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Proto-oncogene c-*myc* is expressed in cerebellar neurons at different developmental stages

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During post-natal cerebellar development the steady-state levels of c-*myc* transcripts exhibit characteristic changes. As determined by the S1 nuclease protection assay the level of c-*myc* transcript, which is very high in the late embryonic cerebellum, decreased to low levels shortly after birth. One week later there is a second period of c-*myc* mRNA accumulation followed by a marked decline to finally reach the low adult value. The second peak of high c-*myc* mRNA level correlates well with the proliferation of granule cell precursors, and it is characterized by a marked change in the ratio of the two types of transcripts started at the known c-*myc* promoters 1 and 2. This indicates a change in the cell population involved in the transcription of the c-*myc* gene. *In situ* hybridization shows transiently elevated c-*myc* mRNA levels in neurons of the cerebellar cortex. At post-natal days 3 and 10 (P3 and P10) c-*myc* transcripts are detectable in the superficial external granular layer composed primarily of mitotically active (neural precursor) cells. Purkinje cell somata show cytoplasmic label at P10. These large postmitotic neurons undergo rapid differentiation at this developmental stage. In the adult cerebellum the low c-*myc* mRNA level is apparently due to Purkinje cells with barely detectable amounts of c-*myc* transcripts. The vast majority of mature cerebellar neurons, the internal granule cells, have no specific hybridization signal for c-*myc*. We conclude that neurons *in vivo* can accumulate c-*myc* messenger during proliferation and/or differentiation, perhaps as a cellular response to an external signal.

Key words: cerebellum/c-*myc*/differentiation/oncogene/proliferation

Introduction

The c-*myc* product, a protein with DNA-binding capacity (Abrams *et al.*, 1982; Bunte *et al.*, 1982; Donner *et al.*, 1982), has been postulated to act as a regulated competence factor necessary to allow the cell to enter the proliferative state of the cell cycle (Kaczmarek, 1985), although in rapidly dividing cells it may be expressed at high or low levels, independently from the cell cycle (Hann *et al.*, 1985; Rabbitts *et al.*, 1985; Thompson *et al.*, 1985). In both immortal cells (Campisi *et al.*, 1984; Lachman and Skoultchi, 1984; McCormack *et al.*, 1984; Leder *et al.*, 1983; Taub *et al.*, 1984) and normal tissues the c-*myc* gene is expressed, although often at a low level (Kelly *et al.*, 1983; Pfeifer-Ohlsson *et al.*, 1984; Stewart *et al.*, 1984; Gonda *et al.*, 1982). Recent data indicate that elevated c-*myc* levels are not necessarily related to cell proliferation. Cells may divide without apparent increase of c-*myc* expression (Pfeifer-Ohlsson *et al.*, 1985; Stewart *et al.*, 1984) and equal amounts of c-*myc* RNA occur in dividing and non-dividing cells of the same type (Hann *et al.*, 1985). Further, elevation of c-*myc* transcript levels may be caused by differentiation stimuli (Curran and Morgan, 1985; Lachman and Skoultchi, 1984).

The murine cerebellum provides a useful model system for the investigation of molecular parameters of cell division and differentiation during neurodevelopment of the mammalian CNS. Shortly after birth external granule cells undergo a phase of proliferation before they migrate inwards, thereby passing the Purkinje cell layer (for general citations see Jacobson, 1978). During this time Purkinje cells, which ceased their final DNA synthesis before the last week of gestation, rapidly differentiate.

In the post-natal murine cerebellum different types of neurons proliferate (granule cells) and differentiate (Purkinje cells) in a manner well coordinated in time and space. In this study, using an S1 nuclease protection assay and *in situ* hybridization, we determined steady-state levels and the promoter usage of c-*myc* transcripts as well as the cellular localization of c-*myc* transcripts during cerebellar development. Our analysis reveals that the time and sites of transiently elevated c-*myc* mRNA levels in the cerebellum are well correlated with the appearance of these developmental events.

