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Retinoic acid induced differentiated neuroblastoma cells show increased expression of the βA4 amyloid gene of Alzheimer's disease and an altered splicing pattern

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Retinoic acid (RA) induced differentiation of SH-SY5Y neuroblastoma cells is associated with more than a tenfold induction of total Alzheimer's disease βA4 amyloid protein precursor (APP) mRNA as analyzed by Northern blot hybridisation. S1 nuclease protection experiments reveal that the splicing pattern of these differentiated cells is altered in favor of APP_{695} mRNA, coding for the shortest amyloidogenic βA4 amyloid precursor protein. Induction of differentiation of SH-SY5Y cells with NGF leads to a fivefold increase of total APP mRNA without change in the splicing pattern. This suggests that RA but not NGF induces factor(s) which are responsible for an APP hnRNA splicing favoring APP_{695} mRNA.

Alzheimer's disease; βA4 amyloid precursor protein; Alternative splicing; Retinoic acid; Neuroblastoma cell line

1. INTRODUCTION

Alzheimer's disease (AD) is an irreversible neurodegenerative disorder of the human central nervous system [1]. The neuronal dysfunction is correlated with massive deposition of fibrillar aggregates of the βA4 protein in the brain [2,3]. The pathological βA4 peptide consists of 42-43 residues and is synthesized as part of a larger N- and O-glycosylated integral transmembrane precursor protein (APP for *a*myloid *p*recursor *p*rotein) [4-8]. Proteolytic processing of these APP proteins in the Golgi apparatus leads to the secretion of the extracellular part the APP protein isoforms [8]. Five different transcripts of the single gene coding for the APP (PAD gene, [9]) have been identified, which arise by alternative splicing [4-6,10-12,14,15]. The three major transcripts with sizes of 3.4 to 3.6 kb code for precursor proteins with 695,751 and 770 amino acids (APP_{695}, APP_{751} and APP_{770}) [10-12]. The APP_{751}/APP_{770} proteins differ from APP_{695} by one or two additional domains of 56 or 56 and 19 amino acids [12]. Both domains are encoded by two adjacent exons on the PAD gene, namely exon 7 (168 bp) and exon 8 (57 bp) [12,13]. The expression of the PAD gene is ubiquitous, being highest in brain and kidney [10]. *In situ* hybridisations as well as Northern blot analysis of brain and peripheral tissues demonstrated a higher expression of the two longer PAD transcripts in peripheral organs, whereas the short APP_{695} mRNA is the dominant form in neuronal tissues [10,16]. In order to study the expression of the PAD gene during neuronal differentiation we used the neuroblastoma cell line SH-SY5Y, which can be induced to differentiate by nerve growth factor (NGF) and retinoic acid (RA) [17-19].

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2. MATERIALS AND METHODS

2.1. *DNA constructs*

A 1.7 kb EcoRI fragment of a partial APP_{770} cDNA clone, where the 5'-EcoRI restriction site is introduced by the adaptor during construction of the cDNA library, and the 3'-EcoRI site is at position +2020 of APP_{770} cDNA, was subcloned into the pUC19 polylinker, to give the recombinant plasmid p2.21 [20,19]. Plasmid p2.21 was digested with *Xho*I, resulting in a 826 bp fragment, spanning the KPI- and OX-2 domain from position +320 to position +1135, and was subcloned into the phage M13mp18 [22] which resulted in the recombinant phage M8.11 (Fig. 1). The recombinant phage M15.36 was constructed by subcloning of a 682bp *Xho*I/*Sac*I APP_{770} cDNA fragment from position +1135 to position +1817 into the phage M13mp19 (Fig. 1) [22]. The 1687bp DraIII/EcoRI cDNA fragment of the APP_{770} cDNA in p2.21 was subcloned into the respective restriction sites of plasmid pSP65 (Promega) containing the 3048 bp *Sma*I/*Sma*I APP_{695} cDNA (pSP65/695), constituting plasmid pSP65/770.

