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Granule cell specification in the developing mouse brain as defined by expression of the zinc finger transcription factor RU49

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SUMMARY

The creation of specific neuronal cell types within the developing brain is a critical and unsolved biological problem. Precedent from invertebrate development, and from vertebrate myogenesis and lymphogenesis, has established that cell specification often involves transcription factors that are expressed throughout the differentiation of a given cell type. In this study, we have identified the Zn^{2+} finger transcription factor RU49 as a definitive marker for the cerebellar granule neuron lineage. Thus, RU49 is expressed in the earliest granule cell progenitors at the rhombic lip as they separate from the ventricular zone of the neural tube to generate a secondary proliferative matrix, and it continues to be expressed in differentiating and mature granule neurons. Proliferating granule cell progenitors isolated from the rhombic lip at E14 or from the external germinal layer at P6 continue to express RU49 in vitro. Both the olfactory bulb and dentate gyrus granule cell lineages also express this factor as they are generated with the developing brain. RU49 binds a novel bipartite DNA-binding element in a manner consistent with chemical rules governing the DNA-binding specificity of this class of transcription factor. The novel biochemical properties of RU49 and its restricted expression within the three lineages of CNS granule neurons suggest that RU49 may play a critical role in their specification. Furthermore, these results raise the interesting possibility that the generation of these three neuronal populations to form displaced germinative zones within the developing brain may reflect their use of a common developmental mechanism involving RU49.

Key words: RU49, cerebellum, granule cells, transcription factor, lineage specification, mouse, brain, zinc finger

INTRODUCTION

The role of cell-type-specific transcription factors in lineage specification during invertebrate development (Jan and Jan, 1993; Labouesse et al., 1994), vertebrate myogenesis (Olson, 1994) and development of the immune system (Georgopolous et al., 1992, 1994) is well established. In each of these cases, critical decisions concerning cell fate involve transcription factors that are expressed in the earliest precursors of a particular cell type. Often, these transcription factors continue to be expressed throughout the differentiation program for that cell type, providing definitive markers for a specific cell lineage in both the developing and adult organism. If this paradigm is pertinent to the generation of specific neuronal lineages in the developing brain, then the identification of transcription factors marking neuronal cell types from their birth in the neural tube and throughout their complex program of differentiation is a critical step in advancing our understanding of these cell fate decisions. To address this issue, we have sought to identify transcription factors that may play a role in granule cell specification in the developing cerebellum.

In the mouse, cerebellar granule cells arise between embryonic days 13 and 15 in a restricted region of the ventricular zone of the fourth ventricle adjacent to the rhombic lip (Miale and Sidman, 1961; Fugita et al., 1966). Proliferating granule cell precursors then migrate out over the rhombic lip to establish a secondary proliferative zone, the external granular layer (EGL), beneath the pial surface of the developing cerebellum. While the embryonic EGL is first recognized as a very thin layer of proliferating cells beneath the pial surface, its expansion continues postnatally both through continued entry of precursors from the fourth ventricle near the rhombic lip and through the proliferation of the migrating granule cells within the EGL to generate a thick layer of rapidly dividing granule cells that gives rise to the enormous numbers of this neuronal cell type in the adult brain (Hanaway, 1967). Recent fate mapping experiments in chimeric chick/quail embryos (Hallonet et al., 1990; Hallonet and Le Douarin, 1993; Otero et al., 1993) have established that the EGL gives rise only to cerebellar granule neurons.

The conservation of transcription factor structure and function in organisms as evolutionarily removed from one another as insects and mammals has allowed conclusions about mammalian development to be drawn directly from information obtained by molecular genetic analyses of critical regulatory events occurring during insect development. We

reasoned that studies of transcription factors that have arisen more recently during evolution may provide important insights into novel developmental mechanisms such as the specification of neuronal cell fates within the vertebrate CNS. Although a modest increase in the number of genes encoding a given type of transcription factor might generally be expected as one ascends the evolutionary tree, available evidence suggests that the Zn^{2+} finger transcription factor family has expanded particularly rapidly. Estimates of the number of Zn^{2+} finger genes in the mammalian genome, for example, range as high as 700 (Bellefroid et al., 1989; Crossley and Little, 1991). It has been argued that the rapid evolution of the Zn^{2+} finger transcription factor family reflects the modular structure of these genes and the relatively simple molecular changes required to alter the DNA-binding specificity of the Zn^{2+} finger. To identify novel transcription factor genes that might play important roles in specification of granule neurons, we chose to screen cerebellar cDNA libraries for Zn^{2+} finger cDNAs that mark the granule cell lineage within the developing cerebellum. In this study, we describe a Zn^{2+} finger cDNA (RU49) that is expressed in the earliest granule cell progenitors at the rhombic lip and whose expression is maintained throughout granule cell differentiation. We determine the DNA-binding specificity of RU49 and demonstrate that it interacts with a novel bipartite DNA-binding site in a manner that is consistent with current models for the specificity of Zn^{2+} finger binding to DNA. The expression of RU49 in cerebellar granule cells and their earliest progenitors in vivo and in vitro, and its novel DNA-binding specificity, suggest that RU49 may play a critical role in granule cell specification within the developing cerebellum. Furthermore, its expression in both the olfactory bulb and dentate gyrus granule cell lineages suggests that these three cell populations may share molecular mechanisms for their specification, and provides an opportunity to investigate molecular mechanisms participating in the creation of a secondary proliferative zone within the developing brain.

MATERIALS AND METHODS

Isolation and characterization of cDNA clones

A probe encompassing the zinc finger domains of the ZNF7 gene (Lania et al., 1990) was used to screen at low stringency a mouse P8 cerebellar cDNA library constructed in λgt11 vector (Promega). Restriction mapping with *Taq*I determined the total number of independent clones. DNA sequencing, Northern blot analysis, in vitro translation and Southern blot analysis were done according to standard protocols.

Reverse transcriptase-PCR reaction

Total RNA (10-20 mg) from adult mouse cerebellum, brain and testis was used for cDNA synthesis. The first strand cDNA synthesis was carried out in a buffer with the following components: 5 mM $MgCl_2$, 1× Reverse Transcriptase Buffer (50 mM Tris-HCl, pH 8.3, 8 mM $MgCl_2$, 10 mM DTT), 1 mM dNTPs, 2.5 μM oligo(dT)16, 5 μM random hexamers, 2.5 U AMV reverse transcriptase (NEB), 10-20 mg total RNA. For each sample, two reactions were set up, one with reverse transcriptase (+RT) and one without (–RT). Samples were first annealed for 10 minutes at room temperature, then reactions were carried out at 42°C for 60 minutes, followed by 95°C for 5 minutes. PCR reactions were carried out in the following reagents: 1× PCR buffer (50 mM KCL,10 mM Tris-HCl, pH 8.4, 1.5 mM $MgCl_2$, 0.1 mg/ml gelatin), 0.1 mM dNTPs, 1 U Taq Polymerase, 100 ng of the 5′ and the 3′ primers and 2 ml cDNAs. PCR was performed for 1 minute at 94°C, 2 minutes at 55°C and 2 minutes at 72°C for 30 cycles. Three different 5′ PCR primers were used: p49-5′ (5′CCAGACTCCAAAGCGGTTCTGAG3′), p38-5′ (5′GGGACGGGTTTAGCGACTGTGG3′), p51-5′ (5′CCGGTGAGCCTGCGAGTCCCAG 3′). A common 3′ PCR primer was used: p3′ (5′CCGGAATTCAGTGTCATGGTACCCAAATTG3′). The PCR products were resolved on a 1% agarose gel and blotted onto Genescreen filter. The filters were probed with oligonucleotide probes including p49-5′, p51-5′, p38-5′ and pAUG (5′ATGCCCACCACCTTAGTCATCACAGATTCC3′).

Binding site selection and mobility shift assays

The binding site selection was done according to the method described by Wilson et al. (1993). Nuclear extracts were made from adult mouse cerebellum. The binding site clone containing the most frequently selected binding site (5′ AAGCACCGCCGTAAGTAC 3′) was digested with *Eco*RI to release the insert as well as the flanking amplification sequences and used for mobility shift assays using standard protocols.