Results

Cerebella of normal C57BL/6 mice were collected at various developmental ages. Their total RNA was extracted and samples of it were hybridized with the untranslated (Persson *et al.*, 1984) exon I of the mouse c-*myc* gene (Kelly *et al.*, 1983) as probe and with β_2-microglobulin (β_2m) as an internal control probe (Figure 1B) (Kelly *et al.*, 1983; Parnes and Seidman, 1982). Hybridized material was then submitted to an S1 nuclease protection assay (Kelly *et al.*, 1983; Stewart *et al.*, 1984; Battey *et al.*, 1983; Leder *et al.*, 1983). This assay reveals mainly three sizes of protected probes (Figure 1A): (i) a fragment (203 nucleotides) which can be assigned to β_2m-mRNA (see Figure 1B) and (ii) two fragments of the c-*myc* probe which correspond to the long transcript of 488 nucleotides initiated at the distal promoter 1 and to the short transcript of 355 nucleotides initiated at the proximal promoter 2, respectively. In control experiments after replacing cerebellar RNA by purified tRNA no fragments of the probes were protected (not shown). Figure 1 indicates marked differences of the three RNA species with respect to different developmental times. Using RNA from adult cerebella only traces of the long transcript were detectable. Increasing amounts of labelled single-stranded probe did not increase the intensity of the autoradiographic signals (data not shown). To exclude the possibility that altered c-*myc* levels are only due to altered levels of *all mRNAs*, we used β_2m as an internal control probe. While steady-state levels of c-*myc* transcripts decrease, the level of β_2m mRNA increased >15-fold (Figure 2).

The bands on autoradiograms, representing the size-separated radioactive DNA probes protected against S1 digestion, were

