Regulation of mRNA levels for microtubule proteins during nerve regeneration

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Received 8 June 1983; revised version received 24 August 1983

The molecular regulation of tubulin synthesis was investigated in the regenerating goldfish retina. Previous in vivo studies pointed to an increase in tubulin synthesis in the retina during regeneration of the injured goldfish optic nerve. Using labeled cDNA probes, we showed that this increase occurs as a result of enhanced tubulin mRNA levels. Analysis of labeled in vivo products revealed enhanced β_2-tubulin synthesis accompanied by an increase in the level of the low-M_r microtubule-associated proteins identified as TAU factors. The results are discussed with respect to the possible involvement of these proteins in the process of nerve regeneration.

Regeneration *Tubulin sequence* *TAU factor*

1. INTRODUCTION

Neurons of the central nervous system (CNS) of lower vertebrates, unlike most of the neurons in mammalian CNS, are endowed with a high regenerative capacity. The goldfish visual system undergoes functional recovery following optic nerve lesion and is therefore often used as a model for investigating the mechanisms underlying the regenerative process [1–5]. Restoration of axonal connections with the target, tectum, is preceded by a series of biochemical changes in the retinal ganglia cells; i.e., the cell bodies of the injured axons [4,6–9]. During the recovery process, changes in the composition of the proteins and the rate of their axonal transport were reported [10–13]. In addition to the events described in the cell bodies, changes in the target of the regenerating fibers were also observed [14,15]. Tubulin, the major proteinaceous component of microtubule, is one of the proteins that undergoes enhanced labeling during regeneration.

We have investigated whether there is any selective increase of certain tubulin isoforms and whether the increased labeling of tubulin is accompanied by an increased labeling of microtubule-associated proteins (MAPs) which may indicate their involvement in the process of nerve regeneration.

To elucidate the regulation of tubulin synthesis in nerve regeneration, we have used cDNA probes specific for tubulin. The results reveal that enhanced tubulin labeling is due to an increase in mRNA sequences specific for tubulin.

2. MATERIALS AND METHODS

Goldfish *Carassius auratus* (10–12 cm) were purchased from Assia Maabaroth (Israel) and maintained at 20 ± 2°C. Fish were anesthetized with 0.05% tricaine methansulfonate (MS222, Sigma). The right optic nerve was then crushed with forceps, taking care to injure the nerve only while the surrounding tissue remained intact [16].

2.1. *Labeling of retinal proteins with [^{35}S]methionine*

Dissected tissues were incubated for 1 h at room temperature in Dunlop buffer (25 mM HEPES, 1.3 mM $MgSO_4$, 2.6 mM $CaCl_2$, 1.2 mM

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K_2HPO_4, 5.9 mM KCl, 106.5 mM NaCl, 12 mM glucose and NaOH to pH 7.4) containing L-[^{35}S]methionine (5 μCi/retina, 600 Ci/mmol) [7]. The reaction was stopped by dilution with L-methionine (2 mM, 2 vol.). The tissue was then homogenized, centrifuged (airfuge, 25 lb.in^{-2}, 10 min) to obtain the S-100 pellets and supernatants. Protein-bound radioactivity was determined by precipitation with trichloroacetic acid. Labeled proteins were electrophoresed on SDS–polyacrylamide gel (8–12% gradient) to separate α- and β-tubulin subunits. Running buffer consisted of 50 mM Trizma base, 384 mM glycine and 0.1% SDS. This was followed by autoradiography.

2.2. *mRNA purification*

The isolation of mRNA from tecta and retinae was performed at 10 days post-operation of the right optic nerve. RNA was extracted using a solution of 3 M LiCl, 6 M urea and 10 mM sodium acetate pH 6 [17]. Poly(A)-containing RNA was purified by passage on oligo(dT)-cellulose column.

