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Protein–DNA Interactions During Phenotypic Differentiation

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Abstract

We have been studying the molecular mechanism of neuronal differentiation through which the multipotent precursor becomes limited to the final transmitter phenotype. Here we focused on the role of the 5' proximal regulatory cassette (–190; +53 bp) of the rat enkephalin (rENK) gene in the developmental regulation of the enkephalin phenotype. Several well characterized *cis*-elements, including AP2, CREB, NF1, and NFkB, reside on this region of the rENK gene. These motifs were sufficient to confer activity-dependent expression of the gene during neurodifferentiation when it was tested using transient transfection assays of primary developing spinal cord neurons treated with tetrodotoxin (TTX). This region was then used as a DNA probe in mobility shift assays, with nuclear proteins derived from phenotypically and ontogenetically distinct brain regions. Only a few low abundance protein–DNA complexes were detected and only with nuclear proteins derived from developing but not from adult brain. The spatiotemporal pattern of these complexes did not show correlation with enkephalin expression which was assessed by RT-PCR. We employed synthetic probes corresponding to consensus as well as ENK-specific sequences of the individual motifs to identify the nature of the observed bands. Although both consensus NF1 and enkCRE1(NF1) formed complexes with nuclear proteins derived from the striatum and cortex at various ages, the appearance of the bands was not correlated with ENK expression. Surprisingly, no complexes were detected if other ENK-specific motifs were used as probes. We also tested nuclear extracts derived from forskolin-induced and control C6 glioma cells, again using the whole proximal regulatory cassette as well as individual motifs. These experiments showed the formation of elaborate protein–DNA bands. There was no direct correlation between the appearance of bands and forskolin-induced ENK expression. Unexpectedly, all ENK-specific motifs formed specific and highly abundant protein–DNA complexes when nuclear extracts from the human tumor cell line (HeLa), which does not express ENK, were used. Based on these observations, we concluded that:

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1. Interactions between the proximal regulatory cassette and additional probably far distant regions of the rENK gene and their binding proteins may be necessary to confer developmentally regulated, cell-specific expression of the ENK gene; and
2. Inducibility of the gene by common *cis*-elements can be governed by this region; however, the cell-specificity of the induction remains elusive.

Index Entries: Protein–DNA interactions; phenotypic differentiation; rENK gene.

Introduction

The neurotransmitter phenotype is characterized by the expression of a set of genes that encode for the transmitter itself, such as ENK, or is involved in specific presynaptic catabolism, storage, release, or reuptake of the transmitter molecule (Scheller, 1992). Any of these gene products can be used as molecular markers for the given phenotype. The peptides *met*-enkephalin and *leu*-enkephalin that act as inhibitory neurotransmitters and pharmacologically compete with and mimic the effects of opiate drugs (such as morphine) (Akil et al., 1984), are encoded by the same single copy enkephalin gene (Litt et al., 1988). The ENK phenotype is characterized by enkephalin immunoreactivity (IR) and the expression of ENK mRNA.

The chemical neuroanatomy of the adult mammalian brain shows that neurons that differentiated into their final neurotransmitter phenotypes are distributed in a spatially strictly defined manner (Nieuwenhuys, 1985). Relatively high abundance of enkephalinergic neurons is present in the hypothalamus but enkephalinergic neurons are most abundant in the neostriatum where ~50% of the medium spiny neurons show ENK IR, and they are especially abundant in the caudate nucleus where >80% of the neurons are enkephalinergic (Gerfen, 1992; Khachaturian et al., 1985; Nieuwenhuys, 1985). Compared to the caudate nucleus, other parts of the adult central nervous system (CNS), e.g., frontal cortex or thalamus contain one or two orders of magnitude lower level of ENK mRNA, because enkephalinergic neurons are in low or in very low abundance (Nieuwenhuys, 1985).

This well-defined neuroanatomical distribution is the result of a developmental process. During neurodevelopment, weak hybridization signals for ENK mRNA are first detectable in the striatal primordium around embryonic d 16 (E16). However, significant signals were apparent only around birth with intensity substantially increasing up to postnatal d 7 (P7). At P14, both hybridization signal intensity and immunoreactivity reach adult levels (McDowell and Kitchen, 1987; Tecott et al., 1989).

In order to gain new insight into the regulation of phenotypic differentiation at the level of protein–DNA interactions, we have developed a novel approach. Our working hypothesis is that each of the genes that encode for neurotransmitter phenotypes, such as ENK, contain DNA elements that interact with DNA binding proteins expressed in a development-specific fashion during phenotypic differentiation. The sum of these interactions governs the phenotypic fate of differentiating neurons and sculptures the chemical neuroanatomy of the adult brain by limiting the expression of the ENK gene to specific subsets of neurons.

Our novel approach is based on a systematic spatiotemporal analysis of developmental-specific protein–DNA interactions ("sample and probe") and the identification of the underlying *cis*-elements and *trans*-factors. We assumed that the spatiotemporal pattern of the protein–DNA complex formation will correspond (negatively or positively) to the phenotypic identity of the given brain region as indicated by the levels of ENK mRNA.

In this work, we focus on the proximal regulatory cassette of the rat ENK gene, which we have used as a model gene. This ~200-bp long

5' region of the ENK gene contains many known regulatory elements in rats, humans, and mice (Comb et al., 1983; Yoshikawa et al., 1984; Santha et al., 1995), including those that can respond to activation of three major intracellular signaling pathways (cAMP, depolarization/Ca^{2+}, and protein kinase C) (Comb et al., 1986, 1988; Hyman et al., 1989; Kobierski et al., 1991) and has been suggested to confer cell specificity (Donovan et al., 1992; Takemura et al., 1992). Thus, this proximal regulatory cassette is essential in responding to any of these intracellular signals through binding specific proteins and consequently regulating the transcription of the gene (Comb et al., 1988).

Experimental Procedures

Cell Cultures

Primary Embryonic Spinal Cord Cultures

Dissociated spinal cord-dorsal root ganglia cultures were prepared from E12–E13 fetal mice, plated and cultured according to methods described previously (Agoston et al., 1991a,b). Cells were cultured in 95% MEM (Gibco BRL, Gaithersburg, MD) containing 5% heat-inactivated horse serum and the nutritional mix of N3. Cultures were maintained by weekly removal and replacement of approx 50% of the media.

Astrocytes

Cortical astrocytes were obtained from 1-d-old neonatal (Sprague-Dawley) rats and cultured as described (Olah et al., 1994). After replating confluent cells, astrocytes were grown in T75 flasks for 2 d to near confluence in Dulbecco's modified Eagle's medium (DMEM) with high glucose and 20 m*M* HEPES (pH 7.4) containing 10% fetal bovine serum. Cells were harvested by removing the medium and placing the flask on dry ice. Frozen cultures were stored at –80°C until subcellular fractionation.

C6 Glioma Cells

C6 glioma cells (ATCC, Rockville, MD) were grown to confluence in T75 flasks in DMEM, 10% fetal calf serum, glutamine, pen/strep as described (Joshi and Sabols, 1991). To induce ENK mRNA expression some cultures were treated with forskolin (20 μ*M*) or with the combination of forskolin and cycloheximide (CHX) (75 μ*M*) according to Joshi and Sabols (1991). Media were removed by aspiration, and flasks were rapidly frozen on dry ice and stored at –80°C until subcellular fractionation.