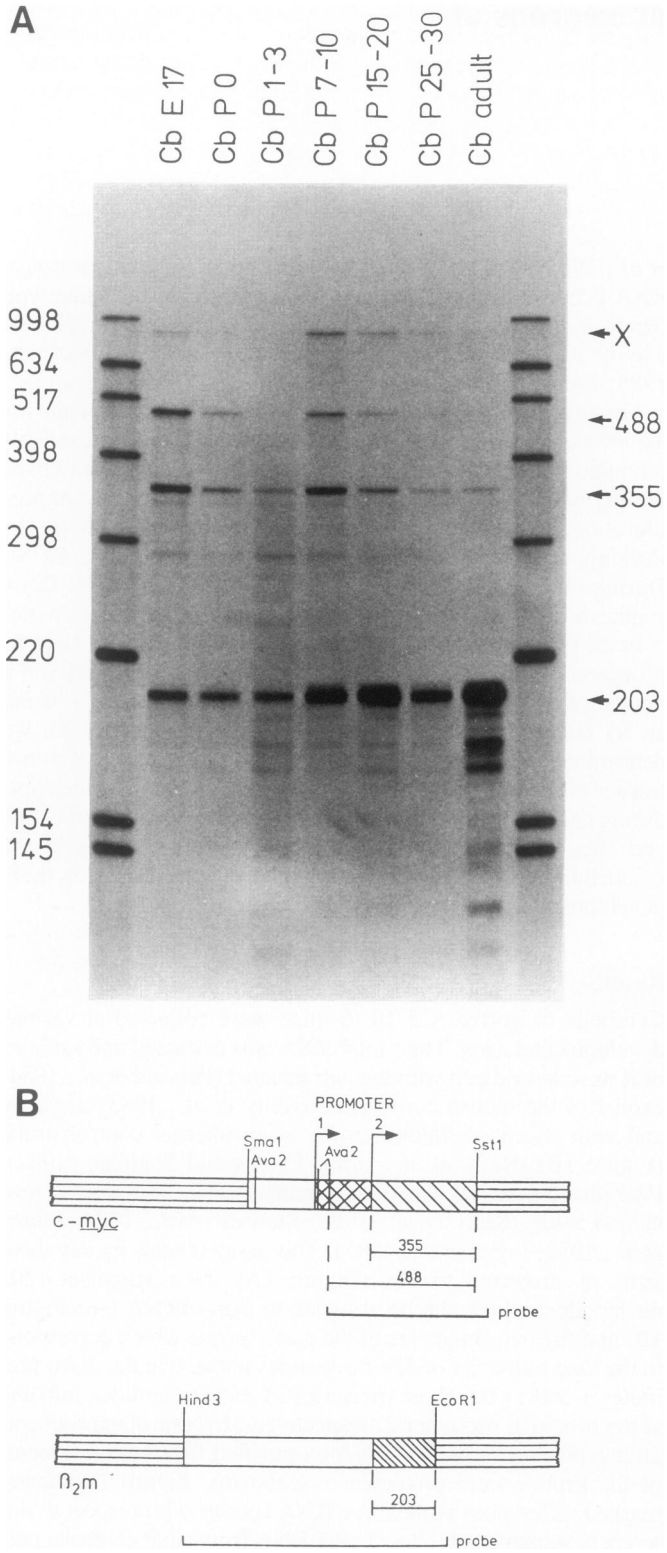

Fig. 1. S1 nuclease protection assay of c-*myc* and β_2-microglobulin (β_2m) probes hybridized to total cerebellar RNA from various developmental stages. As size markers *Eco*RI/*Hin*fI digested pAT153 was applied to either of the flanking lanes. Arrows (**A**) indicate the fragments of the labelled probes protected against nuclease S1 digestion. The band indicated by X probably represents the nuclear precursor RNA encoding β_2m because the single-stranded probe contains part of the first intron and 203 nucleotides of the second exon (Parnes and Seidman, 1982) (**B**). E 17 = embryonic day 17 (appearance of vaginal plug was defined as E0), P = postnatal day, adu = adult (>P100).

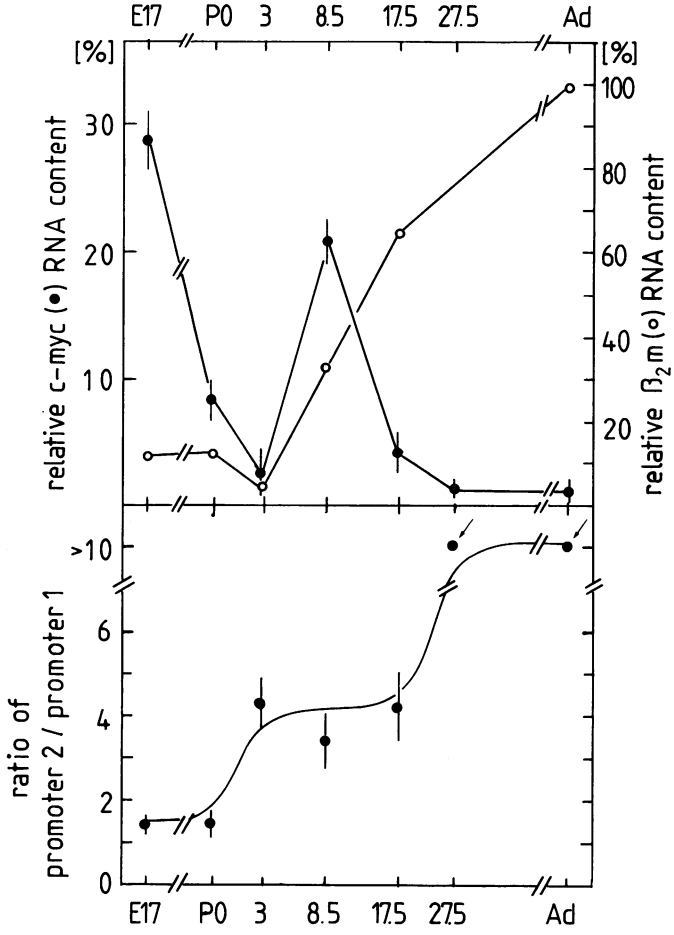

Fig. 2. Steady-state levels of c-*myc* (●) and β_2-microglobulin (β_2m) (○) transcripts (**upper panel**) and promoter usage of c-*myc* transcription (**lower panel**) during cerebellar development. **Upper panel**: the autoradiographs shown in Figure 1A were quantified by densitometric scanning (standardized to 20 μg total RNA). Data are means of at least triplicate experiments (the error tags represent the SD). The value for β_2m in adult cerebellum has been arbitrarily set to 100%. For c-*myc* the sum of the respective quantities for both promoter 1 and 2 transcripts are given. **Lower panel**: the steady-state levels of both c-*myc* transcripts, the long (starting at the distal promoter 1) and the short message (starting at the proximal promoter 2) (see Figure 1), were individually determined. The values are given as means of individual ratios of these two transcripts (the error tags represent the SD). Because of very low amounts of promoter 1-mRNA, the two ratios indicated by arrows can only be defined as >10 (therefore error tags are not given). The age of the donor animals is indicated on the abscissa as a logarithmic scale (E17 = embryonic day 17; P0 = day of birth; pools of post-natal days 1–3 = P3, P7-10 = P8.5 and P15-20 = P18.5, P25-30 = P27.5, Ad = adult = >P100).