2.3. *Electrophoretic analysis of brain mRNA in formaldehyde–agarose gels*

The poly(A)-containing RNA was fractionated by electrophoresis on 1.2% formaldehyde–agarose slab gels. RNA samples (2 μg) were processed as in [17]. Following electrophoresis, the gel was soaked for 30 min in 20 × SSC (1 × SSC contains 0.15 M NaCl, 0.015 M Na-citrate) and blotted during 12–15 h with 10 × SSC onto nitrocellulose filters [17–19]. Hybridization was done with 10^6 cpm/ml of nick-translated pT25 tubulin [^{32}P]cDNA probe [17]. Quantitative determination of the amount of hybridized mRNA was performed by scanning the autoradiograms at 560 nm with a Gilford Spectrophotometer.

2.4. *Preparation of MAPs from goldfish brains and labeled MAPs from goldfish retinae*

Goldfish brains (350) were homogenized in buffer 'A' (MES 0.1 M, 0.5 mM $MgCl_2$, 1 mM EGTA and 0.1 mM EDTA) and after 2 cycles of polymerization the last pellet was resuspended in 100 μl buffer A. Aggregates were removed by airfuge centrifugation (Beckman, 5 min, 25 lb.in.$^{-2}$). The aggregate-free supernatant fraction was loaded on a phosphocellulose column. The flow-through fraction contained mainly tubulin. The column was then washed with 3–4 ml buffer A after which the retained protein was eluted with 0.8 M NaCl in buffer A. The eluted material was then precipitated with 4 vol. acetone at −20°C. The pellet was resuspended in sample buffer and analyzed by SDS–PAGE (8% acrylamide).

Ten days following the lesion of the right optic nerve, retinae from both sides were excised separately and pulse-labeled with [^{35}S]methionine (5 μCi/retina) for 1 h at room temperature in Dunlop buffer. Following labeling the retinae were homogenized in 300 μl buffer A (30 strokes). The homogenate was centrifuged at 100000 × g for 30 min at 4°C. To the supernatant, carrier calf brain microtubule was added and loaded onto a 1 ml phosphocellulose column. MAPs were eluted from the column with 0.8 M NaCl, concentrated with acetone and then subjected to SDS–PAGE.

3. RESULTS

Retinal proteins were pulse labeled with [^{35}S]methionine and analyzed by SDS–PAGE. The results indicated that there is an increase in the labeling of several proteins in the preparation obtained from the regenerating, post-crush (PC) retinae relative to those obtained from control, normal (N) retinae. Tubulin is one of the proteins undergoing increased labeling (fig.1) as reported in [7]. Here, we have found that the β-subunit is preferentially labeled as compared with the α-subunit (fig.1). Moreover, the increase in labeling of the β-subunit was more pronounced in one of the faster migrating β-tubulin isoforms.

The particulate membrane fractions obtained from the in vivo labeled preparations were also analyzed: there were no significant differences in the level and the ratios between α- and β-tubulin subunits of normal and regenerating retinae (fig.1). These results indicate that the major changes in the level and distribution of the β-tubulin in regenerating retinae are confined to the cytoplasmic pool.

To elucidate the regulation of tubulin synthesis in regenerating retinae, we used cDNA probes specific for tubulin to measure the amount of tubulin sequences in mRNA preparations from both regenerating and normal components of the goldfish visual system. From each mRNA prepara-