Transfection Assay in Primary Developing Spinal Cord Neuronal Cultures and Analysis of the Reporter Gene Activities

Seven days postplating the cultures were transfected using lipofectin (Gibco-BRL) according to the manufacturer's instructions. For each 35-mm dish, 10 μg of lipofectin in 0.8 mL OPTI MEM were gently mixed with 2–5 μg of DNA diluted in 0.8 mL of OPTI MEM and incubated for 10 min at room temperature, and for an additional 15 min at 37°C in the tissue culture incubator. Culture media were removed and cells were washed three times with pre-equilibrated OPTI MEM to remove serum. Preincubated lipofectin:DNA mixture in 1.6 mL of OPTI MEM was added per dish and left undisturbed for 4 h in tissue culture incubator. Following incubation, the lipofectin:DNA mixture was replaced by the original conditioned MEM tissue culture medium collected from the same dish. This step was critical in preventing neuronal cell death (Brenneman et al., 1983). Following transfection, some cultures were treated with 1 μ*M* of tetrodotoxin (TTX) to block spontaneous electrical activity (Agoston et al., 1991a). Cultures were harvested 48 h posttransfection and chloramphenicol acetyltransferase (CAT) activities were analyzed using the liquid scintillation overlay technique (Neumann et al., 1987), and luciferase (LUC) activities were measured in a liquid scintillation counter as described (Fulton and Vans, 1993). In order to

Table 1
List of Brain Regions and Developmental Ages for Dissections and Making Nuclear Extracts

	E14	E16	E18	E20	P2	P8	P14	Adult
PoMo[a]	–[b]	–	–	+	+	+	+	++
Striat		–/+	+	+	++	+++	++++	++++
Cortx	–	–	–	–	–/+	–/+	–/+	–/+
Cb	–	–	–	–	+	+	–	–
Liver	–	–	–	–	–	–	–	–

[a]Brain regions are listed in ontogenic appearance: PoMo = pons-medulla oblongata, at E14 = rhombencephalon; Striat = basal ganglia/striatum, at E14 = striatal primordium; Cortx = cerebral cortex, at E14 = prosencephalic wall; Cb = cerebellum, from E18 only; Liv = liver.

[b]Relative ENK mRNA abundancies: – nondetectable; –/+: detectable; +: low abundance; ++: medium abundance; +++: high abundance; ++++: extremely abundant.

correct for varying transfection efficacy, the activity of the reporter gene (CAT) was normalized to the activity of cotransfected LUC.

Reporter Plasmids

The reporter plasmid containing the proximal promoter cassette of the rENK gene (–190; +53) attached to the coding region of the reporter gene CAT (rENK190CAT) was a kind gift from Joshi and Sabol (1991). For cotransfection assays an RSV promoter driven LUC vector was used (Fulton and Vans, 1993).

Microdissection of the Developing Rat Brain

Time pregnant rats (Sprague-Dawley, Taconic Farms, NY) at gestation d 10, 12, 14, 16, 18, and 21, and postnatal d 2, 8, 14, and 28 were used for microdissection. Pregnant animals were handled and anesthetized according to NIH regulations; fetuses were removed and collected into ice-cold Pucks A solution (Gibco BRL). Microdissection was performed under a stereomicroscope; the excised brain regions were collected into liquid nitrogen (Table 1).

Preparation of Nuclear Extracts from Dissected Brain Regions

Nuclear extracts were prepared according to the Dignam-Roeder method (Dignam et al., 1983), or with minor modifications that permit the use of small quantities of tissue. Frozen brain tissues pooled from at least 10 animals were powdered under liquid nitrogen and dissolved in 6 mL buffer A at 4°C. Additional steps in preparing the nuclear extracts were carried out as described (Dignam et al., 1983) except that the protease inhibitor PMSF was replaced by 0.5 m*M* 4-(2-amino ethyl)-benzenesulfonyl fluoride hydrochloride (AEBSF) as serine protease inhibitor (ICN Biomedicals, Aurora, OH). As the final step, nuclear extracts were dialyzed for 2 h at 4°C against buffer D, using a 5000 mol wt cut dialysis cartridge (Bio-Tech, Bellevue, WV); 10-μL aliquots were frozen on dry ice and stored at –80°C. Because nuclear extracts prepared from freshly dissected and frozen tissues resulted in identical DNA binding patterns in our pilot experiments, we routinely used nuclear proteins that were prepared from frozen tissues. For RNA extraction, 400-μL samples of cytoplasmic supernatants were mixed with 2 mL of RNA STAT-60 solution (TelTest, Inc., Inglewood TX), placed on dry ice, and stored at –80°C.

Preparation of Nuclear Extracts from Cultured Cells

One milliliter of buffer A (Dignam et al., 1983) containing 0.5 m*M* AEBSF was added to the flasks just removed from –80°C. Following addition of the buffer, flasks containing the frozen cell layer were equilibrated around 4°C;

Table 2
Enkephalin-Specific Probes Used in Mobility Shift Assay

Name	Sequence	Position
enkAP2:	AGCCCGCTGGCGATTG	–77; –62
enkCRE2:	GGCCTGCGTCAGCTG	–93; –78
enkCRE1–2(NF1):	CGGGCTGGCGTAGGGCCTGCGTCA	–106; –83
enkNFkB:	GTGCTGTGGGGACGTCCCCTCCCG	–143;–120
rENK fragment a/α	GGCGCGCGCCTCTTCGGTTGGGGCTAATTAA TAAAGTGGCTGTGCGGCCGG CCAGAGAGGC	–60; –1

the frozen layer of buffer A containing the cells was incubated on ice for 15 min. Cells were sedimented by centrifugation at 1500*g* for 5 min at 4°C and pellets were resuspended in 1 mL buffer A and sedimented by spinning for 15 s in a microcentrifuge at 4°C. Supernatants were carefully removed and the cell pellets were resuspended in 400 µL of buffer A at 4°C. Cell suspensions were incubated at 4°C for 15 min, following addition of 25 µL of 10% NP-40 vortexed for 10 s and centrifuged at 4°C. Cytoplasmic supernatants (approx 400 µL) were removed and mixed with 2 mL of RNA STAT-60 solution, placed on dry ice, and stored at –80°C. Nuclear pellets were resuspended in 50 µL buffer C at 4°C and mixed on a shaking platform at 4°C for 15 min. Nuclear extracts were spun for 5 min at 4°C; supernatants were dialyzed for 2 h at 4°C against 300-fold excess of buffer D using 5000 mol wt cut dialysis cartridges as described; 10-µL aliquots were frozen on dry ice and stored at –80°C. Protein concentrations were determined with BCA Protein Assay Kit (Pierce, Aug-Beigerland, The Netherlands). Preparations typically yielded 2–4 mg/mL protein.

HeLa cell nuclear extracts were purchased from Stratagene (La Jolla, CA) and CellTech (Minneapolis, MN) at the concentration of 4–6 mg/mL of protein.

Generation of the DNA Fragment for Mobility Shift Assays

The insert containing the ~6 Kb 5' regulatory region and the first intron of the rENK was excised with *Eco*RI digests from the original vector (pRESS1, a kind gift from S. Sabols [Joshi and Sabol, 1991]) and recloned into a pKS-based vector. The DNA fragment containing the proximal regulatory cassette of rENK (–190, +53 relative to the transcriptional start site) was generated by restriction digests with *Sal*I and *Sac*I restriction endonucleases. The fragment was radioactively labeled by filling in the cohesive ends with [^{32}P]dATP according to standard procedures.

Preparation of Synthetic DNA Probes for Gel Mobility Shift Assay

Synthetic probes corresponding to consensus AP2, CREB, NF1, and NFkB sequences were obtained from Stratagene. The design of ENK-specific AP2, CRE2, CRE1–2 (NF1), and NFkB probes corresponding to rat sequences was based on available DNA sequence data (GeneBank access #:X59136) as well as on our own sequence analysis of the pRESS 1 clone (Table 2). Oligonucleotides were chemically synthesized in an Applied Biosystem 380 DNA Synthesizer.

ENK-specific probes were purified on denaturing gel and the single-stranded oligonucleotides were labeled at the 5' end with [γ^{32}P]-ATP, using polynucleotide kinase according to standard procedures. Annealing was done by heating the labeled oligomers in the presence of the complementary strand to 65°C for 20 min and cooling to room temperature for 50 min in the labeling buffer. Annealed probes were gel-purified and eluted in the binding buffer (5% glycerol; 12 m*M* HEPES-

Table 3
Primer Pairs Used in RT PCR Assay

Name	Sequence	Position	Product size, bp	Reference and GeneBank #
NF 68	TGCAGAACGCCGAAGAGTGGT	100		(*J. Cell Biol.* **843,** 1985)
	CTGGTGAAACTGAGCCTGGTC	473	373	K02925
GFAP	GAGCGTGCAGAGATGATGGAG	115		(*Nucleic Acids Res.* **13,** 5527, 1985)
	TCCTCCAGCGATTCAACCTTT	494	379	X02801
ENK	ATGCACACTCGAATGTGAAGG	189		(NIDA, **70,** 43, 1991)
	CTTCTTGGCTAGCAAGTGGC	345	157	M28263
CYC	CGTCTCCTTCGAGCTGTTTGCAGAC	98		(*Nucleic Acids Res.* **18,** 4019, 1990)
	CTTGCCATCCAGCCATTCAGTCTTGG	416	318	X52803

NaOH, pH 7.9; 60 m*M* KCl and 1 m*M* $MgCl_2$). Commercial probes were similarly labeled, gel-purified, and eluted.