measured by densitometry. Figure 2 (upper panel) compares the relative levels of c-*myc* and β_2m transcripts in equal amounts of total cerebellar RNA at different developmental stages. Following a high embryonic level of c-*myc* mRNA the relative amount of this transcript decreased shortly after birth. Between post-natal days 3 and 7 a second increase in c-*myc* mRNAs was observed. At 15–20 days post-natal it dropped to the adult concentration of c-*myc* mRNA which is known to be very low in the brain (Gonda *et al.*, 1982; Stewart *et al.*, 1984). While the percentage of c-*myc* transcripts was diminished the content of β_2m mRNA increased by more than one order of magnitude.

The ratio of c-*myc* transcripts starting at the two promoter sites changed during cerebellar development (Figure 2, lower panel). Both promoters were used almost equally (promoter 2:promoter

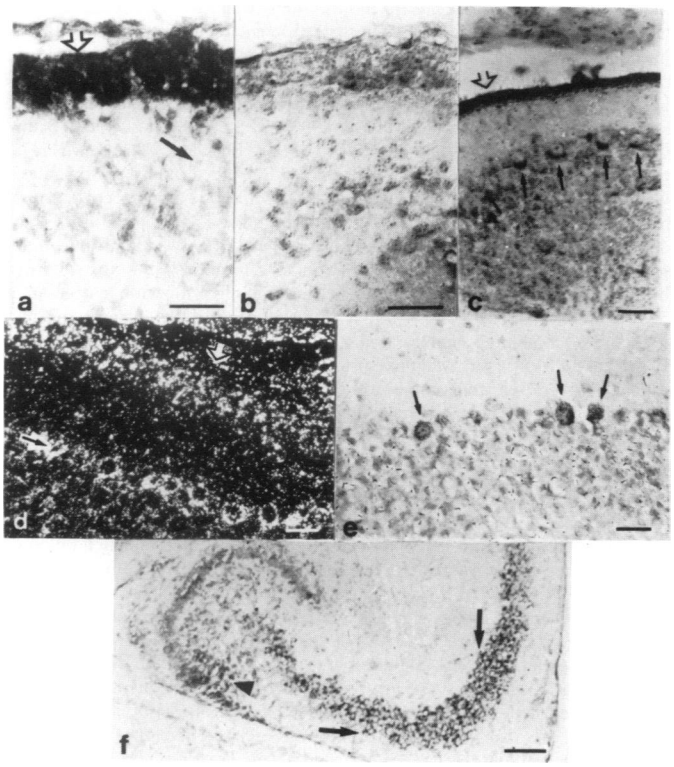

Fig. 3. *In situ* detection of c-*myc* expression during post-natal cerebellar develoment. Biotin-probe used on tissue in **a, b, c, e** and **f**. ^{3}H-probe used on tissue in **d.** (**a**) P3 cerebellum has reaction product associated with the external granule layer (open arrow), which is composed of mitotically active cells (Miale and Sidman, 1961; Fujita *et al.*, 1966; Fujita, 1967), Purkinje cells (arrow) have little if any expression of c-*myc* at this stage of development. (**b**) Adjacent section pre-treated with ribonuclease (see Materials and methods) followed by the normal hybridization protocol. (**c**) P10 cerebellum has reaction product localized over the cells in the germinal matrix zone of the external granule layer (open arrow) but not the mitotically quiescent deeper zone. Hybridization signal is also apparent over the cytoplasm of the differentiating Purkinje cells (arrows). (**d**) Cerebellar section as in **c** (obtained from different brain of the same age; dark field optics). Silver grains are located at the same position as the reaction product in **c** (open arrow marks the matrix zone of the external granule layer; the black arrow indicates the Purkinje cell layer). (**e**) Adult cerebellum has very little signal with c-*myc in situ* hybridization. Some Purkinje cells (arrow) display a light staining for the probe. (**f**) P3 hippocampal formation displays dense staining in the pyramidal cells of regio inferior (arrows) and also some reaction product in the granule cell proliferative region (arrowhead). Scale bars for **a**–**e** = 20 μm; **f** = 100 μm.

