Volume 336, number 1, 8–12 FEBS 13363 December 1993

Induction of stathmin mRNA during liver regeneration

Takashi Okazaki, Toshiyuki Himi**, Christine Peterson, Nozomu Mori*

Division of Neurogerontology, Andrus Gerontology Center, University of Southern California, Los Angeles, CA 90089-0191, USA

Received 21 October 1993

Stathmin is a 19 kDa phosphoprotein, and is proposed to play a role in signal transduction in response to various extracellular stimuli that promote cellular growth and/or differentiation. We examined stathmin mRNA expression during development and liver regeneration in mice. Stathmin mRNA expression declined during the post-natal period and was undetected in adult liver. 36 h after partial hepatectomy, stathmin mRNA was rapidly induced and remained at elevated levels for at least 10 days. In situ hybridization experiments confirmed that stathmin mRNA expression occurred in hepatocytes. These results indicate that the stathmin gene expression appears to be repressed during the post-natal liver development, and is de-repressed by liver regeneration, which suggests that stathmin may be a good molecular marker of liver plasticity.

Hepatic growth; Proliferation; In situ hybridization; Gene expression; Growth-associated protein; p19; Oncoprotein 18; Plasticity

1. INTRODUCTION

Stathmin (also designated p19, oncoprotein 18, pp17, pp20, prosolin, 19 K, and leukemia-associated phosphoprotein, Lap18) is a 19 kDa cytoplasmic phosphoprotein the phosphorylation of which is tightly correlated with the stimulation of cellular growth and differentiation [1–6]. It has been proposed that stathmin plays a general role as an intracellular signal-relay molecule integrating diverse signals regulating cellular proliferation and differentiation [1,2]. Stathmin is a member of a gene family [7–9] that includes a neuronal-specific isoform, SCG10 [10]. In contrast to neural-restricted SCG10 expression, stathmin expression is ubiquitous and its protein and mRNA are found in a wide variety of tissues during development and adult life [3,4,9], but are not detected in mouse, rat [3,4] or *Xenopus* [8] liver.

The mature liver is a post-mitotic tissue, and liver proliferation can be induced by surgical or chemical treatments that damage a large percentage of hepatic parenchyma [11]. Parenchyma loss rapidly induces cellular proliferation in an effort to restore liver mass. Since stathmin expression is associated with cellular proliferation [12–14], we hypothesized that stathmin may play a role during liver regeneration.

We show here that the stathmin gene is apparently repressed in the adult liver, but is de-repressed when parenchymal cell growth was induced by partial hepatectomy. The stathmin mRNA upregulation during liver regeneration supports the hypothesis that stathmin is a growth-associated protein. Our results also indicate that liver regeneration provides a model to study the mechanisms of plastic genetic response of stathmin and its role(s) in cellular proliferation.

*Corresponding author. Fax: (1) (213) 740-8241.

***Present address:* Department of Biochemistry, Tokyo Medical and Dental University, Yushima, Bunkyo-ku, Tokyo 113, Japan.

2. MATERIALS AND METHODS

2.1. *Animals and partial hepatectomy*

The C57BL mice were used in both development and liver regeneration experiments. They were fed ad libitum under a 12 h/12 h light–dark cycle. Embryos and pups were sacrificed at E15, E19, P0, P4, P8, P14, P25 ($n \geq 2$). Three month-old mice were used as adult controls. Partial hepatectomy and sham operations were performed as described for rats [11] on male and female C57BL mice (age: 3–6 months old). The mice were ether anesthetized and subjected to midventral laparotomy with approximately 70% liver resection (left lateral and median lobes). Mice were sacrificed at 1, 3, 6, 18, 24, 36, 48 h and 2, 4, 6, 10 days ($n = 2$) post-operation. The liver was excised, cut into several pieces, and quickly frozen in dry ice–ethanol. Half of the tissue was used for RNA isolation, and half for in situ hybridization.