Gel Mobility Shift Assays

Low stringency binding experiments in the presence of 0.05 μg/μL of poly[d(I-C)] were performed according to the manufacturer's instructions (Gelshift Kit, Stratagene). High stringency assays were typically carried out with 10 fmol of radioactively labeled probe mixed with 3 μg of poly[d(I-C)] in binding buffer *(see earlier);* this was followed by adding 3 μg of nuclear protein into a total volume of 10 μL. In the competition assay, the competitor DNA was added first to the mixture followed by the nuclear extract. The binding mixture was incubated at room temperature for 30 min, and equal volumes of samples were loaded onto a 4% polyacrylamide gel (acrylamide: bisacrylamide 40:1) and run in 1X TAE buffer at 9 W constant power for 2 h at room temperature. Gels were fixed in 10% acetic acid, dried, and analyzed by autoradiography or in a phosphor Imager (Molecular Dynamics, Sunnyvale, CA).

Preparation of Total Cellular RNA, cDNA Synthesis, and PCR

Total cytoplasmic RNA was extracted from frozen cytoplasmic supernatants of nuclear preparations or directly from frozen tissue pieces using the RNASTAT-60 reagent and procedure according to the manufacturer's instructions (TelTest, Inc., Inglewood, TX). Oligo dT-primed cDNAs were generated in Superscript reverse transcription system (Gibco BRL). Radioactive PCR reactions with traces of [^{32}P]-labeled dNTPs and each of the following primer pairs: ENK, the neuronal marker neurofilament (NF68), astroglial marker glial acidic fibrillary protein (GFAP), and cyclophilin (CYC) (Table 3) were carried out using the "hot start" method (Perkin-Elmer/Cetus, Norwalk, CT). PCR conditions for the individual PCR primers were established to obtain amplification over two orders of magnitude of signal intensities in the linear range. Following separation of the radioactive PCR products in a 6–20% gradient minigel system (Novex, San Francisco, CA), band intensities were quantified by a PhosphorImager (Molecular Dynamics, Sunnyvale, CA). Relative abundances were expressed as the ratio between the individual marker and CYC.

Results

Promoter Analysis of the Proximal Regulatory Cassette in Embryonic Spinal Cord Cultures

Primary developing spinal cord cultures were transiently transfected with the plasmid containing the proximal regulatory cassette of

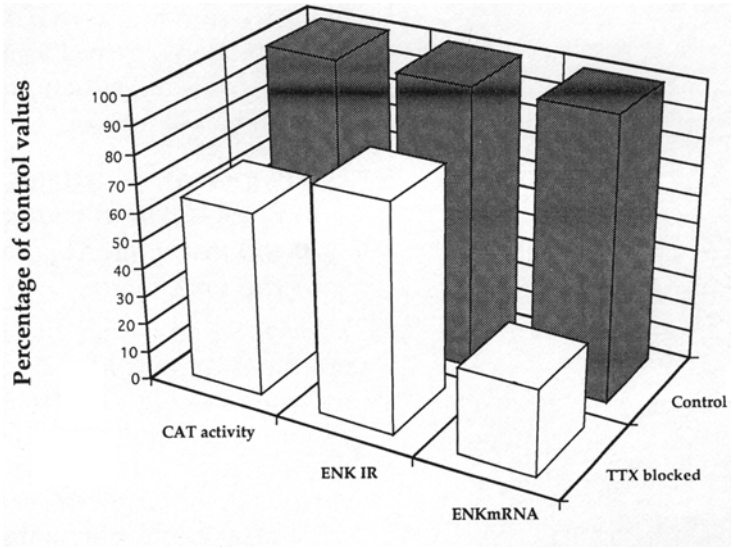

Fig. 1. Analysis of the proximal regulatory region of the rENK gene in transiently transfected control and TTX-treated mouse embryonic spinal cord cultures. In comparison, the effect of TTX blockade on *met*-enkephalin immunoreactivities (ENK IR) and on the abundance of ENKmRNA are included (Agoston et al., 1991a). Changes are expressed as percentages of control values.

the rENK gene. We used the strong promoter (RSV) driven LUC as control in these transfection experiments and CAT activities were normalized to the activities of the cotransfected RSV-LUC. The CAT activity detected in control cultures, where the ENK expression is driven by the ongoing electrical activity (Agoston et al., 1991a), was significantly reduced if the neuronal activity was blocked by TTX treatment (Fig. 1). The decrease in reporter gene activity was similar to the decrease of *met*-enkephalin immunoreactivity as measured by radioimmunoassay (Agoston et al., 1991a). Similar treatment caused an even more significant decrease in ENK mRNA abundance as quantified by Northern analysis (Agoston et al., 1991a,b).

Assessment of the Gene Expression Pattern

We employed RT-PCR to correlate the spatiotemporal pattern of protein–DNA complex formation with the actual abundance of ENK mRNA, the neuronal marker NF6 mRNA, and the glial marker GFAP mRNA in the corresponding cytoplasmic fractions of nuclear preparations used in mobility shift assays. The relative abundance of these markers was normalized to the abundance of CYC mRNA. In the four brain regions investigated, no or very low levels of ENK expression were detected at E14, with the exception of the cerebellar primordium (Fig. 2A). However, this expression was only transient, and at P2, ENK mRNA levels decreased and remained very low compared to the striatum. In the cerebral cortex, ENK mRNA was first detected at P2 and remained low throughout adulthood. By contrast, in pons/medulla oblongata, the abundance of ENK mRNA was detectable at E18 and increased steadily through development, peaking at P8. In the striatum, the ENK message becomes abundant at P2 with levels increasing steady and steeply throughout adulthood (P28). Based on our semiquantitative determination, the relative abundance of ENK mRNA in our dissected adult (P28) striata was approx 14-fold higher than in the cerebral cortex, ninefold higher than in the pons/medulla oblongata, and almost 20-fold greater than in the cerebellum (Fig. 2). Transiently high levels of NF68 mRNA were observed in the cer-

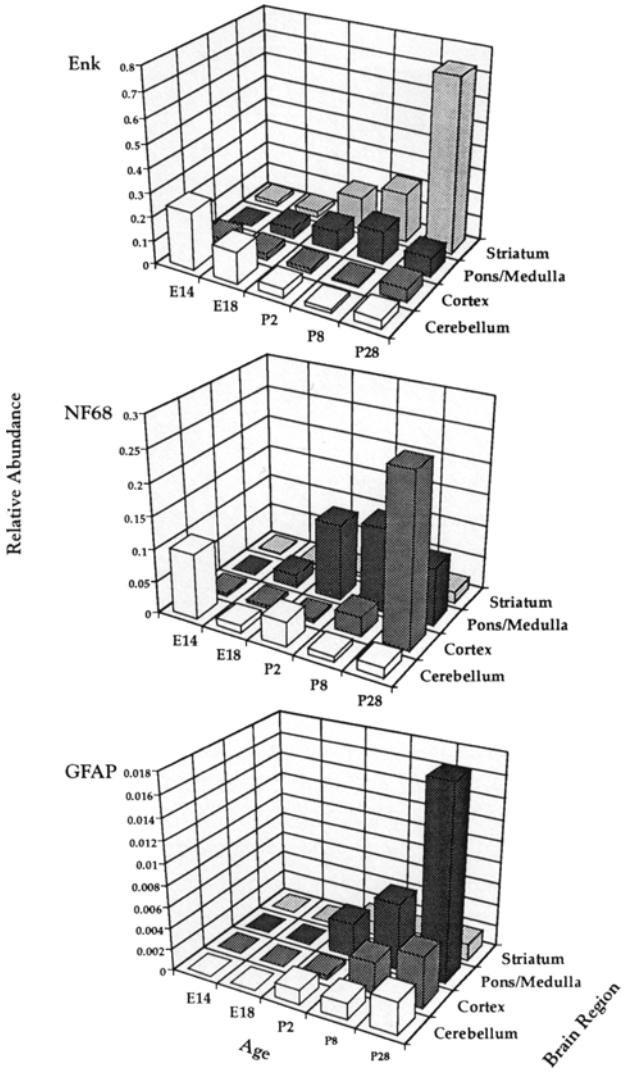

Fig. 2. Relative abundance of ENK mRNA **(A)**, NF68 mRNA **(B)**, and GFAP mRNA **(C)** in selected brain regions at various embryonic (E14 and E18) and postnatal (P2, P8, and P28) ages. The relative abundance of the individual markers was obtained by normalizing their RT-PCR values with the RT-PCR value of CYC mRNA.

ebellar extracts of E14 embryos, but in adult cerebellum, the level of NF68 message was one of the lowest among the brain regions tested (Fig. 2). NF68 mRNA was most abundant in the cerebral cortex, and rapidly increased between P8 and P28. Expression of the astroglial marker GFAP mRNA was greatest in the pons/medulla oblongata in contrast to the striatum, which contained the lowest levels (Fig. 2).