1 = 1.5) until the day of birth. Promoter 2 was predominantly used (promoter 2:promoter 1 = 4) at P7–10. In mature cerebellar tissue almost the entire transcription of the c-*myc* gene initiates at the proximal promoter 2.

To localize the sites of c-*myc* mRNA production, *in situ* hybridization studies were carried out, in which the expression of c-*myc* was visualized for distinct cell types at different post-natal stages of the cerebellar development. At P3, the germinal cells of the external granule layer exhibited a specific hybridization signal that was abolished in controls by RNase pre-treatment of brain sections (Figure 3a and b). Later in cerebellar genesis, post-mitotic granule cells residing in the deep external granular layer, traversing the molecular layer, or residing in the internal granule layer did not exhibit a hybridization signal (Figure 3c–e). The Purkinje cells had the reverse pattern of expression. At P10, all Purkinje cells displayed a strong hybridization signal (Figure 3c and d), while in the P3 cerebellum all Purkinje cells were unlabelled (Figure 3a). As shown in Figure 3e the low basic level of c-*myc* transcripts in the mature cerebellum can be almost entirely assigned to some Purkinje cells which still contained detectable amounts of c-*myc* mRNA. Comparable results were obtained in experiments using different methods of probe labelling and visualization (biotin label and streptavidin-alkaline phosphatase in Figure 3a–c, e and f; ^{3}H-label and autoradiography in Figure 3d). Control experiments hybridizing the sections with the opposite single-stranded probe (identical instead of complementary to the messenger) (data not shown) did not show any specific localization of signal. Thus, mitotically active granule cells, but not migrating or post-migratory differentiating granule cells, exhibited a clear specific hybridization signal. The strongest hybridization of c-*myc* in Purkinje cells is coincident with the establishment of a synapse between the abundant granule cell and the Purkinje cell and Purkinje cell dendritic maturation. A similar pattern was found in the developing hippocampal formation where the large, post-mitotic pyramidal cells and the proliferating granule cells exhibited a relatively high expression of c-*myc* (Figure 3f).

Discussion

We have observed that preceding the early post-natal wave of neuronal proliferation the steady-state level of c-*myc* RNA in the cerebellum is ~25-fold elevated compared to the mature tissue (Figures 1 and 2). Similar factors have been reported from stimulated B- (20-fold) and T-cells (10-fold) (Kelly *et al.*, 1983), fibroblasts after growth induction (20- to 40-fold) (Campisi *et al.*, 1984; Kelly *et al.*, 1983; Müller *et al.*, 1984), regenerating liver tissue (10- to 15-fold) (Makino *et al.*, 1984) and placental development (20- to 30-fold) (Pfeifer-Ohlsson *et al.*, 1984). Although we did not find an internal standard clone for a messenger invariably expressed during neonatal cerebellar development, the accumulation of c-*myc* mRNAs is likely to represent expression in two distinct neuronal cell populations rather than being due to general changes in the poly(A)$^+$ mRNA content of total cerebellar RNA for the following reasons: (i) The changes of the β_2m transcript have a different time pattern as compared to c-*myc*. At both periods when the c-*myc* mRNA level decreases (E17-P3 and after P10) the β_2m level is steady or increases, respectively. (ii) The quantitative data obtained by the S1 protection assay have been confirmed by *in situ* hybridization. (iii) The alterations of the c-*myc* mRNA level are accompanied by changes of promoter usage. One should take into account that even a 10- to 40-fold induction of c-*myc* in a given cell type does not change the promoter usage (Kelly *et al.*, 1983); that different cell lines and tissues seem to have specific individual ratios of long and short c-*myc* mRNAs (Kelly *et al.*, 1983; Stewart *et al.*, 1984), and that neuroblastoma cells and cell hybrids of neuroblastoma and primary cerebella neurons exhibit significantly different promoter ratios (Ruppert and Wille, in preparation). It is therefore unlikely that the alterations in the steady-state levels of c-*myc* messenger were due to general changes in the mRNA-to-total RNA ratio. Combining the results of S1 nuclease assay and *in situ* hybridization, we interpret the three different promoter usage ratios (Figure 2, lower panel) as indicative of c-*myc* production in three distinguishable cell populations: embryonic cells; the mixed neonatal populations of external granule precursor cells and Purkinje cells; and the Purkinje cell population in the adult cerebellum.