2.2. *RNase protection assay and Northern blot hybridization*

RNase protection was performed as previously described [15] using ^{32}P-labeled RNA probes. For quantitation of stathmin mRNA in mouse tissues, a *Hin*dIII-truncated probe was generated using the pCP724 subclone that contained a portion of the second intron and the third exon [7]. Control probes were generated by truncation of a mouse phosphoglycerate kinase (PGK) cDNA [16] and a human γ-actin cDNA (see [15]) with *Sau*3A and *Hin*fI, respectively. All antisense probes were synthesized with SP6 RNA polymerase in the presence of 800 Ci/mmol [^{32}P]UTP (DuPont/NEN). Full-length probes were purified by polyacrylamide gel electrophoresis. Hybridization with 10 μg of total cellular RNA was carried out at 50°C in the presence of 10 μg of *Escherichia coli* tRNA as carrier in a 30 μl reaction volume containing 80% formamide and 40 mM PIPES buffer (pH 6.4). After 12 h of incubation, digestion was carried out with RNase T1 (BRL). Protected fragments were resolved on 4% polyacrylamide sequencing gels containing 7.5 M urea. Radiolabeled size markers were generated by the end-labeling of *Eco*RI–*Hin*fI double digest of pBR322 using Klenow fragment in the presence of [^{35}S]ATP (800

 Published by Elsevier Science Publishers B.V.

Ci/mmol, DuPont/NEN). Intensities of the protected fragments were quantitated on autoradiograms with a densitometer (Bio-Rad, model GS-670). Northern blot hybridization was performed as described previously [18]. c-*fos* cDNA was a gift from Dr. P. Vogt, and the insert was subcloned into pGEM3. A full-length tumor growth factor TGFβ1 clone, which was a gift from Dr. N. Nicols, was cut and a portion of unique region (nucleotide residues 617–1,345) was subcloned intto pGEM3.

2.3. *In situ hybridization*

Whole bodies of embryo and pups were frozen in powdered dry ice. Whole body (15 μm) and liver sections (13 μm) were cut on a cryostat (Hacker Instruments), then mounted onto gelatin coated slides, and stored at −80°C until use. The sections were post-fixed, acetylated, dehydrated, and hybridized as previously described [17,18]. Sections were hybridized with ^{35}S-labeled sense or antisense cRNA probe. Photographs were taken using the X-ray film image as a negative, and emulsion-dipped slides.

3. RESULTS

3.1. *Stathmin mRNA expression in the liver of embryonic and prenatal animals*

A representative expression profile of stathmin mRNA as assayed by in situ hybridization in whole body sections at post-natal day 5 (P5) mouse is presented as Fig. 1. Stathmin mRNA is abundant in nervous tissues including brain, spinal cord, as well as peripheral ganglia, e.g. dorsal root ganglia. In situ hybridization with stathmin sense probe showed negligible signals (not shown). Stathmin mRNA expression was high in neural tissues from E15 and E19 embryos (data not shown), and peaked between late gestation to early post-natal days in the rat [19]. Other non-neuronal tissues also expressed significant amounts of this mRNA during these periods. Notably, developing thymus expressed very high levels of stathmin mRNA, while embryonic (not shown) and post-natal (see Fig. 1) liver expressed moderate levels.

3.2. *Apparent repression of stathmin mRNA expression in the adult liver*

Stathmin mRNA is expressed in embryonic and early post-natal mouse liver but is not detected in the adult liver (Fig. 2). We carefully determined the expression levels of stathmin in mouse tissues and cells by RNase protection assays. Using a 420 nt-long probe which contains a part of intron 2 and exon 3, a 170 nt-long RNA was protected in brain, testis, heart, spleen and kidney,