2.2. *Cell culture methods*

The subclone neuroblastoma cell line SH-SY5Y was grown in equal parts of Minimum Essential Medium (MEM) with Earle's Salts and L-glutamine and Ham's F-12 supplemented with nonessential amino acids (Eagle's formulation), 2.5 mg/ml fungizone, 50 mg/ml gentamycin (all Gibco/BRL) and 10% heat-inactivated fetal calf serum (Boehringer Mannheim). Cells were seeded at a density below 2×10^3 cells/cm^2 (low density) and passaged in log phase at a density of 1 to 3×10^4 cells/cm^2 (medium density). Differentiation was induced by culturing cells in medium supplemented with nerve growth factor (20

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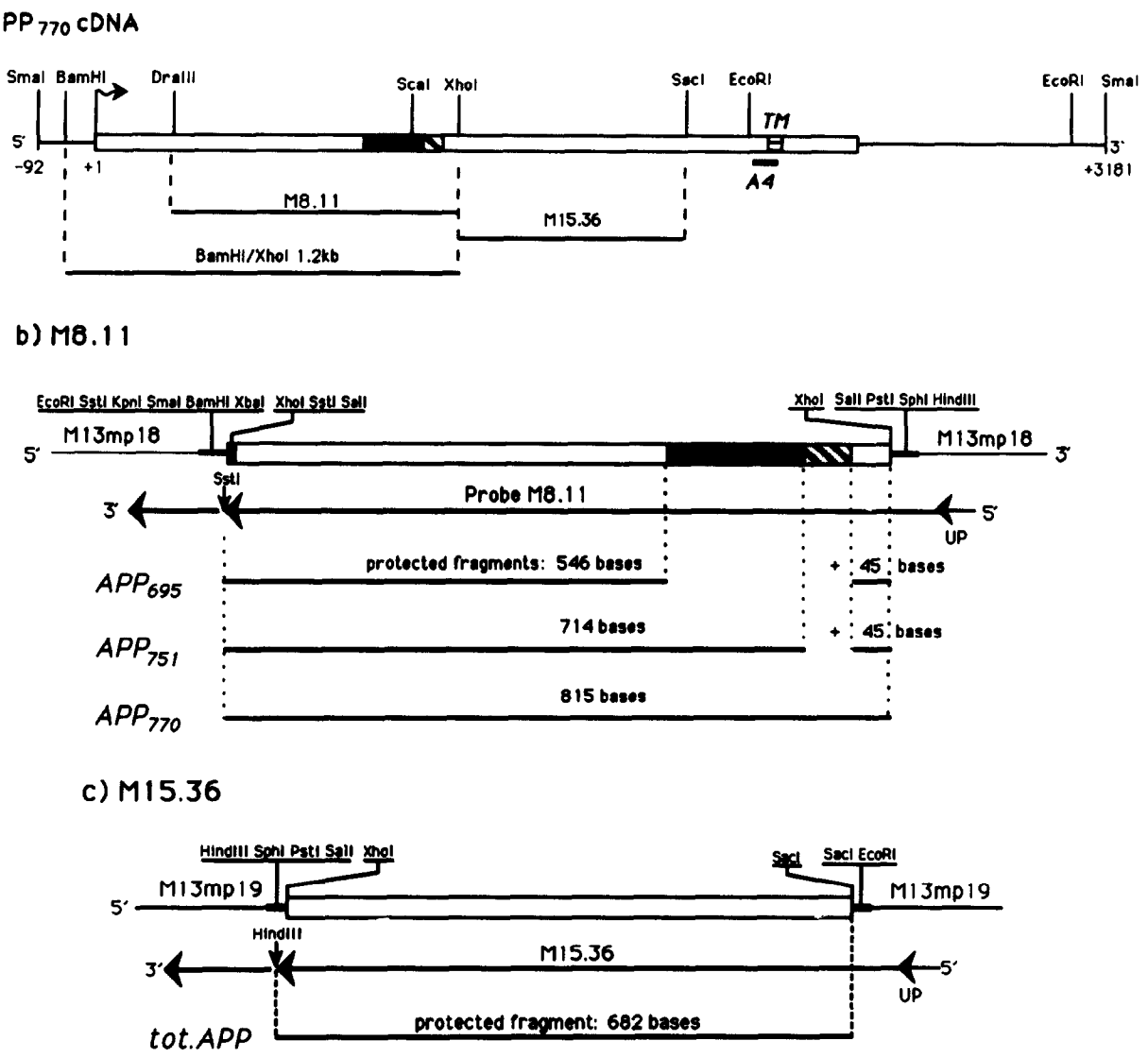

Fig. 1. (a) APP_{770} cDNA in plasmid pSP65. The arrow indicates the translation start site, the coding region is shown as an open box. The KPI-exon is denoted as a black box, the OX-2-exon as a hatched box. *TM* stays for *trans*membrane coding region, the black bar below indicates the βA4 coding portion of the APP_{770} cDNA. (b) Phage M8.11, and protected fragments after S1 digestion. The APP_{770} cDNA is shown as an open bar. The black bars represent the KPI-exon, the hatched bar represents the OX-2-exon. (c) Phage M15.36, and protected fragments after S1 digestion. The APP_{770} cDNA is shown as an open bar.

ng/ml) (kindly provided by Dr. R. Heuman, 5000 fold concentrated stock solution in medium), or retinoic acid (10 μM, Sigma, 1000-fold concentrated stock solution in 10% ethanol) for the time indicated. Medium was exchanged every second day, except for the 2 day incubation times in which medium was changed once after 24 h. Cells were harvested after washing once in phosphate-buffered saline (Ca^{2+}/Mg^{2+}-free) with mechanical agitation. They were immediately used for RNA preparation or stored at −80°C after shock freezing in liquid nitrogen.