In situ hybridizations

In situ hybridization was done according to the method of Schaeren-Wiemers and Gorfin-Moser (1993). Briefly, anti-sense RNA probes for RU49 were prepared using digoxigenin-11-UTP (Boehringer-Mannheim) in the transcription reaction. The template for the reaction was a 0.9 kb *Eco*RI-*Pst*I fragment containing the non-zinc finger domain of RU49. Hybridization was done at 72°C for 16 hours, followed by washes in 0.2× SSC at 72°C for 1 hour. Samples were incubated overnight with alkaline phosphatase-conjugated goat anti-digoxigenin Fab fragment (Boehringer-Mannheim) at 1:2000

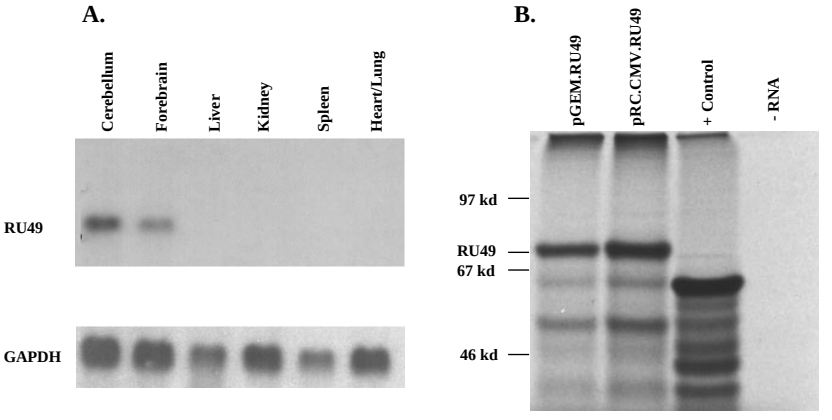

Fig. 1. Northern blot analyses and in vitro transcription and translation of the RU49 cDNA clones. (A) Total RNA (10-20 μg) from adult mouse cerebellum, forebrain, liver, kidney, spleen and heart/lung were electrophoresed and transferred to a filter. The filter was hybridized with a probe encompassing the full length RU49 cDNA. The panel below shows the same blot rehybridized with a GAPDH probe to control for RNA loading. (B) In vitro transcription and translation of RU49 cDNA clones. RU49 cDNA clones in pGEMEX-2 vector and in pRC. CMV vector were translated in vitro in a rabbit reticulocyte lysate system. The positive control (+control) is using a vector containing the luciferase gene. The negative control for in vitro translation was a translation reaction without added RNA (–RNA). The positions of marker proteins are shown.

dilution, washed and processed for colorimetric detection using NBT and X-phosphate.

RESULTS

Isolation of cerebellar Zn^{2+} finger cDNAs

To identify Zn^{2+} finger proteins expressed in the cerebellum, a probe encompassing the finger domains of the human *ZNF7* gene (Lania et al., 1990) was used to screen a cDNA library prepared from postnatal day 8 mouse cerebellum. The *ZNF7* gene is a C_2H_2 Zn^{2+} finger gene, expressed in many human cell lines. It is related to the *Drosophila Kruppel* gene and shares with it a very highly conserved 7-amino-acid hinge region preceding the Zn^{2+} fingers, which is characteristic of this very large subfamily of Zn^{2+} finger proteins (Schuh et al., 1986). From the primary screen of the cerebellar cDNA library, approx. 200 positive plaques were detected. One hundred of these were purified (RU1-100), amplified by PCR and restriction mapped. These results established that there were 68 different cDNAs within this pool, indicating that well over 100 different Zn^{2+} finger genes are expressed in the developing mouse cerebellum. We were initially surprised by the large number of independent cDNAs obtained in this screen, so ten random clones were chosen to determine partial DNA sequences. All of these ten genes encoded previously uncharacterized Zn^{2+} finger proteins (data not shown), indicating that many of these clones encoded novel cDNAs.

Individual cDNAs were used for northern blot hybridization to detect either brain-specific or developmentally regulated mRNAs, resulting in the isolation of three clones from the first 20 screened that appeared to be brain specific. As shown in Fig. 1A, a single 2.2 kb RU49 mRNA is detected in both the adult cerebellum and forebrain, although no RU49 mRNA is present in poly(A)$^+$ mRNA isolated from a variety of other adult tissues. Since the 2.2 kb RU49 cDNA appeared to be nearly full length, synthetic RNA was prepared from the RU49 cDNA and translated in a rabbit reticulocyte lysate. As shown in Fig. 1B, RU49 cDNA encodes a 72×10^3 M_r primary translation product, indicating that the RU49 cDNA probably contains the entire open reading frame. The specificity of RU49 expression in these initial assays stimulated our interest in further pursuing the characteristics of this cDNA.