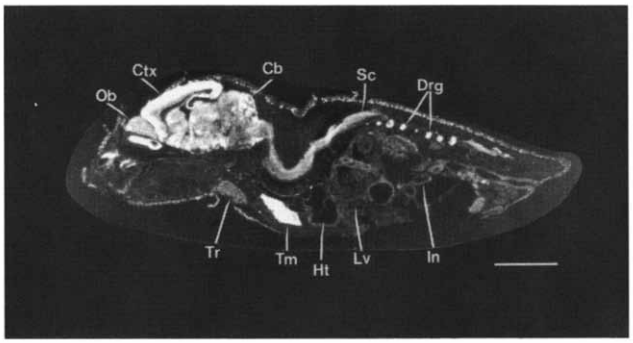

Fig. 1. Stathmin mRNA expression during development. Shown is an in situ hybridization image of stathmin mRNA in a post-natal day 5 mouse whole mount senction. Note strong signals are seen in neural tissues, i.e. olfactory bulb (Ob), cerebral cortex (Ctx), cerebellum (Cb), spinal cord (Sp) and dosal root ganglia (Drg), as well as thymus (Tm), while low levels of signals are seen in other tissues including thyroid (Tr), heart (Ht), intestine (In), and liver (Lv). Bar = 5 mm.

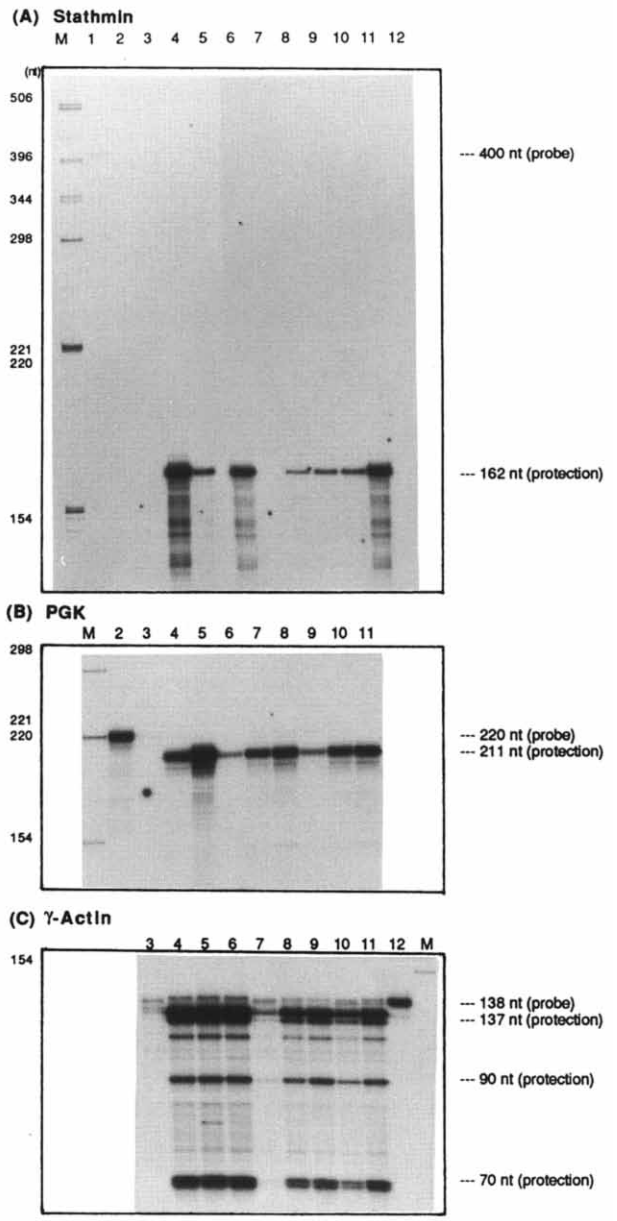

Fig. 2. Stathmin mRNA expression in adult tissues. Probes used for the three sets of experiments: (A) p19/stathmin, (B) PGK, and (C) γ-actin, were run unhybridized in lanes 1, 2, and 12, respectively. RNase protection experiments were carried out with carrier tRNA only (lane 3), or RNAs from cells and tissues: C1300 cells (lane 4), NIH-3T3 cells (lane 5), testis (lane 6), liver (lane 7), kidney (lane 8), spleen (lane 9), heart (lane 10), and brain (lane 11). Sizes of the probes and protected fragments are given at the right side of each panal. Note that the sizes of protected fragments are slightly larger than the corresponding sequences because only RNase T1 was used to digest unhybridized cRNA. Size markers were ^{35}S-labeled *Eco*RI–*Hin*fI double digests of pBR322.