Developmental Changes in DNA–Protein Complex Formation of the Proximal Regulatory Cassette of the ENK Gene

In order to assess the involvement of the proximal regulatory cassette(–190; –/+53 bp fragment a; Fig. 3A) in the developmental expression of the ENK gene, we investigated the formation of DNA–protein complexes at various developmental stages using mobility shift assays and correlated the binding patterns with ENK expression. Nuclear extracts derived from cerebellum, cerebral cortex, pons/medulla oblongata, and striatum at E14, E18, P2, P8, and P28, were probed with radioactively labeled fragment a. In all nuclear extracts tested, we detected several low abundance DNA–protein complexes (Fig. 3B). Surprisingly, the overall DNA–protein binding capacity between fragment a and any of the nuclear extracts tested was three to five times weaker compared to other further upstream DNA fragments labeled to identical specific activity and probed with identical protein extracts (Dobi et al., in preparation). As assessed from R_f values, similar or identical complexes were formed using nuclear extracts derived from the various brain regions; only the intensity and the developmental appearance of the complexes varied significantly (Fig. 3B). Because all complexes had similar temporal patterns, we concluded that formation of DNA–protein complexes probably correlates with other developmental events rather than with the developmental expression of the dominant neurotransmitter phenotype of a specific brain region (i.e., ENK in striatum). Most of these complexes were formed during the course of neurodifferentiation; in adult (P28), only one complex remained detectable in cerebral cortex and striatum (Fig. 3B). Although these brain regions represent phenotypically

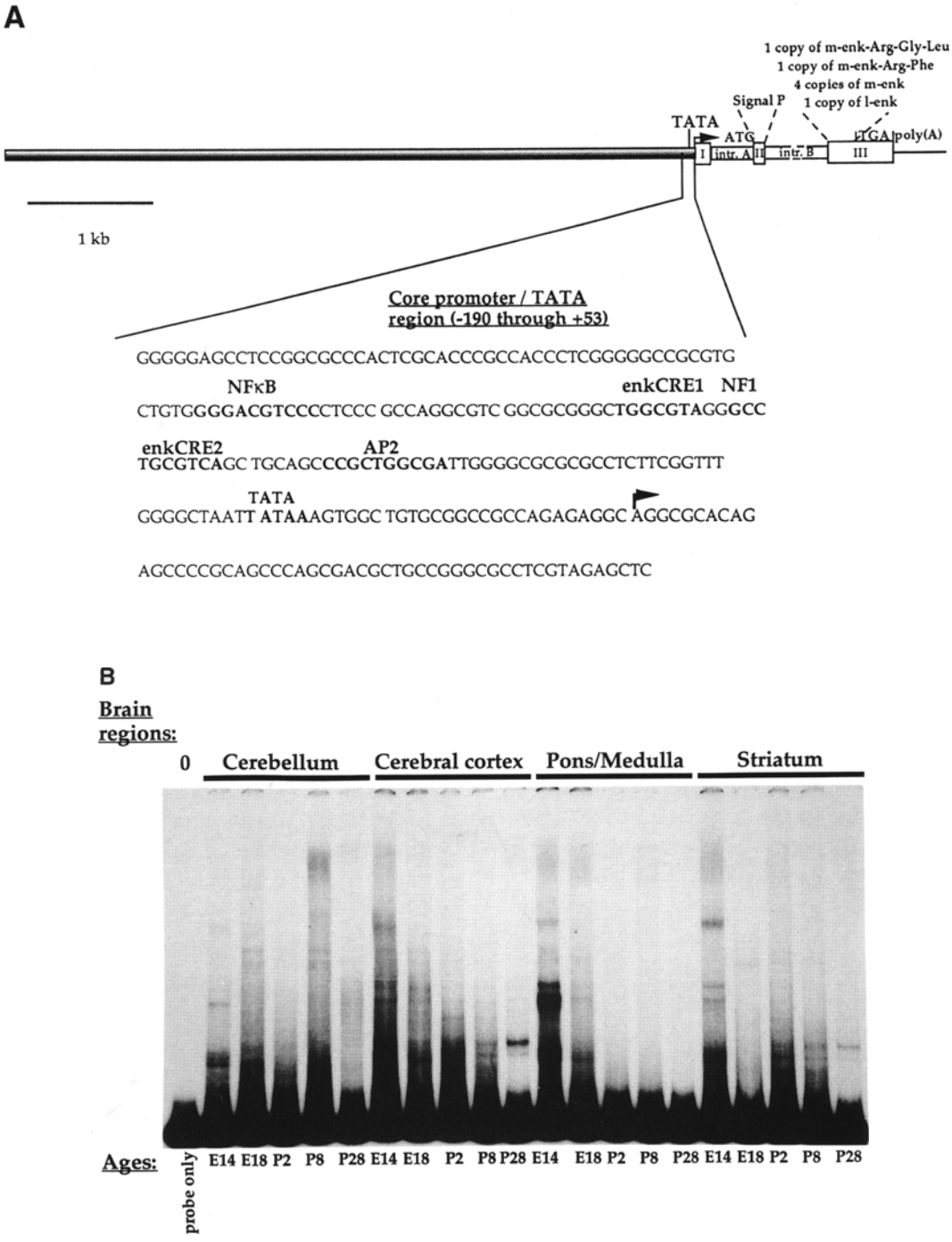

Fig. 3. The structure of the rENK gene and the position and sequence of the proximal regulatory cassette/fragment a. Identified *cis*-elements are in boldface **(A)**; mobility shift screening of nuclear proteins derived from different brain regions of various embryonic (E) and postnatal (P) ages as indicated using fragment a as probe **(B)**.

contrasting parts of the mammalian brain in terms of ENK expression (Table 1 and Fig. 2A), the binding patterns were very similar if not identical, and we were unable to correlate ENK expression with the binding pattern of the nuclear proteins to fragment a at any developmental stages investigated.

Developmental Changes in DNA–Protein Complex Formation of Consensus and ENK-Specific cis*-Elements*

The surprisingly low binding capacity between fragment a and the various nuclear extracts that resulted in very faint bands even after several days of exposure made the application of competitive gel shift assays technically and conceptually unfavorable. Therefore, we used individual synthetic motifs as probes to assess the involvement of known *cis*-elements present on fragment a in the formation of DNA–protein complexes.

Because earlier transient transfection experiments using C6 glioma cells, as well as footprint analysis with purified HeLa proteins and proteins derived from total bovine brain, suggested a key role for the ENK-specific CRE element (enkCRE) in regulating ENK gene expression (Joshi and Sabols, 1991), we tested formation of DNA–protein complexes with the enkCRE1–2(NF1) probe and nuclear extracts derived from striatum, cerebral cortex, cerebellum, and liver at various developmental stages.

A single, low mobility complex with similar mobility to the one also observed with the whole fragment a was formed with nuclear extracts derived from the described three brain regions between E18 and P2 (Fig. 4A). In both striatum and cerebral cortex, the complex was not detectable after P8; however, in the cerebellum it remained relatively abundant even at P28 (Fig. 4A). Again, there was no correlation between formation of DNA–protein complexes at any developmental stage with the enkCRE1–2(NF1) motif and ENK expression. This was particularly striking in the cerebral cortex and striatum, where the abundance of ENK-mRNA differed at least one order of magnitude in the adult, although neither quantitative nor qualitative differences were seen in protein–DNA complex formation. Nuclear extracts derived from liver at all ages formed a complex of low abundance with similar but probably not identical mobility.