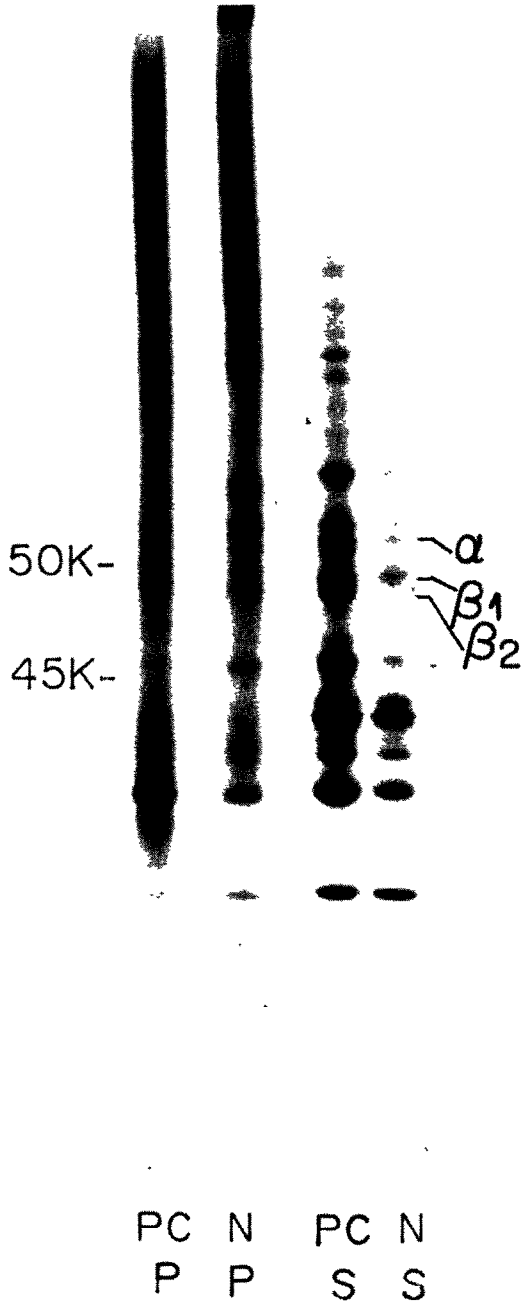

Fig.1. Analysis of in vivo labeled proteins by SDS–polyacrylamide 8–12% gradient slab gel electrophoresis. (A) Post-crush (PC) and control (N) retinae were pulse-labeled with [^{35}S]methionine (5 μCi/retina) 10 days following lesion of the right optic nerve. High-speed supernatants (airfuge, 25 lb.in.$^{-2}$, 10 min) designated (S) and the corresponding pellet fractions (P) were collected. The pellets were resuspended in the homogenizing buffer containing 0.75% SDS and centrifuged (1300 × g, 10 min). Aliquots from all these supernatants were mounted on the gel. 45 k and 50 k are M_r-values ($\times 10^{-3}$) of actin and tubulin, respectively.

tion 2 μg were subjected to 1.2% agarose gel, and hybridized with labeled tubulin cDNA clone. The autoradiogram of the exposed blot was scanned (fig.2) indicating an increased level of hybridization with mRNA from regenerating retinae (PC) as compared to the level observed with mRNA from the control, ipsilateral retinae (N). In addition, mRNA isolated from the regenerating tecta, left tecta contralateral to the side of the injury (PC) showed a higher hybridization level than that obtained with mRNA isolated from the control, ipsilateral right tecta (N).

MAPs are known to enhance assembly of microtubules in vitro [20]. It was therefore of interest to determine whether parallel changes in the synthesis of MAPs during the regenerative process could be detected. MAPs from goldfish brain were isolated by the method described for the isolation of these proteins from several sources [21]. Accordingly, the depolymerized microtubules were applied onto a phosphocellulose column in low salt solution (0.1 M NaCl). The fractions obtained were analyzed on SDS–PAGE followed by staining with Coomassie brilliant blue. The flow-through fraction of the phosphocellulose column consists mainly of tubulin whereas the protein fraction which is retained on the column and eluted with 0.8 M NaCl is enriched in MAPs, and contains only small amounts of tubulin and actin as compared to their initial concentration (fig.3A (2)). Both the high-M_r MAP_1 and MAP_2 as well as the TAU factors are seen in the fraction eluted from the column (fig.3A (2)). The eluted fraction was added to purified tubulin from goldfish brain to examine its ability to induce microtubule assembly. Addition of the protein factors induced tubulin polymerization as well as shortened the lag period (fig.3B).

Under the same experimental conditions, the extent of polymerization was lower probably due to the lower concentrations of tubulin in the assay mixture as compared to the homogenate.