The early elevated level of c-*myc* transcripts appears to be related to cell proliferation. In fact, *in situ* hybridizations (Figure

4a, c, d) confirm the quantitative data in localizing the c-*myc* RNA in the germinal matrix zone of the external granule cell layer, which contains mitotically active cells (Fujita, 1967; Fujita *et al.*, 1966; Miale and Sidman, 1961). Analogous *in situ* hybridization studies have been published for developing placenta and early embryogenesis (Pfeifer-Ohlsson *et al.*, 1984, 1985).

On the other hand, induction of c-*myc* has been demonstrated *in vitro* in cells stimulated towards differentiation. Thus, erythroleukaemia cells (Lachman and Skoultchi, 1984) and the neuronal pheochromocytoma cells PC12 (Curran and Morgan, 1985) raise their c-*myc* RNA levels for a temporary period after stimulation by dimethylsulphoxide and NGF, respectively. A similar early increase preceding the 10-fold suppression of the c-*myc* mRNA level has been observed after receptor-mediated inhibition of growth in a B-cell lymphoma (McCormack *et al.*, 1984). Purkinje cells have ceased their final DNA synthesis by embryonic day 13 and no post-natal [^{3}H]thymidine incorporation has been observed in these neurons (Miale and Sidman, 1961). However, the elevated c-*myc* RNA levels in total cerebellar RNA at P7-10 are evidently also due to high levels in Purkinje cells. This is a time period of active development and differentiation of the impressive Purkinje cell dendritic arbor (for review see Jacobson, 1978). We have thereby demonstrated that there are postmitotic, differentiating cells *in vivo* which contain transient high c-*myc* mRNA levels.

In analogy to other systems it can be suggested that the high steady-state level of c-*myc* transcripts in normal cells is a common cellular consequence of exogenous or autocrine stimulation. Depending on the developmental state of the stimulated target cell, the c-*myc* gene product may interact with DNA to make the cell competent for receiving further signals (Kaczmarek *et al.*, 1985) either progressing them towards cell division (Kelly *et al.*, 1983; Smeland *et al.*, 1985; Rabbitts *et al.*, 1985) or preparing the cells for terminal differentiation (Lachman and Skoultchi, 1984).

These findings may be speculatively interpreted in the light of data from other systems to suggest that granule and Purkinje cells are targets for specific stimuli inducing, among other cellular responses, c-*myc* expression. Granule cells may receive a signal from other neural cells of the cerebellum or stimulate themselves in an autocrine fashion (Goustin *et al.*, 1985) as postulated for neuroblastoma (van Zoelen *et al.*, 1984). Wetts and Herrup (1983) hold the Purkinje cells responsible for the number of granule cells. For Purkinje cells it has been speculated that migrating granule cells may be a source of such a differentiation signal (citations in Jacobson, 1978) although the nature of this hypothetical stimulus is still unknown. Nevertheless, we believe that c-*myc* and other oncogene probes will provide a powerful tool for future investigations of signaling pathways (Heldin and Westermark, 1984; Weinberg, 1985) involved in brain development.