2.3. *RNA preparation and Northern blot hybridisation*

Total RNA was extracted by the guanidinium isothiocyanate method [21,23] and 10 μg of total RNA was run in formaldehyde-agarose gels which were subsequently used for the Northern transfer [20,21,24]. Nytran™ filters (Schleicher and Schüll) were used as the solid support. Prehybridisation and hybridisation of the filters were done in 5× SSPE, 5× Denhardt's solution, 0.2% SDS, 50% formamide and 100 μg denatured calf thymus DNA overnight at 42°C. The 1182 bp *Bam*HI/*Xho*I APP_{770} cDNA restriction fragment was labelled by random priming (Boehringer Mannheim), and used at 1×10^6 cpm/ml for quantitation of total APP expression [25]. Control hybridisations were done with the same filter after stripping off the APP probe and rehybridisation with the randomly primed 0.8kb *Xba*I/*Pst*I fragment of the rat GAPDH cDNA (kindly provided by Francis Stewart [26]). The final stringent washes of the filter were done in 0.2× SSC, 0.1% SDS at 65°C for 5 min.

2.4. *S1 nuclease protection assay*

A uniformly labelled single-stranded DNA probe was synthesized by annealing 1 pmol of M13 phage specific oligonucleotide primer to 250 ng of single-stranded template DNA of M8.11 or M15.36 [27,28]. This primer was extended with 1 unit Klenow polymerase (Boehringer Mannheim) in the presence of 25mM dATP, dGTP, dTTP, 2 mM dCTP and 40 μCi of α-[^{32}P]dCTP (3000 Ci/mmol, Amersham) for 15

minutes at 37°C. Extended products were digested with *Sac*I (for M8.11) or *Hin*dIII (for M15.36) for 45 min, the labelled single stranded DNA probes were purified on a 5% denaturing polyacrylamide gel, cut out and electroeluted (Elutrap, Schleicher and Schüll). Excess probe (50 000 cpm) was hybridised overnight to 5 or 10 μg of total RNA at 53°C in the case of probe M15.36 and at 55°C in the case of probe M8.11 (75% formamide, 0.4 M NaCl, 20 mM Tris/HCl pH 7.4, and 1 mM EDTA). S1 nuclease digestion (Boehringer Mannheim, 1200 U/sample) was performed for 2 h at 37°C (0.3 M NaCl, 3 mM $ZnSO_4$, 60 mM NaAc pH 4.5, 0.5 μg denatured calf thymus DNA). The resulting products phenol/$CHCl_3$-extracted, ethanol precipitated and resolved on a 5% denaturing sequencing gel. Autoradiography was performed using Kodak X-OMAT™ AR films, and appropriately exposed X-ray films with signals in the linear responsive range were used for densitometry on a Hirschmann elscript 400 densitometer.