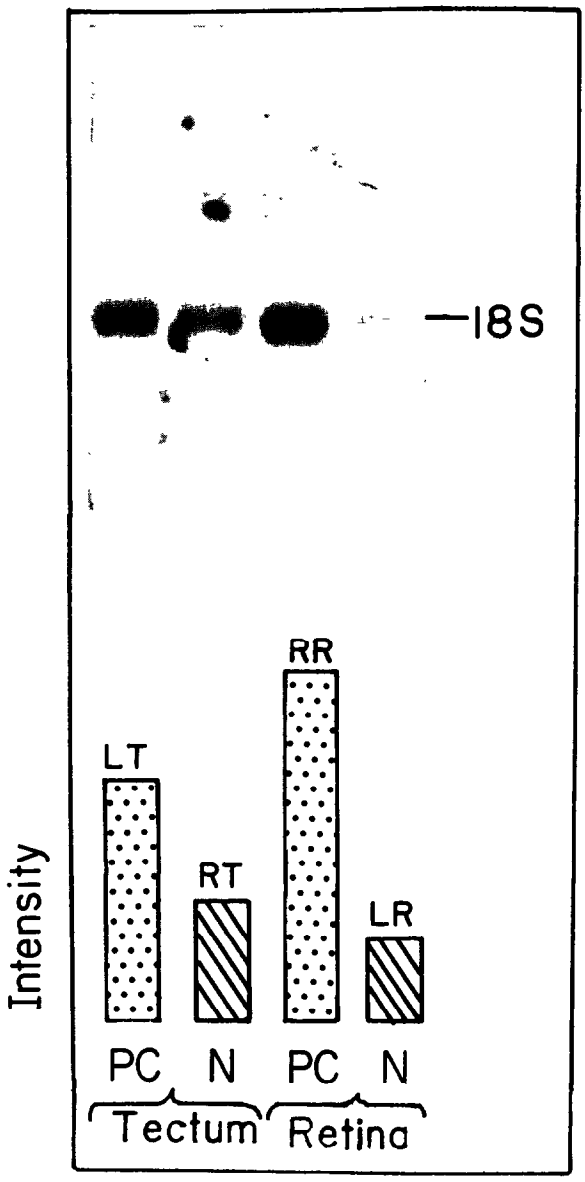

Fig.2. Hybridization of tubulin cDNA to size fractionated RNA isolated from goldfish retinae and tecta. Poly(A)-containing RNA was fractionated by electrophoresis on 1.2% formaldehyde denaturing agarose gels (2 μg). Following the electrophoresis the gel was blotted for 12–15 h with 10 × SSC onto nitrocellulose filters. The hybridization was performed as in section 2. LT and RT are left and right tecta representing regenerating and control tecta, respectively. The autoradiograms presented in the upper part were scanned and the intensity of hybridizable tubulin mRNA in the normal (N) and post-crush (PC) retina and tectum are presented in the bottom part.

The proteins which were eluted from the column were considered as goldfish brain MAPs based on their electrophoretic mobility and on their ability to induce polymerization of tubulin. Therefore, it was adequate to adapt this procedure to obtain factors from retina as well. Fig.3C shows the autoradiogram of the radioactive MAPs isolated from in vivo, [^{35}S]methionine-labeled proteins of post-crush (PC) and control (N) retinae. The relative amount of the labeled TAU factors in the PC and control retina was evaluated by densitometric scanning of the autoradiograms. The results indicate a significant increase in the amount of two TAU species (fig.3C). Moreover, the TAU species from the regenerating retinae migrated at a slightly faster rate than the corresponding TAU species from normal retinae.

The methionine content of mammalian TAU factors is very low [22] as compared to that of tubulin or actin, so that the actual amount of TAU proteins may be higher than reflected from the extent of [^{35}S]methionine incorporation. In these retinal fractions, MAP_1 and MAP_2 could not be detected.