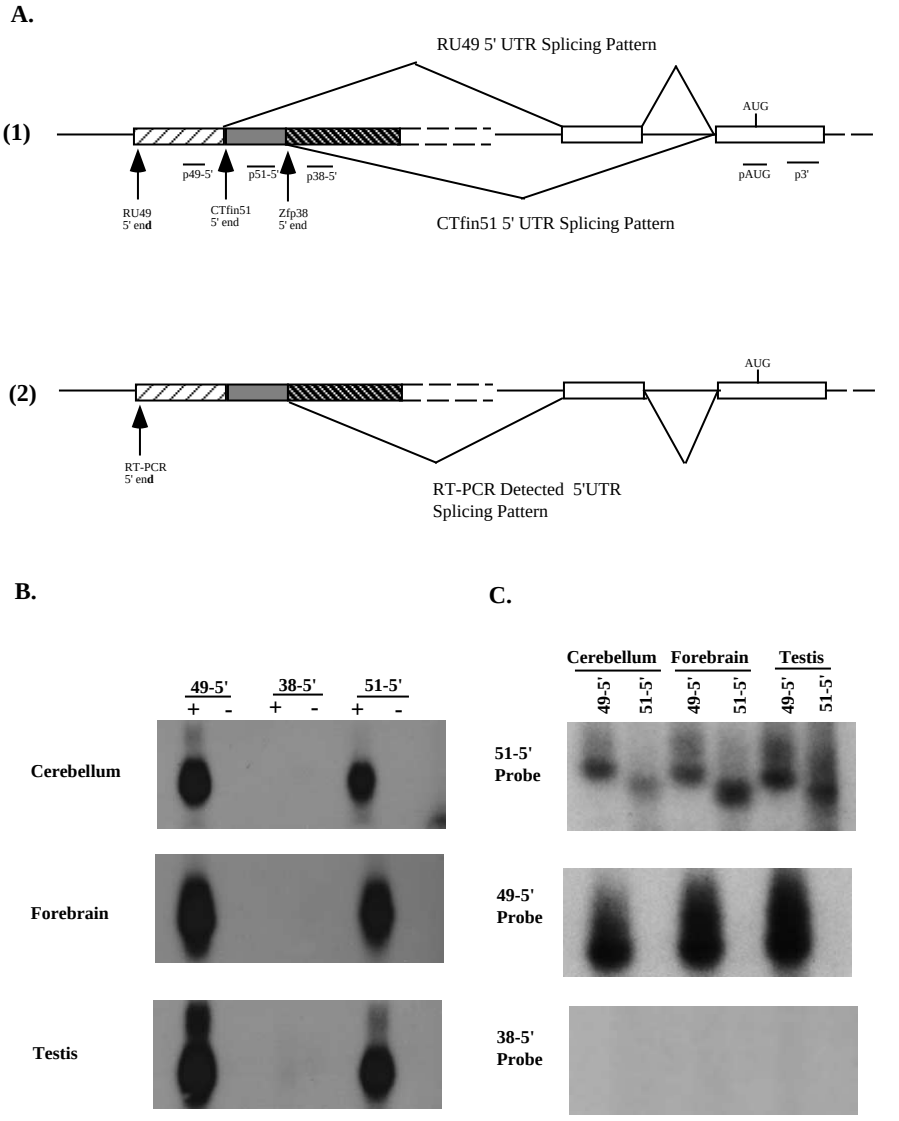

Fig. 2. Reverse transcriptase PCR reaction to determine the 5′ alternative splicing pattern of RU49 transcripts in adult mouse cerebellum, forebrain and testis. (A) The known cDNAs, RU49, CTfin51 and Zfp38 have the same coding region, but different 5′ ends and different alternative splicing patterns at the 5′ untranslated region (5′UTR). These are illustrated in (1), the unique 5′ sequences of RU49 (35 bp), CTfin51 (21 bp) and Zfp-38 (796 bp) lie adjacent to each other on the genomic sequence published by Chowdhury et al. (1992). The arrows indicate the locations of 5′ ends of the three cDNAs. The splicing patterns of RU49 and CTfin51 are indicated. The Zfp-38 cDNA begins downstream from both RU49 and CTfin51, and contains a relatively large first exon not present in the other cDNAs. The locations of the PCR primers, p49-5′, p38-5′, p51-5′ and p3′ and the oligonucleotide probe pAUG, are shown on the diagram. (2) Shows the splicing pattern of the RU49 cDNA, as determined by RT-PCR in adult cerebellum, forebrain and testis. (B) RT-PCR experiments were performed using total RNA from adult mouse cerebellum, forebrain and testis, with different 5′ PCR primers (49-5′, 38-5′ and 51-5′) and a common 3′ PCR primer (p3′). Each reaction was done with (+) or without (–) reverse transcriptase to control for the dependence of the PCR products on the reverse transcriptase reaction. The RT-PCR products were electrophoresed and blotted onto a filter and probed with the end-labeled pAUG oligonucleotide. (C) The RT-PCR products using either 49-5′ or 51-5′ primers from cerebellum, forebrain and testis were electrophoresed and blotted on to a filter. The filter was then probed separately with end labelled 51-5′, 49-5′ and 38-5′ oligonucleotide probes. The GenBank accession number for RU49 is U41671.

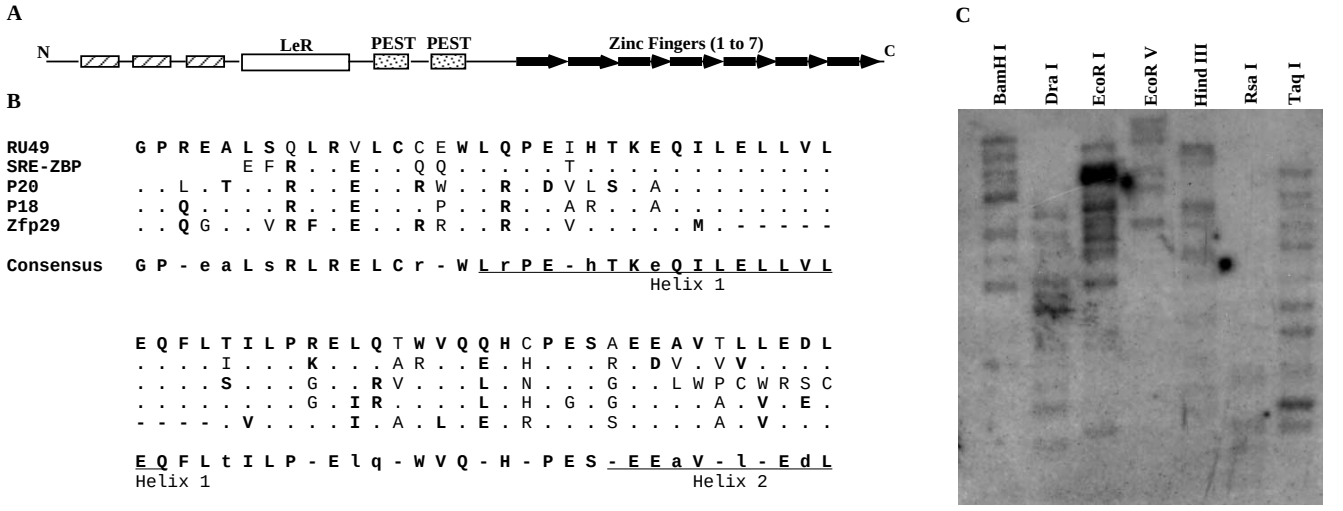

Fig. 3. (A) The modular structure of the RU49 protein, including three N-terminal repeats, the LeR domain, two PEST sequences (Rogers et al., 1986) and seven *Kruppel*-type zinc fingers at the C terminus. (B) The sequence alignment of the RU49 LeR domain with LeR domains of human SRE-ZBP (Attar and Gilman, 1992), human p20 (Pengue et al., 1994), p18 (Pengue et al., 1993) and mouse Zfp29 (Denny and Asworth, 1991). Dots indicate identity and bold letters indicate conservative amino acid substitutes. There are two predicted helical regions, identified by two secondary structure prediction programs, Chou-Fasman and Garnier-Osguthorpe-Roboson. (C) Mouse genomic DNA (15 μg) was digested with different restriction enzymes, electrophoresed and blotted onto filters, and then probed with the RU49 LeR domain.

The primary structure of the RU49 cDNA

To further characterize the RU49 cDNA, its complete nucleotide sequence was determined and used to search GenBank. Two interesting properties of the RU49 clone were revealed in this analysis. First, the deduced protein encoded by the RU49 cDNA is identical to a previously cloned Zn^{2+} finger cDNA designated zfp38 (Chowdhury et al., 1992) or CTfin51 (Noce et al., 1992), which is expressed at very high levels during meiosis in both spermatocytes and oocytes. Chowdhury et al. (1992) reported RNase protection experiments indicating that this gene is expressed in many tissues. However, as shown in Fig. 1, no significant expression in tissues other than brain, testis and ovary (data not shown) was observed in our analysis by northern blotting or in situ hybridization (see below). This is in agreement with the results of Noce et al. (1992), although they also detected low levels of RU49 mRNA in spleen. Second, the structures of the zfp38 and CTfin51 testis cDNAs diverge from the RU49 cerebellar cDNA near the 5′ terminus, suggesting that the RU49 transcript may be alternatively spliced. To examine which of these cDNAs correspond to the major transcript of the gene and to assess the possibility for alternative splicing, we have used RT-PCR. As shown in Fig. 2A, the 5′ ends of the RU49, CTfin51 and zfp38 cDNAs lie adjacent in the genome (Chowdhury et al., 1992; X. W. Yang and N. Heintz, unpublished results). To detect mRNAs containing these sequences, we have prepared primers specific for the 5′ ends of these clones and used them in concert with a common 3′ sequence to amplify PCR products from reverse transcribed mRNA from cerebellum, forebrain and testis. As shown in Fig. 2B, bands of the expected size can be amplified using the RU49 and CTfin51 primers in all three tissues examined, whereas no product can be detected using the zfp38

A. Examples of RU49 Binding Sites
(Total # clones sequenced = 61)

1. **AGTAC**CGCTCCC**AGTAC**CCATC
2. CAACC**AGTAC**CCGCT**AGTAT**CA
3. **AGTAC**TTACTAA**AGCAC**CC
4. **AGTAC**TTACTAA**AGTAC**
5. CGA**AGTAC**TT**AGTAC**TCA
6. A**AGTAC**CTTCTA**AGCAC**TC
7. **AGTAC**CGCCGTA**AGTAC**
8. **AGTAC**CGGAAA**AGCAC**TCA
9. **GGTAC**TGGGAGC**GGTAC**
10. AA**AGCAC**CCTTCC**AGTAC**
11. CATTTGGGATCTG**AGTAC**
12. **AGTAC**TGACA**AGCAC**CCA
13. AG**AGCAC**TAAGTACCCTT
14. CTC**AGTAC**TC**AGTAC**CCA
15. AGGGC**AGTAC**T**AGTAG**ATC
16. **AGTAC**CGCTCCC**AGTAC**CC
17. A**AGCAC**CGCCGTA**AGTAC**
18. A**AGCAC**CGCCGTA**AGTAC**
19. GG**GTGCT**GCGGAT**AGTAC**
20. **AGCAC**CCATACA**AGCAC**TT

B. Orientation and Spacing Preference

Orientation	→ →	← →	→
Frequency	47/61	4/61	10/61
Spacing			
7 bp	26	0	
6 bp	5	2	
5 bp	2	1	
4 bp	1	1	
3 bp	1	0	
2 bp	9	0	
1 bp	3	0	

Fig. 4. DNA-binding-site consensus for the RU49 zinc finger domain and the orientation and spacing preference for the in-vitro-selected RU49-binding sites. (A) 20 of the 61 sequenced RU49-binding site clones are shown. The consensus binding site sequences are shown in bold. The major consensus site is 5′AGTAC3′ although, less frequently, the binding sites can be 5′AGCAC3′ or 5′GGTAC3′. (B) The number of sequenced RU49-binding sites containing direct repeats, everted repeats and single sites are shown. Among the clones with two consensus sites, the spacings between the sites are also listed.