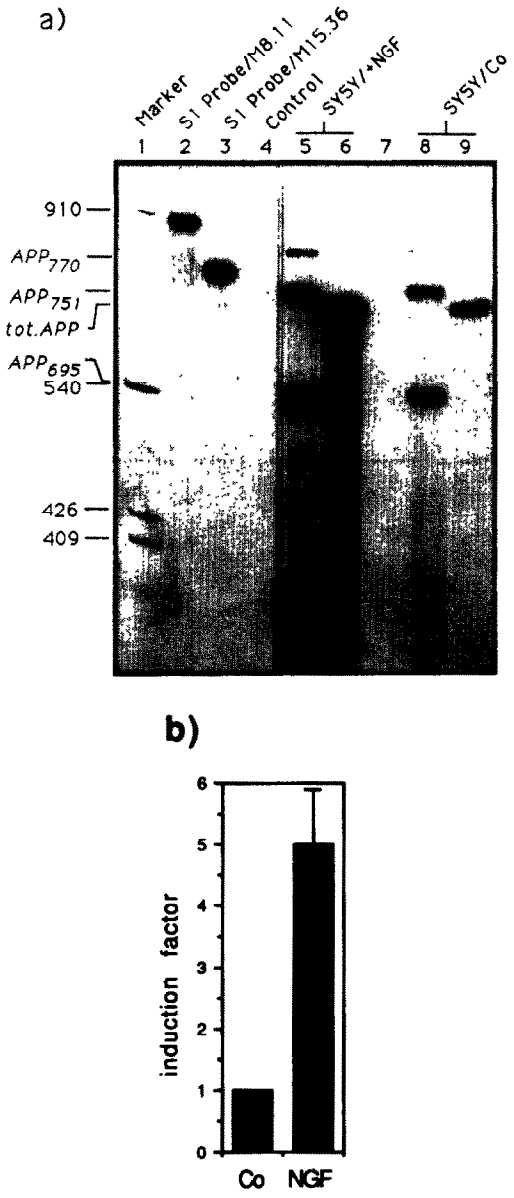

Fig. 2. (a) S1 nuclease protection assay with probe M8.11 and M15.36 of RNA from SH-SY5Y cells in the control and the NGF-induced differentiated state. The protected fragments of probe M8.11 (APP_{770}, APP_{751} and APP_{695}) and of probe M15.36 (tot. APP), are indicated. (Lane 1) molecular weight marker; (2 and 3) S1 probe M8.11 and M15.36, respectively; (4) control hybridisation of 20 μg yeast tRNA to M8.11 and M15.36 together in one sample; (5) protected fragments of M8.11 in RNA of differentiated SH-SY5Y cells after 8 days of NGF treatment; (6) protected fragment of M15.36 in RNA of differentiated SH-SY5Y cells after 8 days NGF treatment; (8) protected fragments of probe M8.11 in RNA of SH-SY5Y cells of the undifferentiated control state; (9) protected fragments of probe M15.36 in RNA of SH-SY5Y cells of the undifferentiated control state. (b) Quantification of the S1 nuclease protection experiments of total APP mRNA induction after 8 days of NGF-treatment in differentiated SH-SY5Y cells ($n = 2$).

3. RESULTS AND DISCUSSION

After 8 days in culture in the presence of NGF (20 ng/ml) the majority of SH-SY5Y cells extend neurites, cease multiplying, aggregate, and have electrically excitable membranes [28]. They resemble closely the phenotype of a primary culture of sympathetic ganglia after treatment with NGF. Total RNA was prepared and subsequently used for S1 nuclease analysis with

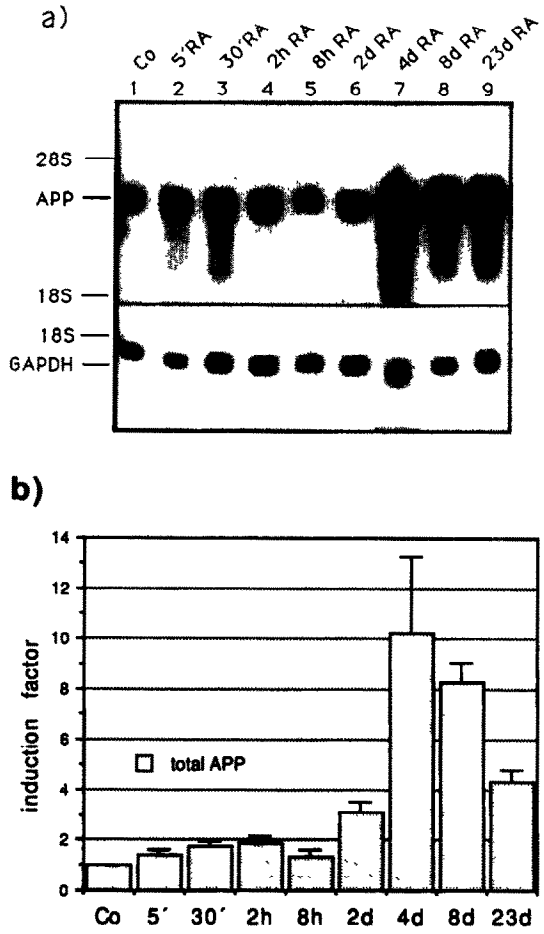

Fig. 3. (a) Northern hybridisation analysis of RNA from SH-SY5Y cells treated with RA to induce differentiation. The signal of the APP mRNA (APP) runs at approximately 3.6kb. (1) RNA from SH-SY5Y cells in the low density control state, (2–9) RNA from SH-SY5Y cells treated with RA for the times indicated. (b) Quantification of the induction of total APP mRNA (total APP) during differentiation with RA.

probe M8.11 as well as with probe M15.36. We observed a fivefold higher expression of the PAD gene in the differentiated cells compared to control cells (Fig. 2a, lanes 6 and 9; Fig. 2a, lanes 5 and 8, Fig. 2b) which is in agreement with the developing hamster brain [30]. Different exposure times to NGF did not result in a stronger response of the PAD gene to this agent (data not shown). In undifferentiated as well as NGF induced differentiated SH-SY5Y cells the APP_{695} transcript accounts for about 49%, the APP_{751} transcript for about 41%, and the APP_{770} transcript for about 10% of total APP mRNA.