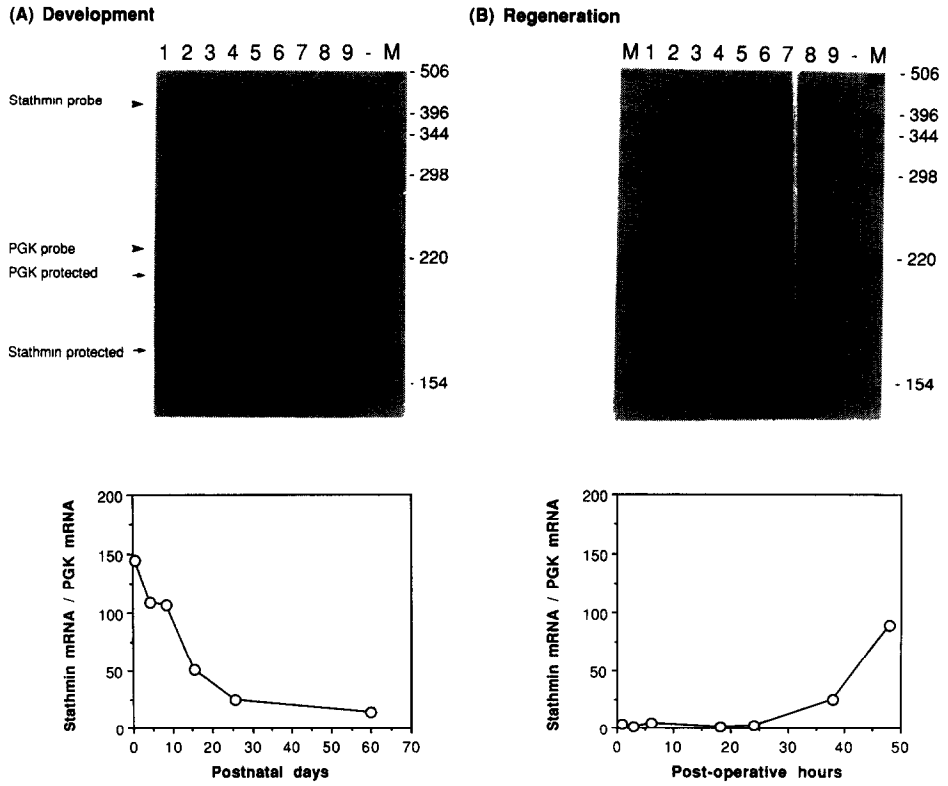

Fig. 3. Repression of stathmin mRNA expression during post-natal development and de-repression during liver regeneration. Upper and lower panels show RNase protection profiles of stathmin and phosphoglycerate kinase (PGK) mRNAs and quantitation of stathmin mRNA levels compared with the PGK mRNA levels. (A) Stathmin mRNA levels decrease during post-natal development. Lane 1, tRNA only; lanes 2–7, mouse liver RNA from post-natal day 0, 4, 8, 14, 25 and 60; lanes 8 and 9, probe only (no hybridization nor digestion). (B) Stathmin mRNA is induced during liver regeneration. Lane 1, control mouse liver RNA; lanes 2–7, liver RNA from 1, 3, 6, 18, 24, 36, 48 h post-operation; lanes 8 and 9, probe only (no hybridization nor digestion). Size markers are as indicated in Fig. 2.