Zinc Fingers		**1**	**2**	**3**	**4**	**5**	**6**	**7**
Key Recog.AAs (-1,2,3,6)		**NS N K**	**HS N L**	**QS D K**	**GG S R**	**QA G S**	**HS N K**	**SS N K**
Finger Specif.		**+++**	**+++**	**+++**	**-**	**+/-**	**+++**	**+++**
Suzuki Code (For -1,3,6)	3′	**A A G** n n **T** A	**G A T** n n **C**	**A C G** n **A T** A	**-** n **G** T A	**A -** n n	**G A G** n n **T** A	n **A G** 5′ n **T** A
Ser(+2)*		**T/C**	**T/C**	**T/C**			**T/C**	**T/C**
Binding Models:								**Frequency**
1.DR(5-7sp.)	3′	**C A T** **C A T** **C A T**	**G A** **G A** **G G**				**C A** (C) **C A T** **C A T**	**G A** 5′ **G A** **G G**	**33**
			C A T/C	**G A**			**C A T**	**G A** 5′	
3.ER	3′	**C A** (C)	**G A**			**T**	**C A T**	G 5′	**4**
4.DR (2sp.)	3′	**C A T**	**G A** T/A	**T C** A/**G**	T G **G**/A 5′				**9**
5.DR (1sp.)	3′	**C A T**	**G A** T	**C A T**	G A 5′				**3**
6.Single Site	3′	**C A T**	**G A**/G 5′						**10**

Fig. 5. A model of RU49 zinc finger interaction with different classes of in vitro selected binding sites. The key DNA recognition residues of the seven RU49 zinc fingers (positions –1, 2, 3 and 6 of the helix of each finger) are shown in the top two lines. According to the rules of Suzuki et al. (1994), the size of the side chains at the key recognition residues can be used to predict whether the zinc finger is specific or non-specific in DNA recognition. RU49 fingers 1-3 and fingers 6 and 7 are predicted to be very specific for DNA recognition (+++), whereas fingers 4 and 5 are predicted to be non-specific (– or +/–). The chemical code for specificity derived from Suzuki et al. (1994) is shown, indicating DNA bases recognized by key recognition residues. The DNA-binding sites are aligned with the fingers in an antiparallel manner, the 5′ base of each subset interacting with the C-terminal recognition residue of each Zn finger. Bold letters indicate potential for specific recognition by a given amino acid. Two modifications of the Suzuki code have been employed. First, according to Jamieson et al. (1994), a T at the 5′ of the triplet DNA subset preferentially interacts with Lys at position +6 of the Zn^{2+} finger recognition helix. Second, the crystal structure of the Tramtrak-DNA complex revealed that Ser at +2 of the recognition helix can specifically interact with either T or C at the 3′ base of the subset (Fairall et al., 1993). According to these rules, in-vitro-selected RU49-binding sites can then be aligned in agreement with the modified chemical code onto respective fingers, as shown. For example, the most frequently selected binding site of two direct repeats with 7-bp-spacing can be aligned in the following way: fingers 6 and 7 recognize the 5′ consensus site and fingers 1 and 2 recognize the 3′ consensus. All the other types of in-vitro-selected binding sites can be aligned in a similar manner. The frequency of each type of binding site is shown on the right.

primer. In each case, the amplified products were dependent upon inclusion of reverse transcriptase in the reaction. To exclude the possibility that the zfp38 product was not amplified due to its larger expected size (1 kb) relative to the other products, a control reaction that amplified a 2 kb fragment from the same mRNA was performed and resulted in an easily detectable product of the expected size (data not shown). These results, the structures of the different cDNAs and the genomic organization of the gene, all suggested the possibility that this gene is alternatively spliced.

To determine whether the zfp38-specific cDNA sequences are contained within the RU49-amplified products, Southern hybridization using exon-specific probes was performed on the amplified RT-PCR products from all three tissues. As shown in Fig. 2C, none of the RT-PCR products hybridize to zfp38-specific oligonucleotide, confirming the results in Fig. 2B and indicating that the zfp38 cDNA is a very minor species. In contrast, the CTfin51 probe hybridized to all observed products, whereas the RU49-specific probe only hybridized to reaction products that had been prepared by using it to amplify the cDNA. These results clearly indicate that the major transcript from this gene in vivo contains the RU49 5′ end fused to the reported CTfin51 sequences, and that the zfp38 cDNA must represent a relatively rare alternatively spliced mRNA (Fig. 2A, diagram 2).

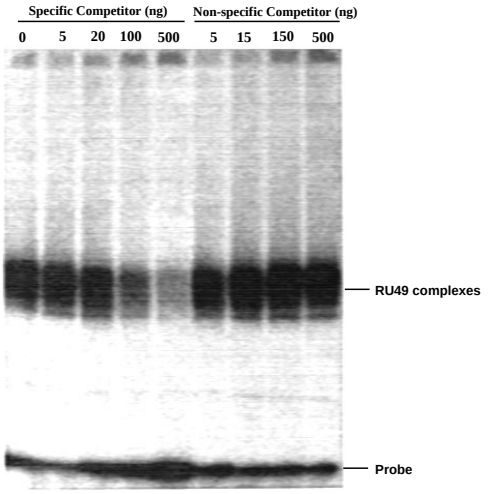

Fig. 6. Gel mobility shift assay demonstrating that there is a binding activity in the cerebellar nuclear extract that interacts with the selected RU49-binding sites. Nuclear extracts were prepared from adult mouse cerebellum and the gel mobility assay was performed using an end-labeled double strand oligonucleotide probe containing the in-vitro-selected RU49-binding site. Unlabeled specific competitor DNA (0 ng, 5 ng, 20 ng, 100 ng and 500 ng) and non-specific DNA (5 ng, 15 ng, 150 ng and 500 ng) were added to each binding reaction to demonstrate the binding specificity.

As shown in Fig. 3A, RU49 contains seven Zn^{2+} fingers and several interesting structural domains that have been identified through sequence analysis. At the amino terminus, RU49 contains three highly conserved tandemly repeated sequences that function as a transcriptional activation domain when assayed by fusion to the Gal4 DNA-binding domain (Chowdhury et al., 1992), providing strong evidence that this protein is a transcription factor. Our initial analysis of the RU49 cDNA also revealed a second region of interest, which has recently been noted by Pengue et al. (1994). This domain, which is referred to as the LeR domain, is leucine rich and contains two predicted alpha helices (Fig. 3B). It is very highly conserved in a subset of proteins that are either known Zn^{2+} finger transcription factors, or contain other structural elements that are characteristic of this class of transcription factors. To obtain an estimate of the number of genes containing this domain within the mouse genome, we utilized a probe specific for the RU49 LeR domain on genomic Southern blots. As shown in Fig. 3C, there are approximately a dozen genes within the murine LeR domain family, implicating this domain as an important and conserved structural motif within this subfamily of Zn^{2+} finger proteins.

RU49 interacts with a novel bipartite DNA-binding element

Although the identification of a transactivation domain at the amino terminus of RU49 provides strong evidence that this protein acts as a transcription factor (Chowdhury et al., 1992), these data do not aid in attempts to identify potential target genes that might be regulated by RU49. To further pursue the functions of RU49, we have determined its DNA-binding specificity using the bacterially expressed RU49/GST-fusion protein to select optimal binding sites from a collection of random 18mer double stranded DNAs (Thiesen and Bach, 1990; Wilson et al., 1993). In this case, double-stranded DNAs were selected from the random population using RU49/GST fusion proteins bound to agarose beads, eluted from the protein and reamplified by the polymerase chain reaction. This procedure was repeated for 5 to 10 cycles, the remaining double-stranded DNAs were cloned and sequenced, and the binding sites aligned to determine a consensus.