It has been reported that retinoic acid (RA) is a potent inducer of differentiation in neuroblastoma cells [31]. When SH-SY5Y cells were cultured in the presence of RA, the majority of the cells developed the characteristic phenotype of differentiated neurons after four to five days (Fig. 5a and b). Between days 6 and 12 the typical morphology of differentiated neuroblastoma cells could be maintained. The RA-induced differentiated SH-SY5Y cells showed consistently a more slender and longer bipolar morphology than cells which were differentiated through NGF. A less than twofold increase in total APP mRNA is seen during the

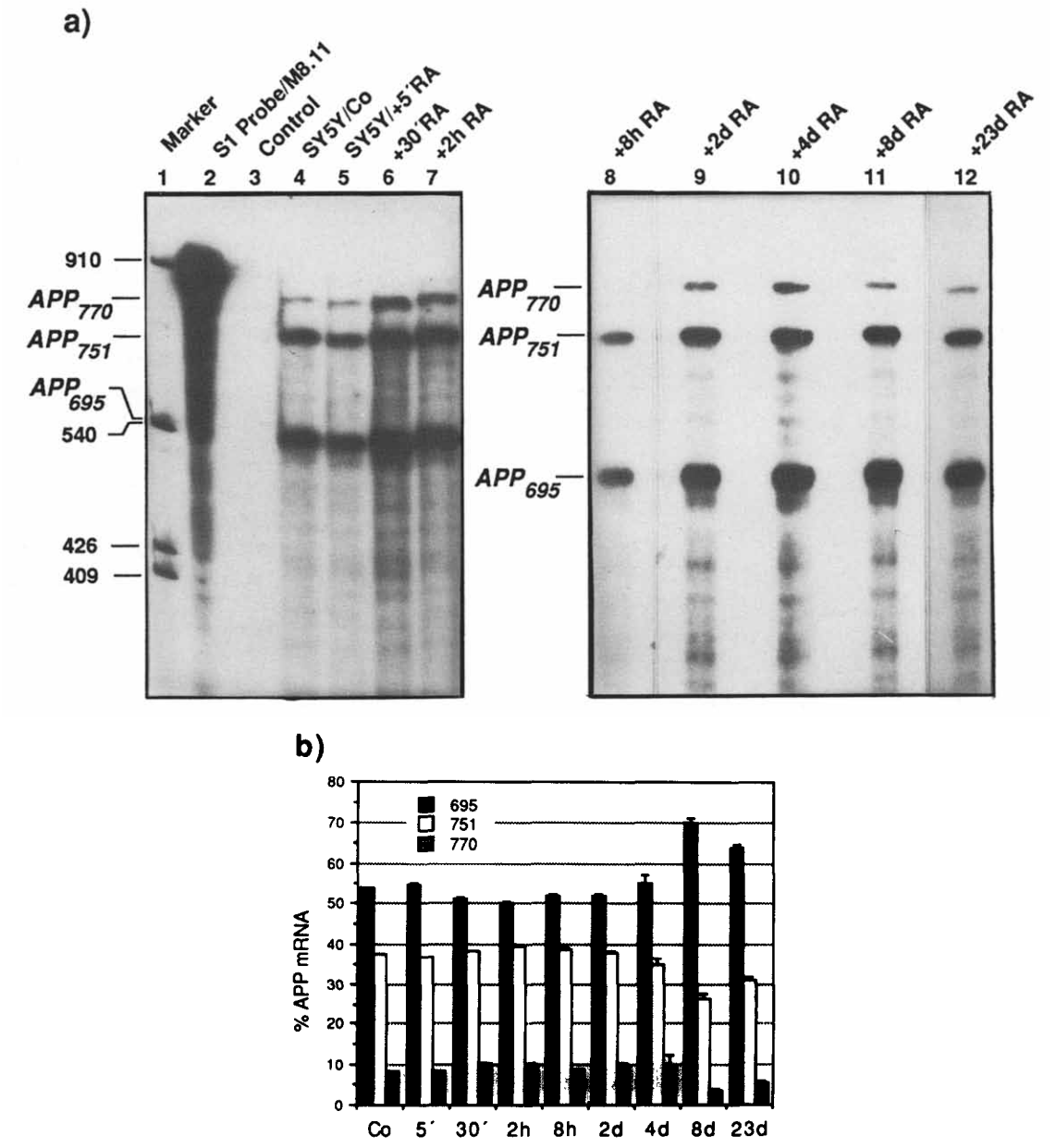

Fig. 4. (a) S1 nuclease protection assay with probe M8.11 of RNA from SH-SY5Y cells treated for various times with RA to induce differentiation. (1) molecular weight marker in bases, (2,3) probe M8.11 and the control hybridisation to 10 μg yeast tRNA; (5–12) the protected fragments in RNA of SH-SY5Y cells treated for various times with RA in comparison to the control state of low density cells (4) (b) Quantification of the S1 nuclease protection experiments on the RNA of SH-SY5Y cells treated for various times with RA to induce differentiation ($n = 3$).

first 8 hours of RA administration (Fig. 3a, lanes 2–5; Fig. 3b). The marked increase in PAD gene expression peaks at day 4 in culture with RA with an induction factor of 10.2. APP mRNA remains elevated through day 23, the longest timepoint tested, with a factor of 4.3, compared to control cells (Fig. 3). The sizes of the PAD transcripts were in the range of 3.6 kb.

The ratios of the different PAD transcripts in the same RNA preparation, measured with probe M8.11 in a S1 nuclease protection assay, reveals a significant shift in the splicing pattern within a slightly different time frame as observed for the increase of total APP mRNA. Whereas in control cells grown at a low density the relative amount of APP_{695} transcript is 54%, in cells which were treated with RA over a period of 8 days, the APP_{695} transcript makes up 70% of total PAD transcripts (Fig. 4a, lanes 4 and 11, Fig. 4b). In contrast