Additional ENK-specific *cis*-elements (enkCRE2, enkAP2, enkNFkB) (Joshi and Sabol, 1991; Comb et al., 1992) located on the proximal regulatory cassette (fragment a) were also tested in an identical gelshift assay system. Surprisingly, none of these ENK-specific variants, enkCRE2, enkAP2, enkNFkB, of consensus elements showed specific binding with any of the nuclear extracts derived from four different brain regions at ages between E14 and P28 (data not shown).

To test our binding conditions, we repeated the experiment with a radioactively labeled consensus NF1 probe. NF1 binding proteins were detected in nuclear extracts from all ages and brain regions (Fig. 4B). These DNA–protein complexes were especially abundant in the nuclear extracts of E18 and P2 striatum and cerebral cortex. As expected, NF1 binding proteins were plentiful in the nuclear extracts derived from adult (P28) liver. The specificity of the binding was confirmed using competition assay (data not shown). As we have demonstrated here, our binding conditions permit the detection of NF1 binding from either brain or peripheral tissue extracts. Similarly, DNA–protein complexes were detected with consensus AP2 (Fig. 4C) or consensus CREB probes (Fig. 4D) in different brain regions. Also, there was considerable AP2 binding in both striatum and cerebral cortex at age P2, but was completely absent in liver (Fig. 4C). This complex showed a medium to high mobility doublet. Similar experiments with a consensus CREB motif showed a shift in the mobility of the complex between ages P2 and P28 in both the striatum and cerebral cortex (Fig. 4D). No complex was found in nuclear extracts derived from liver at any ages.

Protein–DNA Interaction of Consensus and ENK-Specific cis*-Elements with Primary Astrocytes and HeLa Cells*

Transcription factors that interact with elements located on the proximal regulatory cassette of the ENK gene were first characterized by using affinity-purified HeLa nuclear proteins (Comb et al., 1988). However, the report did not give any indication about the specificity of these interactions. We decided to test nuclear extracts derived from HeLa cells as well as from rat primary cortical astrocytes, both with the whole fragment a and with ENK-specific variants of the four major *cis*-elements present on fragment a (enkAP2, enkCRE2, enkCRE1–2(NF1), and enkNFkB). We employed both our stringent and the widely used "kit-conditions" (*see* Materials and Methods). Nuclear extracts derived from primary cortical astrocytes contained no specific binding protein, whether the whole fragment a or individual motifs were used as probes under either stringent or nonstringent conditions. Nonstringent conditions with the whole fragment a as a probe only resulted in a smear with no detectable bands (Fig. 5A). By contrast, nuclear extracts of HeLa cells contained specific binding activities to all four ENK-specific variants of the *cis*-elements AP2, CRE2, CRE1–2(NF1), and NFkB (Fig. 5A).

To further characterize binding specificity, we probed the detected DNA–protein complexes in a competitive mobility shift assay using HeLa cell nuclear extracts (Fig. 5B). These experiments with various cold competitor motifs showed that only the enkCRE1–2(NF1) probe formed specific complexes with the HeLa nuclear extracts. However, enkCRE1–2(NF1) formed two overlapping binding activities with similar or identical R_f values but with a different appearance. The competition assay showed that only one complex is derived from an NF1 motif binding protein(s), whereas the other is owing to an AP2 element binding protein. We found that both NF1 motifs, the ENK-specific (Fig. 5B, lane 10), or the consensus form (Fig. 5B, lane 14) can compete with the labeled enkCRE1–2(NF1) probe, resulting in the disappearance of the fuzzy band (Fig. 5B, lanes 10 and 14). By contrast, neither enkCRE2 nor AP1 nor AP2 could compete with the complex formation. This competition experiment also showed that the AP2 consensus element could compete with the labeled enkCRE1–2(NF1) probe, resulting in the disappearance of the sharp band but retaining the fuzzy band (Fig. 5B, lane 13). By contrast, neither cold enkCRE2 nor enkAP2 motifs could compete with the complex formation; therefore, the binding site(s) of these protein(s) probably was on the distal side of the enkCRE1 element. Sequence analysis of the AP2 consensus sequence revealed homology between this element and the 5' sequence of the enkCRE1–2(NF1) sequence that was used as a probe. Therefore, we believe that this may be a potential binding site for the HeLa AP2 binding factor. Earlier DNaseI footprinting suggested an interaction between purified enkCRE1 binding proteins and TATA element (Comb et al., 1988). Our competition assay revealed that the probe containing the ENK TATA sequence (fragment a/α; –60 to –1) competed with the enkCRE1–2(NF1) probe for these binding proteins (Fig. 5B, lane 15). In contrast to these DNA–protein complexes, no other motif formed specific DNA–protein complexes when tested with HeLa nuclear extracts. As an example, we show that the addition of any cold competitor DNA to labeled enkCRE2 probe prevented complex formation; therefore, the band observed with enkCRE2 and HeLa nuclear proteins is nonspecific (Fig. 5B, lanes 1–8).

Protein–DNA Interactions of Consensus and ENK-Specific cis*-Elements in C6 Glioma Cells*

The C6 glioma cell line has been extensively used as a model to study the transcriptional regulation of ENK expression (Joshi and Sabols, 1991). Thus, we tested the DNA–protein interaction between enkCRE2 and enkCRE1–

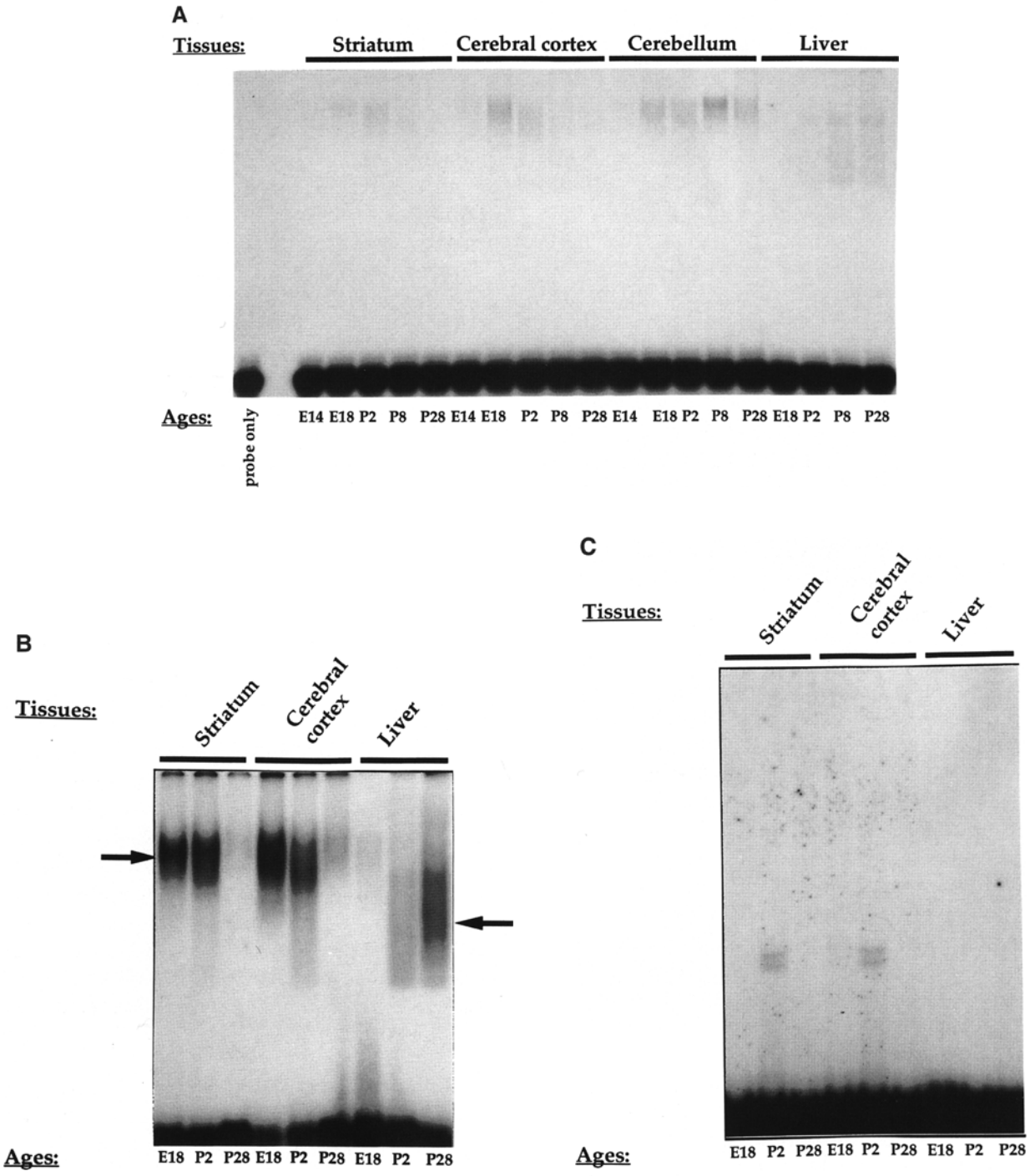

Fig. 4. Mobility shift assay of nuclear extracts derived from cortex, striatum, and liver at ages E14, E18, P2, P8, and P28 with synthetic probes: enkCRE1-2(NF1) **(A);** consensus NF1 **(B)**. Arrows indicate differences between the mobility of brain- and liver-specific protein–DNA complex (B); consensus AP2 **(C);** and consensus CREB **(D)**. Stars indicate the position of the complex at an early postnatal age (P2), whereas dots mark the position of probably the same complex in the adult.