As shown in Fig. 3A, each of the DNA fragments selected by RU49 contained a very close match to the derived consensus binding site 5′-AGTAC-3′. Further inspection of these sequences revealed that most of the RU49-selected DNAs contained two copies of this minimal binding site. To assess the importance of multiples of this sequence for RU49 DNA binding, further selections were performed and analyzed. As shown in Fig. 4B, the results of this analysis revealed that RU49 exhibits a very strong preference for DNA-binding sites containing two RU49 consensus motifs. Thus, 51 of the 61 sequenced RU49-binding sites contained two easily recognized 5′-AGTAC-3′ consensus sites. Among these sites, a strong preference (47/51) for binding to tandem direct repeats of the minimal RU49 consensus binding site was observed. The strong preference of RU49 for binding sites containing two minimal 5′-AGTAC-3′ motifs suggests either that RU49 binds DNA as a dimer, or that it contains multiple DNA-binding motifs with similar specificity. Since the RU49/GST fusion protein used in the selection assays is immobilized on agarose beads, and since RU49 contains seven contiguous Zn^{2+} finger motifs for DNA binding, it seemed probable that these results reflect the similar binding specificity of individual RU49 Zn^{2+} fingers rather than binding of RU49 as a dimer to the selected sites. This conclusion is supported by the fact that

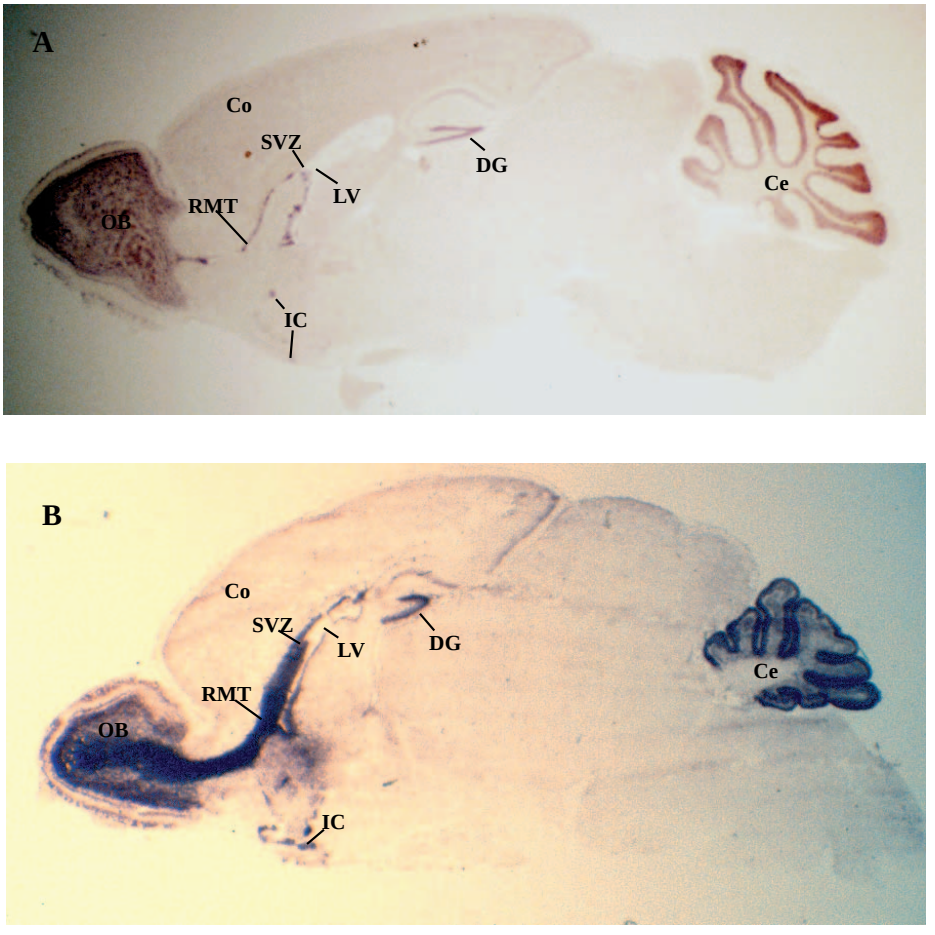

Fig. 7. RU49 expression in the postnatal brain. In situ hybridizations were done in adult (A) and postnatal day 6 (B) sections using a digoxigenin-labelled RNA antisense probe, which encompasses the 800 bp RI-*Pst*I fragment of the non-zinc finger region of RU49. High levels of RU49 expression are observed in the internal granular layer of the cerebellum, the granule cell and periglomerular cell layers of the olfactory bulb and in dentate gyrus granule cells (DG). Expression is also evident in the subventricular zone (SVZ) adjacent to the lateral ventrical (LV), in the rostral migratory tract (RMT) leading from the SVZ to the olfactory bulb (OB) and in Islands of Calleja (IC). No significant levels of expression are observed in other regions of the brain, including the cerebral cortex (Co). The sense probe used as a negative control gave no significant signal above background (data not shown). Scale: A (1:8), B (1:10).

RU49 binding to sites containing either a single 5′-AGTAC-3′ motif or two of these motifs results in an identical pattern of bands in an electrophoretic mobility shift assay (data not shown).

A further feature noted in the analysis of RU49 DNA-binding specificity was that the sites containing two RU49 minimal consensus motifs exhibited a strong preference for the spacing of those sites. Thus, sites containing tandem direct repeats of the RU49 minimal consensus sites revealed a preferred spacing of either 7 bp (26/47) or 2 bp (9/47). It occurred to us that this information, combined with inferences drawn from the cocrystal structures of other Zn^{2+} finger DNA-binding protein/DNA-binding site complexes, might be useful in predicting the alignment of the RU49 on the DNA. As first noted from the cocrystal structure of Zif268 and its cognate binding site (Pavletich and Pabo, 1991), Zn^{2+} finger proteins are modular structures in which each individual finger motif can interact with a 3 bp DNA subsite in an antiparallel manner. Based on the specific contacts present in the Zif268 cocrystal structure (Pavletich and Pabo, 1991), the GLI 1 structure (Pavletich and Pabo, 1993) and the *Drosophila* tramtrak structure (Fairall et al., 1993), a simple code for DNA binding involving four key amino acids (positions –1, 2, 3 and 6 of the helix) in each individual finger that make base-specific contacts in the major groove of the binding site has been proposed (Pavletich and Pabo, 1991; Klevit, 1991; Berg, 1992). This code has been considerably refined, using similar data from a large number of Zn^{2+} finger proteins, to define a set of rules for these interactions (Suzuki et al., 1994). Given these considerations, a RU49 finger/DNA-binding model can be constructed to explain the interaction of this factor with DNA. Since fingers 1, 2, 6 and 7 have similar amino acid residues in key positions for interaction with DNA, we suggest that RU49 is aligned on DNA such that fingers 1, 2, 6 and 7 are critical in determining DNA-binding site selectivity, due to base-specific contacts with the RU49 minimal binding sites (Fig. 5). Since fingers 4 and 5 have glycine in key recognition positions, and therefore lack a side chain for specific interaction with DNA bases, it is unlikely that they participate in optimal DNA binding. This model for RU49 interaction with DNA is consistent with the chemical code for recognition proposed by Suzuki et al. (1994) and explains the preference of a single RU49 molecule to interact with repeated 5′-AGTAC-3′ subsites, as demonstrated by the binding site selection data. Alignment of RU49 on tandem repeats separated by only 2 bp is probably through specific contacts with fingers 1, 2, 3 and 4, since alignment of RU49 on those sites, as shown in Fig. 4B, is also consistent with the Suzuki code (Suzuki et al., 1994).