APP_{770} mRNA is reduced to 3.5% and APP_{751} mRNA to 26.5% of total APP mRNA compared to 37.5% of APP_{751} mRNA and 8.5% of APP_{770} mRNA in undifferentiated cells grown at low density (Fig. 4a, lanes 4 and 11, Fig. 4b). This change of the splicing pattern is only seen after an initial maximal increase of total APP mRNA at day 4 and is associated with an increase of total PAD expression. The more immediate response of the total PAD gene expression by a factor of about 2, which peaks at 2 h after RA-administration, does not lead to a significant change in the splicing pattern (Fig. 4a, lanes 4–7, Fig. 4b).

The total increase of APP_{695} mRNA is tenfold at days 4 and 8. The APP_{751} mRNA is increased by a factor of 9.5 at day 4, and 6 at day 8, and the APP_{770} mRNA by a factor of 12 at day 4 and only 3.4 at day 8. These data demonstrate an alternative splicing pattern of the APP hnRNA in differentiated SH-SY5Y cells, induced by RA, in favor of the APP_{695} mRNA.

The neurotrophic factor NGF is known to be important in maintenance and survival of sympathetic and sensory neurons of the peripheral nervous system [32]. It has been shown that NGF stimulates the outgrowth of sympathetic neurons into the central nervous system [33]. Retinoic acid however is a potent morphogen which plays an important role in the development of the CNS of vertebrates [34]. RA has been shown to be involved in the establishment of an anterior-posterior axis in the CNS and during the limb development in vertebrates [35,36]. It binds to nuclear receptors (hRAR) which are ligand-dependent transcription factors, thereby activating the DNA binding capability of these receptors and regulating gene expression through responsive elements in the promoter region [37–40].

A possible explanation for the differences observed in the APP expression in RA-induced differentiated cells could be a regulation of genes by RA involved in the expression of specific splicing factors responsible for the alternative splicing of the PAD gene. These RA-target genes would not be affected by NGF, thus not involving a change in the splicing program in these cells.