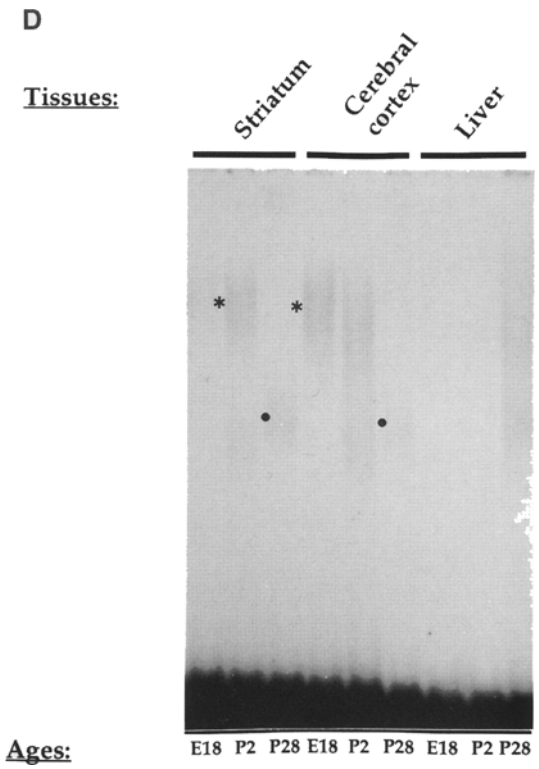

Fig. 4D

2(NF1) motifs and nuclear extracts derived from C6 cells. Similar to HeLa nuclear extracts, proteins of C6 nuclear extracts did not form a specific DNA–protein complex with enkCRE2 (Fig. 6A, lanes 1–6). The medium mobility band observed with HeLa extracts (lane 7) is identical to the nonspecific complex displayed in Fig. 5B. Like C6 glioma cells, primary cortical astrocytes also lack any binding activity with enkCRE2 (Fig. 6A, lane 8). On the other hand, nuclear extracts of C6 cells, just like HeLa cells, contained similar if not identical binding activity with the probe enkCRE1–2(NF1) (Fig. 6A, lanes 9–13).

In order to induce ENK expression, some C6 cultures were treated with forskolin in the presence and/or absence of CHX according to Joshi and Sabols (1991). Although no significant change was observed in binding intensities between forskolin-induced and control cultures in the presence and/or in the absence of CHX (Fig. 6A, lanes 9–13), CHX treatment completely eliminated the binding activity with the enkCRE1–2(NF1) probe (Fig. 6B, lane 14). This CHX-induced loss of binding capacity suggests that the half-life of the DNA binding protein is very short (<7 h) and, therefore, this binding required uninterrupted protein synthesis. Surprisingly, forskolin treatment prevented this CHX-induced loss of binding activity (Fig. 6B, lanes 12,13); however, the mobility of the complex was changed as compared to control or just forskolin-treated cells (Fig. 6B, lane 13, star). Nuclear extracts derived from primary cortical astrocytes formed similar, however less abundant, complexes with the enkCRE1–2(NF1) (Fig. 6A, lane 16).

Using the enkCRE1–2(NF1) and consensus NF1 motifs as probes, we also tested nuclear extracts derived from adult rat striatum under identical binding conditions. Although there was no binding activity with the ENK-specific probe (enkCRE1–2[NF1]), nuclear extracts from adult striatum formed an abundant complex with the consensus NF1 probe (Fig. 6B, lanes 1, 6). However, this complex displayed a slightly different mobility, was similar to that of astrocytes, but was much less abundant than complexes of C6 cells (Fig. 6B, lanes 6, 8–10). As controls, we used nuclear extracts from primary astrocytes and C6 glioma cells with and without forskolin treatment that formed a single abundant band with both enkCRE1–2(NF1) and consensus NF1 probes (Fig. 6B, lanes 3–5, 8–10).

In a competitive experiment, we tested the specificity of DNA–protein complexes formed between nuclear extracts derived from C6 cells and the NF1 probe (Fig. 6C). Using increasing amounts of cold competitor DNA, we confirmed that the protein binding was specific for the NF1 motif. As expected, slight competition was observed with the CRE2 probe that contained half of the NF1 consensus site.

Discussion

The well-defined chemical neuroanatomy of the adult mammalian CNS is the result of a developmental process during which neuro-

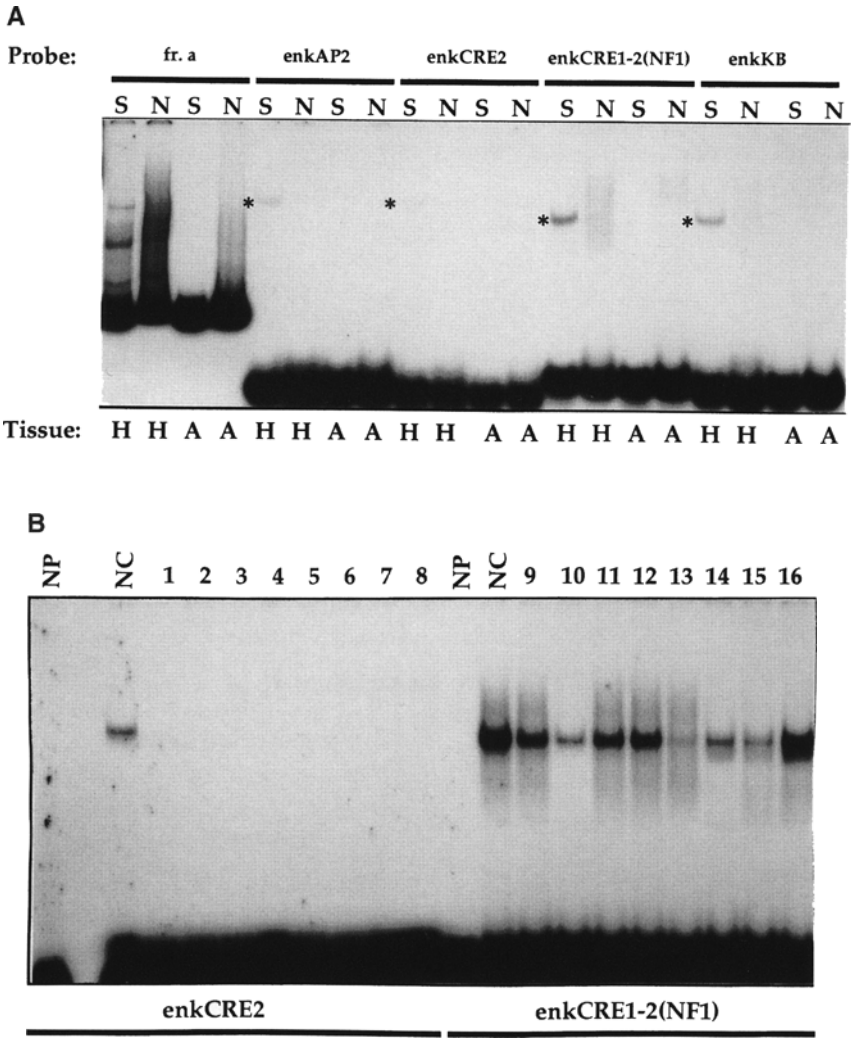

Fig. 5. Mobility shift screening of nuclear extracts derived from primary cortical astrocytes (A) and HeLa cells (H) with fragment a and synthetic enkAP2, enkCRE2, enkCRE1–2(NF1), and enkNFκB under standard (N) and stringent (S) binding conditions. Stars indicate the position of the resulting complexes **(A)**; competition assay of DNA–protein complexes using HeLa extracts and enkCRE2 or enkCRE1–2(NF1) probes for competition with enkCRE2 (lanes 1 and 9), enkCRE1–2(NF1) (lanes 2 and 10), enkAP2 (lanes 3 and 11), consensus AP1 (lanes 4 and 12), consensus AP2 (lanes 5 and 13), consensus NF1 (lanes 6 and 14), probe a/α (containing the TATA box) (lanes 7 and 15), and nonrelated single-stranded DNA (lanes 8 and 16) motifs. NP = no protein and NC = no competitor, respectively **(B)**.

glial precursors differentiate into their final cellular identity. For cells that are in the neuronal lineage, cellular identity includes the choice of the neurotransmitter phenotype. At the genomic level, this is achieved by limiting the expression of transmitter-specific genes to a set that is necessary to synthesize the chosen neurotransmitter biosynthetic machinery and represses all others (Anderson, 1993).