RU49 DNA-binding activity in the adult mouse cerebellum

The determination of the RU49 DNA-binding specificity allowed us to investigate whether a transcription factor with the same functional specificity as the bacterially expressed RU49 protein is present in the cerebellum. Nuclear extracts were prepared from adult mouse cerebellum and used in electrophoretic mobility shift assays to assess whether a DNA-binding protein that specifically interacts with the derived RU49 consensus site is present in the cerebellum. As shown in Fig. 6, the labeled RU49 DNA probe interacts strongly with a

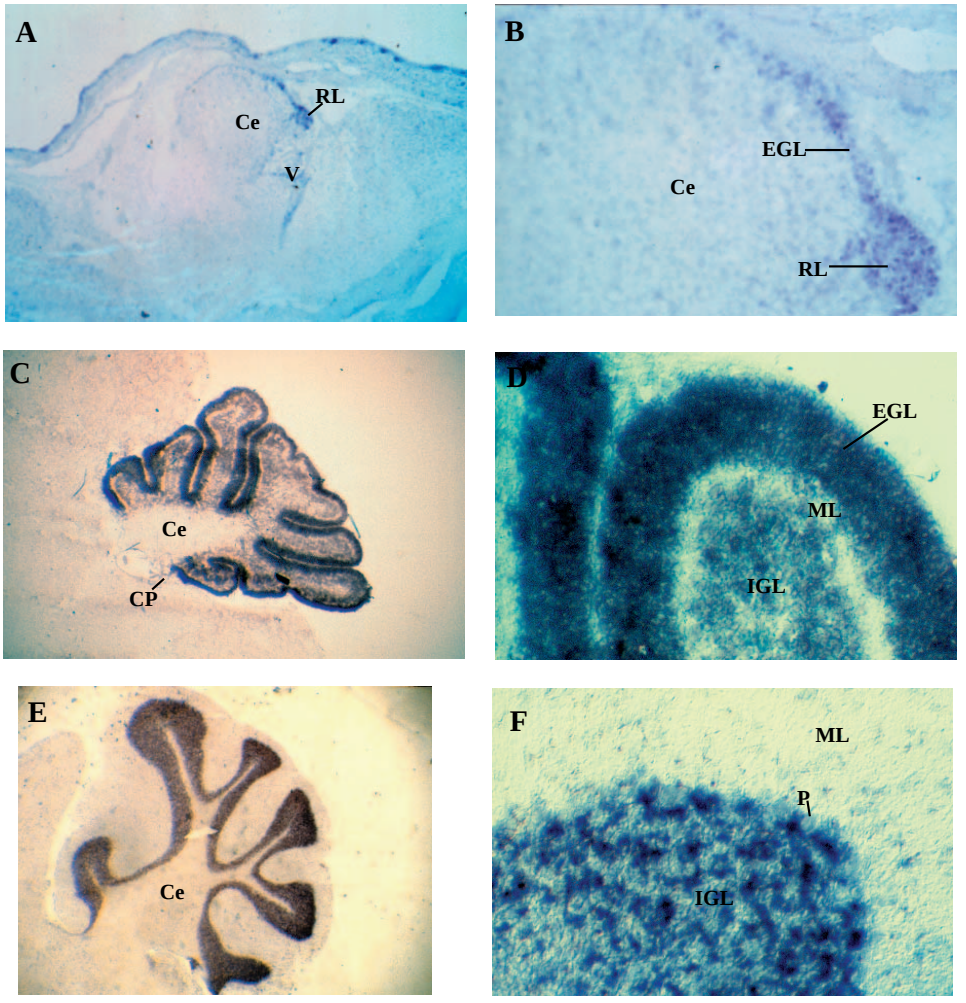

Fig. 8. Expression of RU49 in the developing cerebellum. This figure shows parasagittal mouse brain sections from embryonic day 14.5 (A and B), postnatal day 7 (C and D) and adult (E and F). All the left side panels (A, C, D) are low magnification pictures while the right side panels are corresponding higher magnification pictures. The sense probe used as a negative control gave no significant signals above background (data not shown). Abbreviations: Ce, cerebellum; EGL, external granular layer; IGL, internal granular layer; RL, rhombic lip; V, fourth ventricle; ML, molecular layer; CP, choroid plexus. Scale: A, C, E, (1:15); B, D, F, (1:150).

protein present in adult cerebellar nuclear extracts. This interaction is specifically lost when the unlabeled RU49-binding site is used as a competitor DNA, but is not affected by competition using a control binding site for an unrelated Zn^{2+} finger protein. Western blots using antibody to the bacterial fusion protein established that a single approx. 75×10^3 M_r protein is detected in the cerebellar extracts (data not shown), consistent with the detection of a similar sized band in testis, as reported by Noce et al. (1992). These results establish that functional RU49 DNA-binding activity is present in adult cerebellum.

RU49 expression in postnatal development

Having demonstrated that functional RU49 protein is expressed in adult cerebellum, we were next interested in determining its pattern of expression in the adult brain. As shown in Fig. 7A, in situ hybridization reveals that the major sites of RU49 expression in adult brain are granule cells of the cerebellum (Cb), olfactory bulb (OB) and dentate gyrus (DG). RU49 is also expressed in olfactory bulb periglomerular cells (PG), the subventricular zone (SVZ) of the lateral ventricle (LV) and the rostral migratory tract (RMT) leading from the SVZ to the OB, which continue to supply both granule cells and periglomerular cells to the olfactory bulb in the adult animal (Altman, 1969b; Lois and Alvarez-Buylla, 1994; Luskin, 1993). Finally, RU49 mRNA is observed in small groups of cells between the SVZ and the olfactory tubercle. Both the positions of these cells in the adult brain, and the discrete boundaries of these groups of cells as revealed by RU49 expression, suggest that they may be the Islands of Calleja (IC; Calleja, 1893), although additional studies must be done to confirm this identification.

Since one of the most interesting properties of olfactory bulb, dentate gyrus and cerebellar granule cell differentiation is that these three cell populations continue to be generated postnatally, we next examined the expression of RU49 in postnatal day 6 animals. As shown in Fig. 7B, the expression of RU49 is exquisitely specific to these three cell lineages as they continue to differentiate in the developing brain. Two differences between early postnatal RU49 expression and that seen in adult brain are particularly noteworthy. First, it is immediately clear, even at low magnification, that RU49 marks cerebellar granule cells, both as they assemble into the internal granular layer and during their proliferation and differentiation within the external granular layer. Second, in early postnatal animals, the rostral migratory tract carrying granule cells and periglomerular cells from the subventricular zone to the olfactory bulb is extremely heavily labelled. In addition, our impression from many such analyses is that the diffuse staining between the fourth ventricle and the olfactory tubercles may represent cells migrating from the ventricle to take up positions in the Islands of Calleja, since this diffuse staining becomes more focused as these structures become increasingly more refined later in development.

RU49 expression in cerebellar granule cells

The pattern of RU49 expression within the developing cerebellum is striking. Classical studies have established that cerebellar granule cells arise between embryonic days 13 and 15 at the caudal edge of the developing cerebellum in a structure called the rhombic lip (Miale and Sidman, 1961; Fugita et al., 1966; Altman, 1969a). In sagittal section, this structure appears as a thickening of the caudal edge of the developing cerebellum. At these very early stages, proliferating granule cell precursors spread rostrally from the rhombic lip beneath the pial surface as a thin layer of rapidly proliferating cells that will eventually cover the entire surface of the developing cerebellum. As shown in Fig. 8A,B, in situ hybridization experiments reveal that the expression of RU49 during this period precisely marks cerebellar granule cell precursors as they are generated at the rhombic lip and during their migration beneath the pial surface to establish the external germinal layer (EGL). Thus, at embryonic day 14.5, RU49 mRNA is present in a well-defined population of cells at the caudal edge of the developing cerebellum (Fig. 8A). At high magnification, it is evident that at this age RU49-positive cells have just begun to spread rostrally from this position along the superficial surface of the cerebellar anlage (Fig. 8B). The restriction of RU49 expression to a discrete population of progenitor cells at the rhombic lip in E14.5 embryos is consistent with the birth-dating studies of Miale and Sidman (1961), which placed the first birth of mouse cerebellar granule cells between embryonic days 13 and 15. Furthermore, its presence in a thin layer of granule cells emanating from the rhombic lip and migrating rostrally directly beneath the pial surface is precisely as was expected from the detailed anatomical studies describing the formation of the rodent EGL (Altman and Bayer, 1985a,b,c).