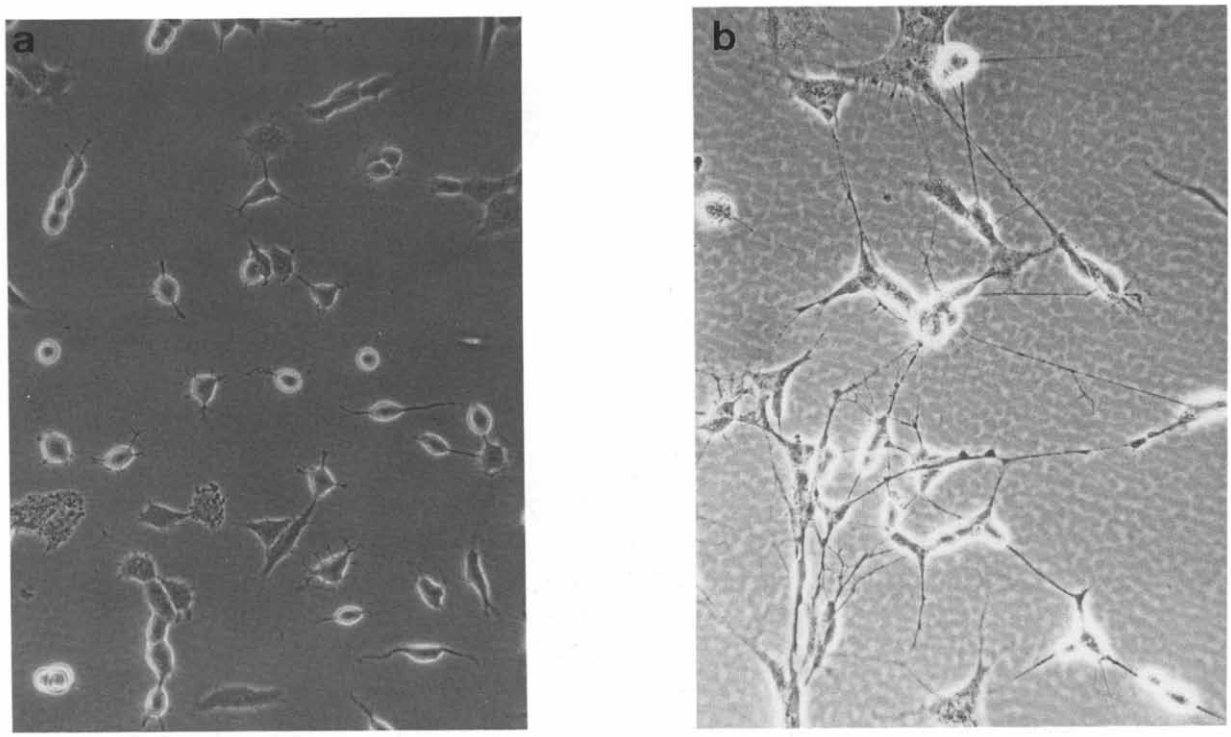

Fig. 5.(a) Effect of RA on the morphology of SH-SY5Y cells (phase contrast). (a) Cells were seeded at a density below 2×10^3 cells/cm^2 and grown for 4 days. Medium was changed after the second day. (b) Early log phase SH-SY5Y cells were treated with RA (10 μM) for 8 days. Medium was changed every second day.