We used a transient transfection assay to assess the involvement of the proximal regulatory cassette of the rENK gene in governing ENK gene expression. Earlier results indicated that during neurodifferentiation the expression

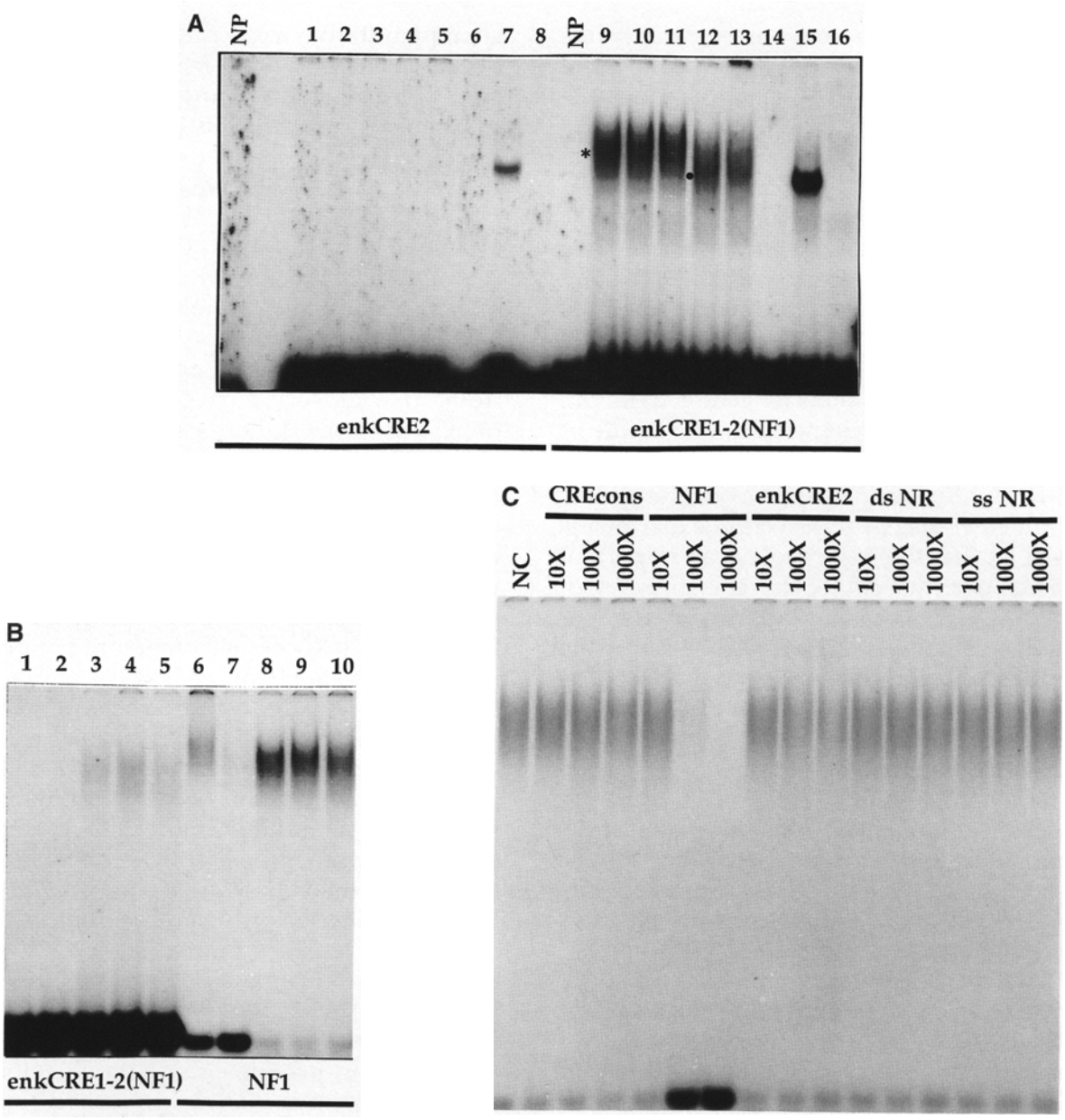

Fig. 6. Mobility shift assay of radioactively labeled enkCRE2 or enkCRE1–2(NF1) probes with nuclear extracts derived from: control C6 cells (lanes 1 and 9); forskolin-treated (1 h) C6 cells (lanes 2 and 10); forskolin-induced (6 h) C6 cells (lanes 3 and 11); C6 cells pretreated with CHX for 1 h followed by forskolin treatment for 1 h; (lanes 4 and 12); C6 cells pretreated with CHX for 1 h followed by forskolin treatment for 6 h (lanes 5 and 13); C6 cells cotreated with forskolin and CHX for 7 h (lanes 6 and 14); HeLa cells (lanes 7 and 15); primary cortical astrocytes (lanes 8 and 16); and with no protein (NP). The original mobility of the complex is marked by a star and the altered mobility seen after combined forskolin-CHX treatment is indicated by the dot **(A);** gel mobility shift assay of enkCRE1–2(NF1) or consensus NF1 probes with nuclear extracts derived from adult striatum (lanes 1 and 6); rat cortical primary astrocytes (lanes 2 and 7), untreated C6 cells (lanes 3 and 8); C6 cells treated with forskolin for 1 h (lanes 4 and 9); C6 cells treated with forskolin for 1 h and CHX for 1 h (lanes 5 and 10) **(B);** competition assay of C6 cell nuclear extracts with radioactively labeled consensus NF1 as probe and consensus CREB, consensus NF1, enkCRE2, nonrelated double-stranded (dsNR) and nonrelated single-stranded (ssNR) as competitors **(C)**. The numbers indicate the fold excess of the cold competitors.

of the enkephalin gene and other neuropeptide genes in spinal cord neurons are critically influenced by the ongoing TTX-sensitive spontaneous neuronal (electrical) activity (Agoston et al., 1991a,b) as the major epigenetic factor in this part of the CNS. Accordingly, TTX blockade caused an almost complete blockade in the transcription of the rENK gene as judged from earlier experiments (Agoston et al., 1991a), and significantly decreased the activity of the reporter gene CAT. The discrepancy on the extent of TTX effect on ENK mRNA abundance vs ENK IR and reporter gene activity can be attributed to the differences in the nature of markers (enzyme activity vs immunoreactivity vs mRNA abundance). However, the results from these transient transfection experiments suggested that this region plays an important role in conferring activity-driven expression of the rENK gene. As earlier experiments demonstrated, this activity-driven regulation of the gene during early neurodifferentiation activates L-type voltage-dependent calcium channels (Agoston et al., 1991a) and likely involves the calcium responsive element located within the region (Comb et al., 1988). An identical region of the human ENK gene was used for making transgenic animals and afferent stimulation of the spinal cord in transgenic animals caused marked upregulation of the reporter gene (Donovan et al., 1992; Takemura et al., 1992), which also underlines the importance of this region in induced expression of the gene.

Because our goal is to identify the *cis-* and *trans-* elements that control the differentiation of ENK phenotype, we have started to use our "'sample' and 'probe' approach." In this study we used nuclear proteins derived from phenotypically distinct brain regions dissected at various stages of neurodifferentiation and the proximal regulatory cassette of the rENK gene as probes.