but not liver total RNA (Fig. 2A). Since the RNase protection assay is highly sensitive and discriminates cross-hybridization with other RNAs, including rRNAs, these results strongly suggest that the normal adult liver does not express stathmin mRNA. Quantitation by laser densitometory indicated that the brain and testis expressed at least 4-fold more transcripts than other moderately expressing tissues, such as heart, spleen and kidney (data not shown). Similarly, the mouse neuroblastoma C1300 expressed 4-fold more transcripts than NIH-3T3 fibroblasts (Fig. 2A). Transcripts of phosphoglycerate kinase (PGK) and γ-actin, used as controls, were more evenly distributed among the various tissues and cells examined (Fig. 2B,C). These results indicate that the stathmin gene is actively transcribed during development, but its expression ceases or may become repressed at some time during post-natal development.

To determine when stathmin mRNA expression declined during post-natal development, we measured the mRNA levels by the RNase protection assay (Fig. 3A). Stathmin mRNA diminished gradually during this period and decreased by more than 90% by P25 when compared to the newborn level. Thus, stathmin mRNA expression ceased by about 1 month after birth, which corresponds to hepatocytes becoming post-mitotic. This suggests that stathmin expression correlates with maturation, i.e. completion of differentiation or proliferation.

3.3. *De-repression of stathmin gene expression during liver regeneration*

Since stathmin expression is commonly observed in rapidly growing tissues and cells, i.e. developing brain, thymus (see Fig. 1), cytokine-induced T-cells, and tumor cells [12–14], we determined whether stathmin mRNA expression can be induced in partially hepatectomized mice. We measured stathmin mRNA levels in the liver at various times after surgery. By 24 h stathmin mRNA was undetectable, however, it rapidly increased by 2–3 days post-operation to the level of newborn animals (Fig. 3B). Northern blot hybridization experiments showed that the sizes of the induced mRNA was the same as that in newborn liver and brain (i.e. 1 kb and 3 kb) (data not shown). In situ hybridization experiments revealed that the stathmin mRNA was induced in hepatocytes (Fig. 4). Only background levels of the mRNA encoding SCG10, a neural-specific isoform of stathmin, were detected in either control or regenerating liver (data not shown).

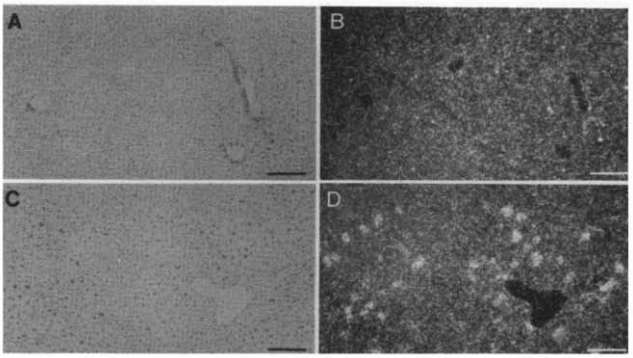

Fig. 4. Stathmin mRNA is induced in regenerating hepatocytes. Shown are sections hybridized with stathmin antisense probe; liver sections of control mouse (A,B) and day 6 after partial hepatectomy (C,D). Photomicrographs were taken under bright-field (A,C) and dark-field (B,D) illumination. Bars = 70 μm.

Stathmin mRNA induction in regenerating liver occurred 24 h after partial hepatectomy. The kinetics of this induction is rather slow in comparison to other genes that are involved in liver regeneration. For example, the mRNA of the so-called immediate-early gene [20], c-*fos*, was transiently up-regulated by 1 h post-surgery (data not shown). Messenger RNA encoding TGFβ1 that acts on differentiation and regeneration in a variety of tissues [21,22] and is involved in repressing DNA synthesis in regenerating liver [23], appeared at low but significant levels 6 days after surgery (data not shown). Thus, stathmin appears to be a slow-responding gene but may not be a late-responding gene.