At postnatal day 7, the developing cerebellum is characterized by the presence of a very robust population of rapidly proliferating granule cells, which expand beneath the pial surface to create an EGL approximately 8-10 cells thick. Relatively few cells during this period have completed their migration via Bergmann glial guides to the developing internal granular layer (IGL). At this age, the RU49 staining is evident in the EGL, both in the most superficial layer containing rapidly proliferating granule cell precursors, and in the deep EGL containing postmitotic, differentiating granule cells (Fig. 8C,D). It is readily apparent from this data that RU49 continues to be expressed in maturing granule cells as they begin to populate the IGL. The fact that RU49 mRNA is not detected in any other cerebellar cell type (Fig. 8E,F), in particular stellate and basket cells within the molecular layer, is of interest because it is consistent with the fate mapping experiments of Hallonet et al. (1990) and Otero et al. (1993), indicating that the EGL gives rise only to granule neurons.

RU49 expression in olfactory bulb and dentate gyrus granule cells

In addition to the abundant expression of RU49 in the cerebellum, as noted above, high levels of RU49 mRNA are readily apparent, both in the crescent-shaped hippocampal dentate gyrus and in the olfactory bulb (Fig. 7). Furthermore, the presence of RU49 mRNA in the rostral stream emanating from the subventricular zone of the inferior horn of the lateral ventricle and projecting into the developing olfactory bulb in adult animals is particularly interesting, given the expression of this transcription factor in cerebellar granule cell at all stages of their development.

Detailed autoradiographic and anatomic studies by Altman (1969b) first established that the major target for cell production in the subventricular zone adjacent to the lateral ventricle is the olfactory bulb. This work provided a detailed description of the generation of the cells in the subventricular zone, the precise pathway taken by these precursors as they continue to divide and migrate out to the bulb, and as they become integrated into the olfactory granular layer. In situ hybridization

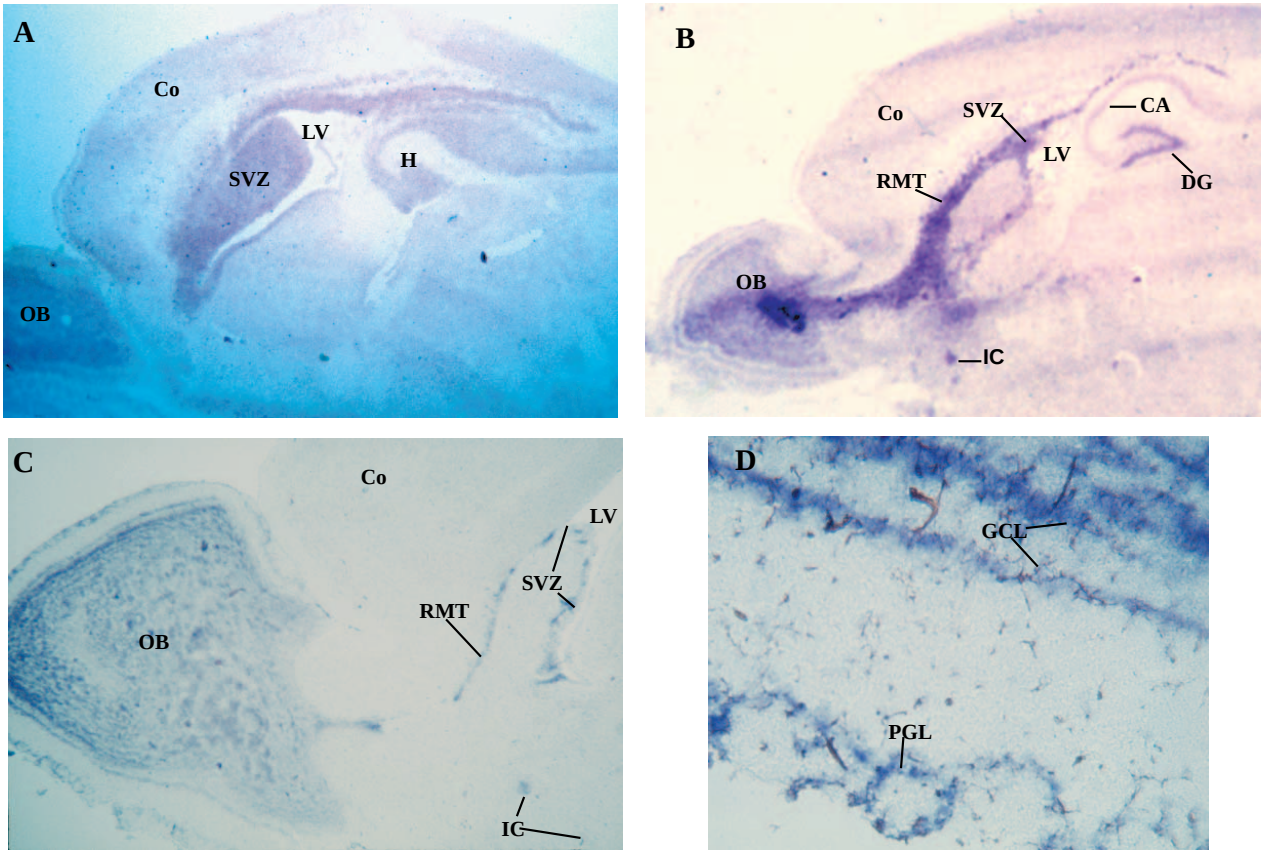

Fig. 9. Expression pattern of RU49 transcripts in the developing mouse forebrain. In situ hybridizations were done on mouse parasagittal sections from P0 (A), P7 (B) and adult (C,D) mouse brain to delineate patterns of expression in the olfactory and dentate gyrus granule cells. (A) At P0, RU49 mRNA is detected in the subventricular zone (SVZ), the olfactory bulb (OB) and the hippocampal region (H). (B) At P7, RU49 is expressed in the subventricular zone (SVZ), the rostral migratory tract (RMT), the olfactory bulb and the dentate gyrus (DG), but is not present in the cortex (Co), nor in Ammon's horn (CA) of the hippocampus. (C) In the adult brain, RU49 is expressed in the subventricular zone (SVZ), the rostral migratory tract (RMT), the olfactory bulb (OB) and Islands of Calleja (IC). (D) High magnification examination of the olfactory bulb in adult animals demonstrates that RU49 is present in both granule cells (GCL) and periglomerular cells (PGL). Scale: A (1:30), B (1:18), C (1:16), D (1:160).

for RU49 mRNA in the developing and adult olfactory bulb (Figs 7,9) reveals that its pattern of expression precisely reproduces Altman's original description of this cell population. Furthermore, close inspection of these data reveals that RU49 is also expressed in periglomerular cells in the olfactory bulb (Fig. 9D). This is consistent with recent transplantation (Lois and Alvarez-Buylla, 1994) and retroviral lineage marking (Luskin, 1993) experiments that have definitively established that this 'rostral migratory stream' contributes both granule cells and periglomerular cells to the developing and adult olfactory bulb.

Although we have not undertaken a detailed examination of RU49 expression in the granule cell lineage of the dentate gyrus, we believe that it is also expressed throughout the differentiation program of this cell type. Thus, at birth (Fig. 9A), RU49 is strongly and asymmetrically expressed in the germinal pool adjacent to the lateral ventrical that contributes heavily to formation of the dentate gyrus granule cell layer (Altman and Das, 1965). These observations, and the refinement of this pattern in postnatal animals to local expression in the dentate gyrus (Fig. 9B), are consistent with the changing pattern of hippocampal granule cell neurogenesis after birth.