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REFERENCES

[1] Alzheimer, A. (1907) Allg. Z. Psychiat. Psych. Gericht. Med. 64, 146–148.
[2] Glenner, G.G. and Wong, C.W. (1984a) Biochem. Biophys. Res., Commun. 120, 885–890.
[3] Masters, C.L., Simms, G., Weinman, N.A., Multhaup, G., McDonald, B.L. and Beyreuther, K. (1985) Proc. Natl. Acad. Sci. USA 82, 8245–4249.
[3] Kang, J., Lemaire, H.-G., Unterbeck, A., Salbaum, J.M., Masters, C.L., Grzeschik, K.-H., Multhaup, G., Beyreuther, K. and Müller-Hill, B. (1987) Nature 325, 733–736.
[5] Tanzi, R.E., Gusella, J.F., Watkins, P.C., Burns, G.A.P., St George-Hyslop, P., van Keuren, M.L., Patterson, D., Pagan, S., Kurnit, D.M. and Neve, R.L. (1987) Science 235, 880–884.
[6] Goldgaber, D., Lerman, M.I., McBride, O.W., Saffiotti, U. and Gajdusek, D.C. (1987) Science 235, 877–880.
[7] Dyrks, T., Weidemann, A., Multhaup, G., Salbaum, J.M., Lemaire, H.-G., Kang, J., Müller-Hill B., Masters, C.L. and Beyreuther, K. (1988) EMBO J. 7, 949–957.
[8] Weidemann, A., König, G., Bunke, D., Fischer, P., Salbaum, J.-M., Masters, C.L. and Beyreuther, K. (1989) Cell 57, 115–126.
[9] Salbaum, J.M., Weidemann, A., Lemaire, H.-G., Masters, C.L. and Beyreuther, K. (1988) EMBO J. 7, 2807–2813.
[10] Tanzi, R.E., McClatchey, A.I., Lamperti, E.D., Villa-Komaroff, L., Gusella, J.F. and Neve, R.L. (1988) Nature 331, 528–530.
[11] Ponte, P., Gonzalez-DeWhitt, P., Schilling, J., Miller, J., Hsu, D., Greenberg, B., Davis, K., Wallace, W., Lieberburg, I., Fuller, F. and Cordell, B. (1988) Nature 331, 525–527.
[12] Kitaguchi, N., Takahashi, Y., Tokushima, Y., Shirohiri, S. and Ito, H. (1988) Nature 331, 530–532.
[13] Lemaire, H.G., Salbaum, J.-M., Multhaup, G., Kang, J., Bayney, R.M., Unterbeck, A., Beyreuther, K. and Müller-Hill, B. (1989) Nucl. Acid. Res. 17, 517–522.
[14] De Sauvage, F. and Octave, J.-N. (1989) Science 245, 651–653.
[15] Kang, K. and Müller-Hill, B. (1990) Biochem. Biophys. Res. Commun. 166, 1192–1200.
[16] Neve, R.L., Finch, E.A. and Dawes, L.R. (1988) Neuron 1, 669–677.
[17] Ross, R.A., Spengler, B.A. and Biedler, J.L. (1983) JNCI. 71, 741–747.
[18] Cole, G.M., Wu, K. and Timiras, P.S. (1985) Int. J. Dev. Neurosci. 3, (1), 23–32.
[19] Pennypacker, K.R., Kuhn, D.M. and Billingsley, M.L. (1989) Mol. Brain. Res. 5, 251–258.
[20] Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A. and Struhl, K. (1989) (ed). In Current Protocols in Molecular Biology, Wiley, New York.
[21] Maniatis, T., Fritsch, E.F. and Sambrook, J. (1982). Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, New York.
[22] Yanish-Perron, C., Vieira, J. and Messing, J. (1985) Gene 33, 103–119.
[23] Chirgwin, J.D., Przybla, A.E., Mac Donald, R.J. and Rutter, W.J. (1979) Biochemistry 18, 5294–5299.
[24] Thomas, P. (1980) Proc. Natl. Acad. Sci. USA 77, 5201–5205.
[25] Feinberg, A.P. and Vogelstein, B. (1983) Anal. Biochem. 132, 6–13.
[26] Fort, P., Marty, L., Piechaczyk, M., Sabrouty, S.E., Dani, C., Jeanteur P. and Blanchard, J.M. (1985) Nucl. Acid. Res. 13, 1431–1442.
[27] Berk, A.J. and Sharp, P.A. (1977) Cell 12, 721–728.
[28] Ruppert, C., Goldowitz, D. and Wille, W. (1986) EMBO J. 5, 1897–1901.
[29] Perez-Polo, J.R., Wehrbach-Perez, K. and Tiffany-Castiglioni, E. (1979) Dev. Biol. 71, 341–355.
[30] Mobley, W.C., Neve, R.L., Prusiner, S.B. and McKinley, M.P. (1988) Proc. Natl. Acad. Sci. USA 85, 9811–9815.
[31] Pahlman, S., Ruusala, A., Abrahamson, L., Mattson, M.E.K. and Esscher, T. (1984) Cell Differ. 14, 135–144.
[32] Barde, Y.-A., Edgar, D. and Thoenen, H. (1983) Annu. Rev. Physiol. 45, 601–612.
[33] Levi-Montalcini, R. (1975). In The Neuroscience: paths of discovery. Worden, F.G., Swazey, J.P. and Adelman, G. (eds.). Cambridge, MA, MIT Press, 245–265.
[34] Durston, A.J., Timmermans, J.P.M., Hage, W.J., Hendriks, H.F.J., de Vries, N.J., Heideveld, M. and Nieuwkoop, P.D. (1989) Nature 340, 140–144.
[35] Eichele, G. (1989) Trends Genet. 5, 246–251.
[36] Dolle, P., Ruberte, E., Kastner, P., Petkovich, M., Stoner, C.M., Gudas, L.J. and Chambon, P. (1989) Nature 342, 702–705.
[37] Petkovich, M., Brand, N., Krust, A. and Chambon, P. (1987) Nature 330, 444–450.
[38] Giguere, V., Ong, E.S., Segui, P. and Evans, R.M. (1987) Nature 330, 624–629.
[39] Green, S. and Chambon, P. (1988) Trends Genet. 4, 309–314.
[40] Evans, R.M. (1988) Science 240, 889–895.