4. DISCUSSION

Our results demonstrate that the mRNA encoding the 19 kDa phosphoprotein, stathmin, is induced in adult regenerating liver [1,2]. The induction is not an immediate response but correlates with the growth and proliferation of hepatocytes. Stathmin mRNA induction in regenerating liver serves as an excellent example of the correlation of its expression and cellular proliferation [1,12–14]. Stathmin may not be involved in the maturation of liver cell function, since its mRNA induction decreases by day 10 post-surgery. Direct correlation of the stathmin gene expression and cellular proliferation suggests that stathmin may be involved in cell cycle control. Recent evidence that stathmin is phosphorylated by MAP kinase and p34cdc2 kinase [24,25] and that it is expressed and phosphorylated in *Xenopus* oocytes [8] favours this idea. It will be of interest to examine whether the stathmin protein levels and the degree of its phosphorylation change during liver regeneration.

The precise mechanism of stathmin gene induction during liver regeneration is unknown. Since stathmin mRNA is undetected in normal adult liver, its induction may involve transcriptional activation. The proximal promoter region (−730 bp) of the mouse stathmin gene can activate transcription in vitro in nuclear extracts prepared from adult mouse liver (T. Okazaki and Y. Nakanishi, unpublished observation), which suggests that a transcriptional silencer(s) may exist further upstream or downstream of the promoter region, as in the case of the SCG10 gene [15,26], the neural-specific homolog of stathmin. Understanding the precise mechanisms of stathmin gene induction during liver regeneration requires further analysis of transcriptional regulation.

Stathmin gene expression is induced by cellular growth stimulation, but it is also induced by certain types of cell death. Our preliminary experiments indicated that stathmin and its mRNA are significantly induced in hippocampal neurons that were treated by colchicine injection [28]. Cellular proliferation and death may therefore share some common mechanisms, and stathmin may locate at one of those processes.

Stathmin may play different or multiple roles in different tissues. For example, stathmin expression occurs at high levels in tissues that contain proliferating cells [4,12,13], but it is also highly expressed in post-mitotic neurons [18,19]. Indeed, the massive expression of stathmin mRNA in developing central and peripheral nervous systems (see Fig. 1) would suggest that stathmin plays a different role(s) in nervous tissues than merely cellular proliferation. It may, for example, work for neuronal growth as SCG10 does in developing neurons, while it may also play a role in signal transduction processes in neurons. In the brain it has been shown that mRNAs encoding SCG10 and stathmin are expressed in different subset neurons. Stathmin is preferentially expressed in short process-bearing neurons, while SCG10 is more abundantly expressed in neurons with long processes, large terminal fields, or extensive dendrites [18]. Thus, stathmin seems to have a role in post-mitotic neurons probably for neuronal differentiation and plasticity, which is independent of the role for cellular proliferation.

The molecular signals controlling liver regeneration are being rapidly defined. Several growth factors, such as epidermal growth factor (EGF), TGFα, acidic fibroblast growth factor (aFGF) and hepatocyte growth factor (HGF), have been defined as mitogens for hepatocyte growth [27]. In contrast to those growth factors, the role of TGFβ1 in inhibiting the growth of parenchymal cells is also of importance for the cessation of liver growth to complete the regeneration [23]. The induction of stathmin mRNA occurs between the transient induction of the immediate-responding genes and the increase in TGFβ1, serving as a new marker of this plastic growth response seen in the liver. It is a future interest to determine signals and factors controlling the stathmin gene induction both in the liver and the brain, which preserve a remarkable plasticity of growth responses.

Acknowledgements: We wish to thank Dr. S. Goto (Toho University, Japan) for providing us with a protocol for hepatectomy, and Gui Xia Yu and Huang Guo for technical assistance. This research was supported in part by grants from NIH (AG07909) and Max Factor Foundation.