4. DISCUSSION

Following crush injury of the optic nerve several changes occur in the respective retina [4–6,9,10,23–25]. Since changes in tubulin labeling [7] were shown to occur only in the retina of the injured side, we used the retinae of the contralateral side as an intrinsic control in these experiments.

Increased tubulin labeling was shown to reach its maximal level about 10 days following the optic nerve injury [7]. Therefore we analyzed the synthesis of tubulins in the retinae at this period post injury. Our results (fig.1) demonstrate an increase in both α- and β-tubulins in the regenerating retina; however this increase is more pronounced in the β-subunits. The observation that the β-isoform of tubulin is increased in the process of regeneration may suggest that the β-tubulin has a role in neuritic extension. This concurs with evidence that neuritic extensions from neuroblastoma cells are associated with increased synthesis of β_2-tubulin [26].

An increase in tubulin synthesis in cell-free system was directed by mRNA isolated from

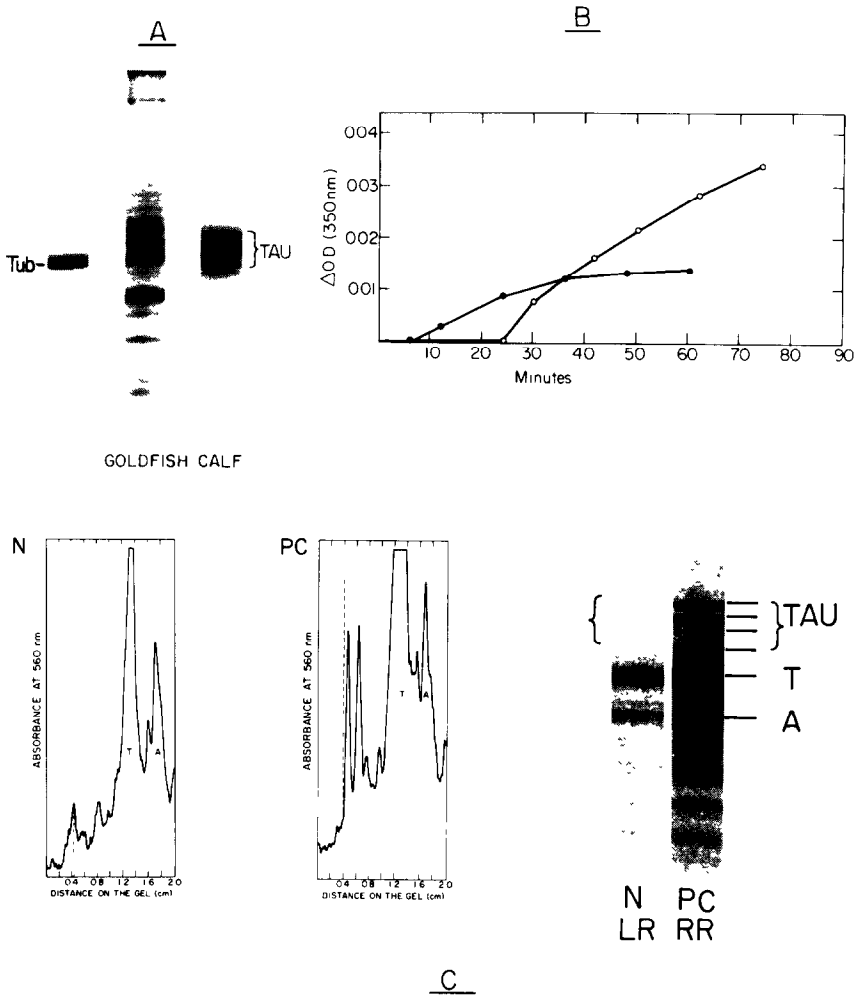

Fig.3. Analysis of MAPs isolated from brain and regenerating retinae of goldfish. (A) Gel analysis of goldfish brain MAPs stained with Coomassie brilliant blue. Tubulin (Tub) and TAU factors of calf brains were run in parallel as markers. (B) The functional activity of the factors prepared from the goldfish brain was assayed by their ability to induce polymerization of goldfish brain tubulin [32]. This graph measured the formation of microtubules based on turbidimetric assay in preparation of: (○) goldfish brain homogenate (5 mg protein/ml); (●) a mixture of goldfish brain tubulin (effluent from phosphocellulose column, 840 μg/ml) and goldfish MAPs (0.8 M NaCl eluate from phosphocellulose column 396 μg/ml). (C) Autoradiogram of labeled TAU factors from regenerating (PC) and normal (N) retinae. Ten days following lesion of the right optic nerve, retinae from both sides were excised and pulsed labelled with [^{35}S]methionine (5 μCi/retina) for 1 h at room temperature in Dunlop buffer. The factors were isolated as in section 2. Following electrophoresis and fluorography the gels were autoradiographed. The densitometric traces of the autoradiograms were obtained by scanning at 560 nm: T, tubulin; A, actin; (– – –) position of the slower moving TAU band from normal retinae.

regenerating retinae [26]. Thus tubulin synthesis during the regeneration process could be controlled by an increase in tubulin mRNA levels. However, these studies lack evidence that this variation in tubulin synthesis is due to an increase in mRNA sequences specific for tubulin.