DISCUSSION

Classical neuroanatomical studies of mammalian cerebellar development (Ramon y Cajal, 1911) and analysis of developmental perturbations resulting from experimental manipulations or evident in neurologic mutant mouse strains (Sidman and Rakic, 1973; Sidman, 1983; Altman, 1976) have contributed to an exquisite description of the complex cellular events that occur during formation of this brain structure, and have demonstrated a variety of cellular interactions that must occur for normal development. From these studies, one might consider cerebellar development as consisting of three major phases: the initial patterning of the cerebellar plate and creation of the cerebellar anlage from the roof of the anterior metencephalon; the sequential specification of neuronal and glial cell types within the developing cerebellar anlage; and the differentiation of these cell types and concurrent generation of the mature cerebellar circuitry in response to local interactions between these distinct differentiating cell populations (reviewed by Hatten and Heintz, 1995). Although a variety of recent cellular and molecular genetic studies have begun to provide mechanistic information concerning both the initial patterning of the cere-

bellar plate (Joyner and Hanks, 1991; McMahon, 1993) and the differentiation of specific cell types within the developing cerebellum (reviewed by Hatten and Heintz, 1995), very little is known about cell specification within the developing cerebellum or elsewhere in the vertebrate central nervous system. In this study, we have identified RU49 as a biochemically novel transcription factor that precisely marks granule cells in the cerebellum, olfactory bulb and dentate gyrus. Its properties suggest that it may play a key role in granule cell specification, and that its further analysis could provide critical insights into formation of secondary proliferative zones within the developing brain.

Zn^{2+} finger transcription factors as novel developmental regulators in the developing CNS

The involvement of transcription factors in the specification of cell types from pluripotent precursors has been established in a variety of systems and cell lineages. Although the search for specific transcription factors that play key roles in cell fate decisions in the vertebrate CNS has begun, the appropriate molecules have not yet been identified. To address this problem during development of the cerebellum, we reasoned that the recent, rapid evolution of the vertebrate Zn^{2+} finger transcription factor family (Bellefroid et al., 1989; Crossley and Little, 1991) and its easily modified DNA-binding specificities may indicate a role for these genes in novel mechanisms of vertebrate development. The results that we have presented in this study provide general support for this idea. The facts that (1) a very large number of Zn^{2+} finger genes are expressed in the developing cerebellum and (2) most of these genes have not been previously identified, are of particular interest because of the complex and largely unexplored regulatory mechanisms that must be utilized by each cell type to create the very precise functional and architectural properties characteristic of the adult cerebellum. Furthermore, (1) the demonstration that RU49 binds to directly repeated 5′-AGTAC-3′ half sites preferentially spaced 7 bp apart, (2) the ability to model RU49-binding site specificity according to simple rules for interaction of the finger domains with DNA (Suzuki et al., 1994) in a manner that highlights the modular nature of this transcription factor family and may reflect its rapid evolutionary expansion and (3) the novelty of the RU49 consensus DNA-binding site, are all indicative that this class of regulatory proteins may be particularly important in recently evolved mechanisms of development.

Specification of granule neurons within cerebellar anlage

Precedent from a wide variety of systems has established that transcription factors play key roles in cell fate decisions in many tissues and organisms, and that these transcription factors are typically expressed within the developing and adult animal in a restricted pattern that marks a given cell lineage. For example, recent studies of the mammalian *Ikaros* gene, which encodes mammalian zinc finger transcription factors expressed both in mature T and B lymphocytes and natural killer cells, and in their earliest progenitors, demonstrate that this gene is required for development of all lymphoid lineages in the mouse (Georgopoulos et al., 1992, 1994).

In this study, we have shown that the expression of RU49 in the cerebellum precisely marks the cerebellar granule cell lineage from its birth at the rhombic lip, during its migration beneath the pial surface to form the external germinal layer and throughout the complex differentiation program of this cell type that culminates in terminal differentiation of granule cells within the internal granular layer. While several other transcription factors are known to be expressed in cerebellar granule cells, in no case has the pattern of expression been restricted to granule neurons. For example, Frantz et al. (1994) have reported that the expression patterns of *Otx1* and *Otx2* in developing cerebellar granule cells suggest a role for these factors in regionalization of the cerebellar cortex. Furthermore, Aruga et al. (1994) have demonstrated that the mammalian Zn^{2+} finger transcription factor *zic* is expressed at high levels in cerebellar granule cells. However, in both of these cases these factors are widely expressed outside the granule cell lineage, suggesting that their functions are not restricted to granule neurons.

Our previous studies of gene expression during granule cell differentiation have revealed several stages in this process that are distinguishable by changing patterns of gene expression (Kuhar et al., 1993). It is important to note that expression of RU49 does not correlate with any particular stage in granule cell differentiation and thus is not likely to play a role in a specific subprogram of granule cell differentiation. Rather, one might expect RU49 to participate in the transcription of genes that are fundamental to the identity of the granule neuron. We have attempted to identify putative RU49 target genes by searching Genbank for optimal RU49-binding sites. These efforts have so far failed to reveal cellular genes as putative targets for RU49.

A common genetic mechanism for specification of cerebellar, hippocampal and olfactory granule neurons?

One of the most provocative results obtained in this study is the demonstration that RU49 is expressed in the granule cell lineages of the olfactory bulb and dentate gyrus, in addition to its expression in the cerebellum. Classical studies of these three cell types in the adult brain clearly demonstrate that they are quite different. Hence, the arrangement of dendrites and axons on each of these cell types is highly characteristic of the brain structure in which they are found (Ramon y Cajal, 1911), and more recent physiologic studies have clearly demonstrated their distinct properties. For example, cerebellar granule cells relay mossy fiber afferents to the cerebellar cortex and excite all other cerebellar cell types using glutamate as a neurotransmitter (reviewed by Ito, 1984). In contrast, olfactory granule cells use GABA as a neurotransmitter and provide inhibitory input to both mitral and tufted cells in the olfactory bulb (Ribak et al., 1977; Quinn and Cagan, 1981). Finally, as discussed above, these cell types arise at very different locations in the developing brain. Thus, neither anatomical nor physiological considerations of fully mature granule cells in these three brain regions would lead one to predict common genetic mechanisms for specification of these cell types.

In spite of these differences, as noted principally by Altman (1969a,b), granule cells of the cerebellum, olfactory bulb and dentate gyrus do share many properties that may point to a common developmental origin. Firstly, each of these granule cell types is generated in a secondary germinal matrix that remains mitotically active well into the postnatal period.

Secondly, each of these cell types must first migrate as a proliferating precursor population in the absence of radial glial guides prior to their subsequent differentiation and migration in close apposition to glia. For both cerebellar and olfactory granule cells, this migration covers very long distances from their site of birth to their final location in the adult structure and occurs through the agency of sialic-acid-modified N-CAM (Tomasiewicz et al., 1993). Thirdly, each of these cell populations differentiate into microneurons with exceedingly small soma, in which the nucleus occupies essentially the entire volume of the soma and the nuclear chromatin is highly condensed (Ramon y Cajal, 1911). Fourthly, each of these cell types eventually forms an extremely tightly packed cellular layer in a phylogenetically ancient cortical structure of the brain. As noted by Altman (1969b), "This *tight packing* of neurons in *these conspicuously laminated cortices* stands in sharp contrast to the *loose packing* of neurons in the neocortex and allocortex". Finally, we have noted that several of the genes that mark specific stages of cerebellar granule cell differentiation (Kuhar et al., 1993) are expressed in the olfactory granule cell lineage at corresponding points in its differentiation (data not shown). Given these considerations, and the shared expression of RU49 in these three neuronal lineages, one might propose that the developmental mechanisms responsible for the generation of these cell types are similar. It should also be noted RU49 expression in the Islands of Calleja would be consistent with this idea, because it is thought that these cells may also arise from the subventricular zone of the lateral ventrical (Meyer et al., 1989).

A definitive demonstration that RU49 plays an important role in specification of these three granule cell lineages will require genetic ablation of its function in the developing embryo. Given the high conservation of this transcription factor across vertebrate evolution (data not shown), these studies could be pursued in several different systems. A demonstration that RU49 is critical for granule cell function, coupled with the present demonstration that RU49 interacts with a well defined consensus DNA-binding site, should allow the initiation of efforts to identify RU49 target genes and their roles in granule cell development.

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