We used RT-PCR to quantify the actual abundance of the ENK message in our tissue samples, which enabled us to correlate the pattern of DNA–protein complex formation and also to verify the neuroanatomical correctness of our microdissection technique. This semiquantitative assay showed the expected development- and brain region-specific expression pattern of ENK mRNA in our microdissected samples identical to previously published results obtained by other techniques (Khachaturian et al., 1983; Yoshikawa et al. 1984; Maderdrut et al., 1986; Ylikoski et al., 1989; Cimino et al., 1991; Ge et al., 1993). The relative abundance of the general neuronal marker NF68 mRNA and the astroglial marker GFAP mRNA measured by RT-PCR in the cytoplasmic supernatants of our nuclear preparations also closely correlated with results published earlier (Julien et al., 1986; Lieberburg et al., 1989; Schlaepfer and Bruce, 1990; Tardy et al., 1990; Riol et al., 1992).

The mobility shift screening of nuclear extracts derived from different brain regions with the proximal regulatory cassette showed surprisingly low overall DNA–protein binding activity, much lower than other 5' regions of the rENK gene tested (Dobi et al., 1995, in preparation). However, almost all the protein–DNA complexes were formed with nuclear extracts that were derived from embryonic to early postnatal brain regions; only one complex was still present in the adult brain. Other 5' regions of the rENK gene tested with identical nuclear extracts formed distinct complexes, but the overwhelming majority of these protein–DNA complexes were also formed during the same developmental period (Dobi et al., 1995, in preparation). These observations suggest that a large number of DNA binding proteins that recognize various elements on the 5' regions of the rENK gene are being expressed temporarily during the period of neurodifferentiation. Many of these binding proteins are probably novel because we found very few homologous regions to known DNA motifs during sequence analysis. Also, the stringent binding conditions routinely used in our mobility shift assays significantly reduced any chance for nonspecific binding. Because secondary modifications such as phosphorylation can substantially modify DNA binding (Karin, 1991; Hagiwara et al., 1992; Ginty et al., 1993; Annweiler et al., 1994), we do not know

whether these proteins are genuinely embryonic-specific in their expression pattern, or whether they merely lose their ability to bind DNA at the end of neurodifferentiation as a result of such modifications.

We have hypothesized that both negative or positive correlation between the spatiotemporal pattern of the DNA–protein complexes and ENK mRNA abundance suggest involvement of elements located on fragment a. This ~200 bp upstream region of the rENK gene contains all the necessary elements (AP-1, NF-1, enkCRE1, enkCRE2, AP-2, and [NF]kB)(Comb et al., 1986, 1992; Hyman et al., 1989; Van et al., 1990; Chu et al., 1991; Jacobson, 1991; Kobierski et al., 1991) that can respond to the activation of any of the major second messenger pathways (cAMP, PKC, Ca^{2+}/depolarization). However, based on our mobility shift assays using the individual motifs as probes, the identity of the observed complexes cannot be fully associated to the *cis*-elements residing on fragment a. In both the striatum and cerebral cortex, the difference in ENK mRNA abundance at P2 is already three- to fourfold, which increases to at least 10-fold in the adult as shown by our RT-PCR assays. However, nuclear proteins derived from the striatum and cortex, as well as the other two brain regions, showed remarkably similar overall binding patterns with fragment a, and no obvious correlation with ENK mRNA expression. One explanation for these seemingly identical DNA–protein complexes appearing in all brain regions at various ages is that they may mark certain general developmental stages.

Several ubiquitous DNA binding proteins, such as NF-1, have been shown to bind their motifs present on the proximal regulatory cassette of the rENK gene (Chu et al., 1991). Others belonging to the CRE-class of transcriptional factors including several CREB proteins isolated from HeLa extracts can interact with the (ENK)CRE-1 element (Konradi et al., 1993). Proteins of the AP-1 *(Fos/Jun)* family and AP-4 bind to the other distinct CRE elements termed (ENK)CRE-2 (Sonneberg et al., 1989). Binding of these proteins to their recognition site is triggered by activating various intracellular signaling pathways and is critical for inducing ENK expression in various model systems (Chu et al., 1991; Joshi and Sabols, 1991; Kobierski et al., 1991; MacArthur et al., 1993).

Most of the experimental evidence pointing toward the critical involvement of this region in ENK expression has been derived from experiments using various tumor model cell lines and the transient transfection paradigms (Chu et al., 1991; Joshi and Sabols, 1991; Kobierski et al., 1991). There are no experimental data available on the involvement of this region in conferring phenotypic establishment in differentiating primary neurons. Not surprisingly, activation of intracellular signaling pathways induced expression of the reporter gene attached to this region in every transient transfection assay. In these elegant experiments, the precise *cis*-requirements for induced ENK gene expression were precisely mapped. These experiments amply demonstrated the essential role of this proximal regulatory region in conferring inducibility of the ENK gene (Comb, 1992; #100). Similar conclusions can be drawn from recent transgenic experiments (Donovan et al., 1992; Takemura et al., 1992), as well as from our transient transfection analysis.

Our results presented here, as well as earlier experiments, suggest that developmental expression (establishment of a given phenotype during neuronal differentiation) and upregulation of the transcriptional rate of that gene in adult neurons may require distinct mechanisms.

Different genes contain variants of common DNA motifs that require diverged *trans*-factors while performing the ubiquitous task associated with the backbone of the motif (Karin, 1992). The ENK gene is one of these and several of its *cis*-elements are ENK-specific variants of common motifs (Comb et al., 1986, 1988). Accordingly, we tested ENK-specific variants of these motifs in our mobility shift system, assuming that enkephalin expressing cells do manufacture ENK-specific versions of these binding proteins. Surprisingly, none of the ENK-variants of major *cis*-elements (enk-

AP2, enkNFkB) tested showed specific binding with any of the nuclear proteins derived from different brain regions. There are several factors that could explain this unexpected result. The stability of nuclear proteins could affect binding; however, identical extracts did bind with other DNA probes (Dobi et al., 1995, in preparation). Secondary modifications could also affect binding, but as our preliminary experiments showed with another 5' region of the rENK gene, conditions used in our nuclear protein purification procedure preserve the in vivo extent of phosphorylation (Dobi et al., unpublished). Sensitivity may be insufficient to detect binding; however, our detection system is extremely sensitive and allows the detection of 50 attomoles of protein–DNA complex.

Because binding conditions can greatly affect detectability and specificity, we tested two different binding conditions: One was widely recommended by manufacturers for the use of their kits; the other was a more stringent, high-specificity condition we had developed. Generally, "kit conditions" did not yield higher sensitivity but resulted in similar complexes, often showing as smears rather than distinct sharp bands. Because we failed to detect ENK-specific binding in any brain region with any of the ENK-specific variants of *cis*-elements we used HeLa nuclear extracts, the original model system for characterizing these elements (Comb et al., 1988). To our surprise, stringent conditions resulted in specific bands that can be competed with the individual motifs. However, no published data indicate that HeLa cells expressed ENK.

In another set of experiments, we also tested nuclear extracts derived from C6 cells, a rat glioma cell line. C6 cells express ENK at an extremely low basal level; however, ENK expression can be increased by elevated intracellular cAMP (Joshi and Sabols, 1991). Forskolin treatment of C6 cells did not coincide with any significant change in the binding pattern of the ENK-specific CRE1–2 element, whereas ENKCRE2 had no binding. One can only suspect that subtle changes in the phosphorylation pattern affecting DNA binding at an undetectable level could be responsible for the forskolin-induced elevation in ENK mRNA expression.

In summary, our present studies have focused on the proximal regulatory cassette of the rENK gene in which we found no in vitro evidence for the previously proposed sole involvement of the region in specifying the ENK phenotype, but supported the role of this region in induced expression of the gene (Comb et al., 1992; Donovan et al., 1992). Because of the lack of biologically meaningful and experimentally accessible model systems, our current knowledge on the precise *cis*- and *trans*- requirements for developmental specification of the neurotransmitter phenotype remains elusive. However, our "sample" and "probe" technique offers a novel approach to identify developmental-specific intranuclear molecular components that govern phenotypic differentiation. Indeed, preliminary results derived from ongoing experiments have identified such components and suggest that specification of the ENK phenotype requires additional far-distant DNA elements that should interact with the proximal regulatory cassette in a developmental and cell-specific manner.

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