Therefore, we have investigated the molecular regulation of tubulin synthesis using labeled cDNA probes specific for tubulin. It should be noted that under these experimental conditions, clone pT 25 recognizes both α- and β-tubulin mRNA species [19]. The results strongly suggest that the pro-

moted synthesis of tubulin in the course of the regenerative process is regulated mainly by increased mRNA levels and not by increased translatability of pre-existing mRNA.

The increase in tubulin sequences in the regenerating tectum may be associated with cell division which occurs when the regenerating fibers reach their target; i.e., the contralateral tectum [14,15]. In the retina the increase in tubulin mRNA could be related to the formation of new microtubules required for axonal growth and elongation.

We have also found that enhanced synthesis of tubulin is accompanied by a selective increased labeling of TAU factors. These particular isoforms may be associated with neurite extension. These TAU proteins could participate in establishing microtubule organizing centers, in determining the spatial organization of the growing microtubules or in their stabilization within the axon. Both tubulin and TAU factors undergo a similar rate of axonal transport [28].

The selective enhanced labeling of the TAU proteins may be directly associated with the enrichment of the β-tubulin subunit in the regenerating retina. If each isotubulin species has its own specific binding site for a certain TAU species, then the selective synthesis of TAU species could be brought about by an increase in the level of the β-tubulin subunit. Developmentally determined changes in the composition and distribution of tubulin and TAU proteins have been described [29,30]. Expression of the genes coding for TAU proteins is regulated at the mRNA level [31]. Therefore, the various isotubulin and TAU isoforms could be non-randomly distributed within a given microtubular system.

The process of optic nerve recovery is probably a result of several molecular events. The increased synthesis of tubulin in the cell body of the axotomized nerve may be a prerequisite for the regenerating process. This study demonstrates the molecular regulation of tubulin synthesis, and may throw some light on the control mechanism of other proteins associated with nerve regeneration.

ACKNOWLEDGEMENTS

This work was supported by grants to M.S. from the Binational Foundation, the Muscular Dystrophy Association and by a grant from the Muscular Dystrophy Association to U.Z.L. M.S. is an incumbent of the Helena Rubinstein Career Development Chair. The authors wish to thank Mr Ivan Rachailovitch for his contribution to this work.