Materials and methods

S1 nuclease protection assay

Uniformly labeled single-stranded DNA probes were prepared (Ley *et al.*, 1982). The primer-extended *Sma*I–*Sst*I subclone M13-38 of the murine c-*myc* gene in M13mp11 (B) (gift from Dr L.Hennighausen and Dr K.Kelly) (Stewart *et al.*, 1984) was digested with *Ava*II generating five fragments. The 521-bp fragment was used for the assay. This probe contains 488 bp of the c-*myc* exon I sequence and 33 bp of vector sequence. The *Eco*RI–*Hin*dIII mouse β_2-microglobulin subclone in M13mp8 (Kelly *et al.*, 1983; Parnes and Seidman, 1982) was digested with *Hin*dIII which releases a fragment of ~870 bp (B). The single-stranded fragments were separated on 5% polyacrylamide gels (8 M urea). Cut out gel slices were re-run on 1% agarose gels, transferred electrophoretically on NA45 DEAE-cellulose membranes (Schleicher and Schüll, FRG) and eluted from these filters (Lizardi *et al.*, 1984). Total RNA has been extracted from pooled cerebella dissected from C57BL/6J wild-type mice of different age-groups (E17, P0, P1-3, P3, P7-10, P15-20, P25-30, >P100) by a modified CsCl method (Kaplan *et al.*, 1979). Steady-state levels of transcripts (the long and short c-*myc* mRNAs starting at promoter 1 and 2, respectively, and β_2m mRNA) were determined using a modified S1 nuclease assay (Kelly *et al.*, 1983; Stewart *et al.*, 1984; Battey *et al.*, 1983; Leder *et al.*, 1983; Taub *et al.*, 1984; Berk and Sharp, 1977). The radioactive probes were used to assay 20 μg total RNA (determined photometrically) from each age group of pooled tissues. Hybridization reactions contained an excess of ^{32}P-labelled probe (1.5×10^4 c.p.m. sp.act. 10^8 c.p.m./μg), 20 μg total RNA, 75% formamide, 400 mM NaCl, 1 mM EDTA and 20 mM Tris-HCl (pH 7.4) (Battey *et al.*, 1983). The probes for c-*myc* and β_2m were hybridized for 18 h in separate tubes according to their different hybridization temperatures (58°C for c-*myc* and 51°C for β_2m). Hybridizations were terminated by placing tubes on ice, combining samples, and subsequently digesting them with 1500 U S1 nuclease (Boehringer, Mannheim) for 2 h at 37°C. After phenol extraction and ethanol reprecipitation samples were separated electrophoretically on 0.4 mm thick 5% denaturing polyacrylamide gels (8 M urea). Gels were exposed to X-ray film for 11 days at −70°C in presence of intensifying screens.

In situ hybridization

Mice were perfused with saline followed by a mixture of 75% ethanol and 25% glacial acetic acid. Brains were removed, dehydrated in alcohol, cleared in xylene and embedded in paraffin. Sections 4 μm thick were cut and mounted on poly-L-lysine coated slides. Deparaffinized sections of tissue were pre-hybridized at 40°C for 1 h with hybridization solution (2.5 × SSC, 30% formamide, 1 × Denhardt's, 250 μg/ml salmon sperm DNA and 100 μg/ml poly-U) without probe but containing 5 μg/ml M13 DNA (BRL). Tissue sections were rinsed in ascending alcohol solutions, air dried and hybridized at 40°C overnight with 50–100 ng labelled DNA probe in 10–20 μl hybridization solution. Probes were nick translated with either biotinylated dUTP (Enzo) of [^{3}H]dCTP and dTTP (Amersham). Also single-stranded probes (as described above) of both strands uniformly labelled with ^{3}H- and ^{35}S-nucleotides were used for hybridization. Only the strand complementary to the mRNA displayed hybridization patterns identical to nick-translated probes. Probes were typically labelled to an equivalent spec. act. of $\sim 1 \times 10^7$ c.p.m./μg DNA. Labelled probes were separated from free nucleotides in a G-50 column. For control experiments sections were pretreated with 100 μg/ml ribonuclease (Sigma) at 37°C for 1.5 h. After hybridization, tissue sections were rinsed twice in 2 × SSC, and 1 × SSC at 40°C and twice in 0.1 × SSC at room temperature. Slides were finally rinsed in H_2O prior to detection. Tissue exposed to biotinylated c-*myc*-DNA was reacted with Enzo Detek I-alk signal generating system to yield a blue reaction product at sites of hybridization. Tissue that was hybridized with a ^{3}H- or ^{35}S-probe was coated with Kodak NTB-2 emulsion, exposed for 3 weeks at 4°C and developed with Kodak D-19 at 16°C for 4 min. Clusters of silver grains that were twice or more than background grain density were presumed to be sites of specific c-*myc* hybridization.

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