REFERENCES

[1] Sobel, A. (1991) Trends Biochem. Sci. 16, 301–305.
[2] Sobel, A., Boutterin, M.C., Beretta, L., Chneiweiss, H., Doye, V. and Peyro-Saint-Paul, H.J. (1989) Biol. Chem. 264, 3765–3772.
[3] Koppel, J., Boutterin, M.-C., Doye, V., Peyro-Saint-Paul, H. and Sobel, A. (1990) J. Biol. Chem. 265, 3703–3707.
[4] Schubart, U.K. (1988) J. Biol. Chem. 263, 12156–12160.
[5] Zhu, X.-X., Kozarsky, K., Strahler, J.R., Eckerskorn, C., Lottspeich, F., Melhem, R., Lowe, J., Fox, D.A., Hanash, S.M. and Atweh, G.F. (1989) J. Biol. Chem. 264, 14556–14560.
[6] Gullberg, M., Noreus, K., Brattsand, G., Friedrich, B. and Shingler, V. (1990) J. Biol. Chem. 265, 17499–17505.
[7] Okazaki, T., Yoshida, B.N., Avraham, K.B., Wang, H., Wuenschell, C.W., Jenkins, N.A., Copeland, N.G., Anderson, D.J. and Mori, N. (1993) Genomics, in press.
[8] Maucuer, A., Moreau, J., Mechali, M. and Sobel, A. (1993) J. Biol. Chem. 268, 16420–16429.
[9] Schubart, U.K., Banerjee, M.D. and Eng, J. (1989) DNA 8, 389–398.
[10] Stein, R., Mori, N., Matthews, K., Lo, L.-C. and Anderson, D.J. (1988) Neuron 1, 463–476.
[11] Higgins, G.M. and Anderson, R.M. (1931) Arch. Pathol. 12, 186–202.
[12] Cooper, H.L., Fuldner, R., McDuffie, E. and Braverman, R. (1991) J. Immunol. 143, 956.
[13] Hanash, S.M., Strahler, J.R., Kuick, R., Chu, E.H.Y. and Nichils, D. (1988) J. Biol. Chem. 263, 12813–12815.
[14] Melhem, R.F., Strahler, J.R., Hailat, N., Zhu, X.-X. and Hanash, S.M. (1991) Biochem. Biophys. Res. Comm. 179, 1649–1655.
[15] Mori, N., Stein, R. and Anderson, D.J. (1990) Neuron 4, 583–594.
[16] Mori, N., Singer-Sam, J., Lee, C.-Y. and Riggs, A.D. (1986) Gene 45, 275–280.
[17] Mori, N., Tajima, Y., Sakaguchi, H., Vandenbergh, D.J., Nawa, H. and Salvaterra, P.M. (1993) Mol. Brain Res. 17, 101–111.
[18] Himi, T., Wang, H., McNeill, T.H. and Mori, N. (1993) Neuroscience, in press.
[19] Amat, J.A., Fields, K.L. and Schubart, U.K. (1991) Dev. Brain Res. 60, 205–218.
[20] Mohn, K.L., Laz, T.M., Hsu, J.C., Melby, A.E., Bravo, R. and Taub, R. (1991) Mol. Cell. Biol. 11, 381–390.
[21] Sporn, M.B. and Roberts, A.B. (1988) Biofactors 1, 89–93.
[22] Finch, C.E., Laping, N.J., Morgan, T.E., Nichols, N.R. and Pasinetti, G.M. (1993) J. Cell. Biochem., in press.
[23] Fausto, N. (1991) Digest. Dis. Sci. 36, 653–658.
[24] Leighton, I., Curmi, P., Campbell, D.G., Cohen, P. and Sobel, A. (1993) Mol. Cell. Biochem., in press.
[25] Beretta, L., Dobransky, T. and Sobel, A. (1993) J. Biol. Chem. 268, in press.
[26] Mori, N., Schoenherr, C., Vandenbergh, D.J. and Anderson, D.J. (1992) Neuron 9, 45–54.
[27] Michalopoulos, G.K. (1990) FASEB J. 4, 176–187.
[28] Mori et al. (1993) Soc. Neurosci. Abstr. 19, 1713.