REFERENCES

[1] Sperry, R.W. (1944) J. Neurophysiol. 7, 5–70.
[2] Jacobson, M. and Gaze, R. (1965) Exp. Neurol. 13, 418–430.
[3] Murray, M. (1976) J. Comp. Neurol. 168, 175–195.
[4] Grafstein, B. and McQuarrie, I. (1978) in: Neuronal Plasticity (Cotman, C.N. ed) pp.155–195, Raven, New York.
[5] Agranoff, B.W., Feldman, E., Heacock, A.M. and Schwartz, M. (1980) Neurochemistry 1, 487–500.
[6] Murray, M. (1973) Exp. Neurol. 39, 489–497.
[7] Heacock, A.M. and Agranoff, B.W. (1976) Proc. Natl. Acad. Sci. USA 73, 828–832.
[8] Giulian, D., Des Ruissearx, H. and Cowburn, D. (1980) J. Biol. Chem. 255, 6494–6501.
[9] Kohsaka, S., Dokas, L.A. and Agranoff, B.W. (1981) J. Neurochem. 36, 1166–1174.
[10] Heacock, A.M. and Agranoff, B.W. (1982) Neurochem. Res. 7, 771–788.
[11] Lasek, R.Y., McQuarrie, I.G. and Wujek, J.R. (1981) in: Exp. Basis and Clinical Implications (Gorio, A. et al. eds) pp.59–70, Raven, New York.
[12] Benowitz, L.I., Shashoua, V.E. and Yoon, M.G. (1981) J. Neurosci. 1, 300–307.
[13] Skene, J.H.P. and Willard, M. (1981a) J. Cell Biol. 89, 86–95.
[14] Stevenson, J.A. and Yoon, M.G. (1978) Brain Res. 153, 345–351.
[15] Stevenson, J.A. and Yoon, M.G. (1981) Neuroscience 1, 862–875.
[16] Heacock, A.M. and Agranoff, B.W. (1977) Science 198, 6466–6469.
[17] Fellous, A., Ginzburg, I. and Littauer, U.Z. (1982) EMBO J. 1, 835–839.
[18] Ginzburg, I., De Baetselier, A., Walker, M.D., Behar, L., Lehrach, H., Freschauf, A.M. and Littauer, U.Z. (1980) Nucleic Acid Res. 8, 3553–3564.
[19] Ginzburg, I., Behar, L., Givol, D. and Littauer, U.Z. (1981) Nucleic Acid Res. 9, 2691–2697.
[20] Weingarten, M.D., Lockwood, A.H., Hwo, S. and Kirschner, M.W. (1975) Proc. Natl. Acad. Sci. USA 72, 1858–2162.

[21] Lenon, A.M., Francon, J., Fellows, A. and Nunez, J. (1980) J. Neurochem. 354, 804–813.
[22] Cleveland, D.W., Spiegelman, B.M. and Kirschner, M.W. (1979) J. Biol. Chem. 254, 12670–12678.
[23] Murray, M. and Grafstein, B. (1969) Exp. Neurol. 23, 544–560.
[24] Murray, M. and Forman, D. (1971) Brain Res. 32, 287–298.
[25] Burrell, H.R., Dokas, L.A. and Agranoff, B.W. (1978) J. Neurochem. 31, 289–298.
[26] Edde, B., Jeantet, L. and Gros, F. (1981) Biochem. Biophys. Res. Commun. 103, 1035–1043.
[27] Burrell, H.R., Heacock, A.M., Water, R.D. and Agranoff, B.W. (1979) Brain Res. 168, 628–632.
[28] Black, M.M. and Lasek, R.J. (1980) J. Cell Biol. 86, 616–623.
[29] Gozes, I. and Littauer, U.Z. (1978) Nature 276, 411–413.
[30] Mareck, A., Fellous, A., Francon, J. and Nunez, J. (1980) Nature 284, 353–355.
[31] Ginzburg, I., Sherson, T., Giveon, D., Behar, L. and Littauer, U.Z. (1982) Proc. Natl. Acad. Sci. USA 79, 4892–4896.
[32] Nunez, J., Francon, J., Lennon, A.M., Fellous, A. and Mareck, A. (1980) in: Microtubule and Microtubule Inhibitors (De Brabander, M. and De Mey, J. eds) pp.213–225, Elsevier, Amsterdam, New York.