. This complex expression pattern argues for a different mechanisms of COUP-TF II gene regulation in different cell types. Our data demonstrate that the effects of RA and dBcAMP on the COUP-TF II promoter activity are different or even opposite in different cell types. Also, treatment of mouse embryos with all-*trans* RA results in stimulation of COUP-TF II expression in the spinal cord and suppression of expression in the telencephalon [33]. Additionally, it has been demonstrated that expression of COUP-TF I and COUP-TF II is induced during neuronal differentiation of teratocarcinoma P19 cells [19,32] but not in several other teratocarcinomas (our unpublished data). Our data support the hypothesis that COUP-TF II gene expression is regulated differently in different cell types and that the same stimuli may regulate its expression in opposite directions depending on the cell type.

Analyses of COUP-TF II promoter activity localized the functional cAMP response element 74 nucleotides upstream from the major transcriptional start site. This CRE is responsible for the stimulatory effect of dBcAMP in teratocarcinoma PCC7 cells. The suppressive effect of dBcAMP on COUP-TF II promoter activity in C6 and U373 cells is not mediated by the same CRE since mutation in this sequence or deletion of it does not change the response of COUP-TF II promoter to dBcAMP in these cells. Sequence analyses of COUP-TF II promoter also identified a possible HRE localized 68 nucleotides from the major transcriptional start site. The sequence of this element does not correspond exactly to HRE, but considering the high variability of HRE sequences, it is possible that this sequence functions as a binding site for RARs or RXRs. Also, CAT assays mapped the RA response element to

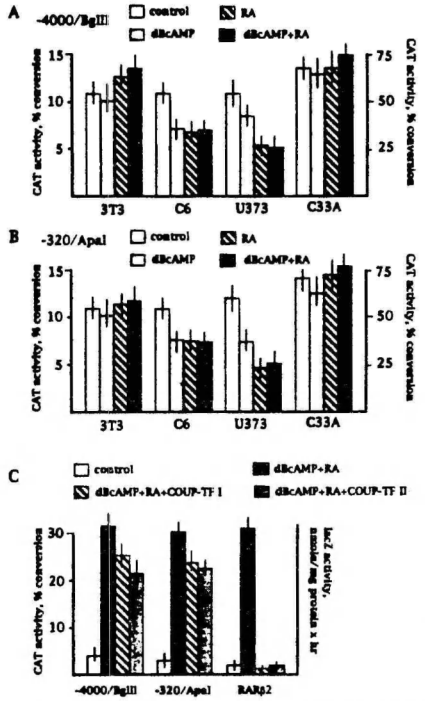


Fig. 4. Effects of all-*trans* retinoic acid, dibutyryl cAMP (A and B), and COUP-TF I and COUP-TF II (C) on COUP-TF II promoter activity in CAT assay using mouse fibroblast (3T3), rat glioblastoma (C6), human glioblastoma (U373), human cervical carcinoma (C33A), and mouse teratocarcinoma (PCC7) cells. (A and B) Mouse COUP-TF II −4000/*Bgl*II (A) and −320/*Apa*I (B) CAT plasmids were transfected into 3T3, C6, U373, and C33A cells. Cells were treated with all-*trans* RA (0.5 μM), dBcAMP (1 mM) or combination of all-*trans* RA and dBcAMP and CAT activities were measured. Left side scale represents CAT activity for 3T3, C6, and U373 cells and right side scale for C33A cells. (C) Mouse COUP-TF II promoter reporter plasmids −4000/*Bgl*II, −320/*Apa*I and RARβ–2-*lacZ* reporter plasmid were cotransfected with expression plasmid pRcCMVneo without any cDNA (control and dBcAMP+RA) or containing mCOUP-TF I (dBcAMP+RA+COUP-TF I) or mCOUP-TF II (dBcAMP+RA+COUP-TF II) cDNA into PCC7 cells. Cells were treated with all-*trans* RA (0.5 μM) and dBcAMP (1 mM) and CAT and *lacZ* activities were measured. Left side scale represents CAT activity for −4000/*Bgl*II and −320/*Apa*I constructs and right side scale represents *lacZ* activity for for RARβ–2-*lacZ* construct. The data shown are representative of at least three independent experiments.

the region −97 to −40. The putative HRE localized 68 nucleotides from the major start site is the only sequence that has certain homology to HRE in this region. Mutations in this HRE or deletion of this sequence do not affect inducibility of COUP-TF II promoter activity by RA. Also, coexpression of COUP-TF I or COUP-TF II, which completely blocks RA inducibility of RARβ2 promoter activity, only slightly reduces RA inducibility of COUP-TF II promoter activity. These data suggest that the effect of RA on COUP-TF II promoter activity is not directly mediated by the binding of RARs or RXRs to the response element, but is indirect. This hypothesis is supported by the data which demonstrate that the induction of the COUP-TF II gene expression by RA in teratocarcinoma P19 cells is a relatively slow process. Induction of COUP-TF II gene expression occurs 24–40 h after treatment of P19 cells with all-*trans* or 9-*cis* retinoic acid [19,34]. In contrast, induction of genes that are directly regulated by the RARs or RXRs, for example RARβ and midkine [31,35,36], occurs in less than 5 h.

Analyses of COUP-TF II promoter activity in different cell lines revealed that all-*trans* RA either stimulates, suppresses or has no effect on transcription. These results suggest that COUP-TF II is involved at least in two different functions of retinoids. First, in cells where COUP-TF II promoter activity is stimulated by retinoids, it may function as a part of the negative feedback loop to suppress effects of retinoids. Second, in cells where COUP-TF II promoter activity is suppressed by retinoids, the retinoid response may be long lasting. At the same time, COUP-TF II may also be involved in timing the switches of retinoic acid mediated gene regulation. Detailed analyses of regulation of COUP-TF II expression in different cell types, and characterization of its role in cell type specific gene regulation are required to understand the logic of COUP-TF II functioning during development and in the adult organisms.

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