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Purification and partial characterization of *Xenopus laevis* tenascin from the XTC cell line

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We report here the purification of tenascin, an extracellular matrix molecule involved in the control of morphogenesis, from the conditioned medium of the *Xenopus* XTC cell line. Tenascin was purified by affinity chromatography on a column of the monoclonal antibody mAb TnM1; the molecule eluted from this column has a relative molecular mass of 210 kDa after reduction. Electrophoretic analysis under non-reducing conditions shows that the purified components are oligomeric disulfide-linked complexes which barely enter a 4% polyacrylamide gel. Upon rotary shadowing these molecules appear to possess a central globular domain to which pairs or triplets of arms are attached. Polyclonal antibodies have been raised against purified *Xenopus* tenascin. They recognise specifically the antigen on Western blots of XTC conditioned medium and adult brain. by immunofluorescence, these antibodies reveal large amounts of tenascin in the secretory vesicles as well as in the extracellular matrix of XTC cells. In the *Xenopus* tadpole, they stain the developing cartilage, the basal lamina of skin epidermis, myotendinous ligaments and restricted regions of the central nervous system.

Tenascin; Antibody; Extracellular matrix; *Xenopus*

1. INTRODUCTION

Tenascin is a component of the extracellular matrix (ECM) whose expression during development and regeneration is closely associated with important morphogenetic events [1–4]. Originally described in the chicken as myotendinous antigen [5,6], tenascin is identical or homologous to molecules discovered simultaneously by other laboratories: cytotactin in the chicken [7–9] and glioma mesenchymal extracellular matrix protein in human [10]. It appears furthermore that tenascin is the predominant mouse component recognized by the J1 antibody [11].

Electron microscopy studies have shown that tenascin has a six-armed structure with a central core named hexabrachion [9,12–14]. The complete sequence of chicken tenascin [15–18] as well as part of that of human tenascin [19] have been determined. They show that tenascin contains EGF-like sequence repeats, fibrinogen- like sequences and type III fibronectin homologies. The latter can undergo alternative splicings giving rise to various molecular forms which have different patterns of expression [18–20]. Tenascin affects differently the adhesion of various cell types [7,21–24] and inhibits the attachment of cells to FN substrates [24–26]. Using fusion proteins containing different regions of the molecule it has been shown that tenascin contains both cell binding sites and sequences with antiadhesive effects [18] providing a possible interpretation of its versatile behavior as it appears plausible that tenascin modulates cell–substrate adhesion by several different molecular mechanisms.

Tenascin expression is strongly modulated where morphogenetic rearrangements take place both in the developing embryo and in differentiating tissues [8,22,27]. Tenascin has been implied in the development of muscle and myotendinous structures [5,6], cartilage and bone [28], central nervous system [1,7,29,30], intestine [31], kidney [32], mammary glands [33], tooth [34] as well as in regenerative processes [3,35,36]. Tenascin is deposited in the ECM bording the neural crest cell migration pathways in mammal, avian and amphibian embryos where it might modulate the interactions of migrating cells with fibronectin [8,22,27,37–39]. The amphibian provides a suitable system for studies on early embryogenesis, metamorphosis and regeneration [40–42]. Several studies have already appeared on tenascin distribution in amphibians [3,27,37,38]. However, all of them have made use of cross-species antibodies and no study has so far been performed using anti-amphibian tenascin antibodies. In this study we report purification and characterization

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of tenascin from the amphibian *Xenopus laevis*. We have used the conditioned medium of the *Xenopus* established cell line XTC as a source of tenascin. The production and specificity of anti-*Xenopus* tenascin antibodies are described.

2. MATERIALS AND METHODS

2.1. *Preparation of conditioned media*

We used the conditioned media produced by four *Xenopus* cell lines: XTC [43], XL [44], XL177 and KR [45] provided by Dr J.C. Smith (National Institute for Medical Research, London). The cells were cultured at 25°C in Leibovitz L-15 medium (Gibco, France) diluted to 61% and supplemented with foetal calf serum to 10%. Conditioned media of these cells were prepared from confluent cell cultures. No need was found of using serum-free media as, in control experiments, we did not detect the presence of any contaminating tenascin in the batch of serum that we used.

2.2. *Antibodies, chicken tenascin*

The production and specificity of antiserum to chicken fibroblast tenascin and of monoclonal antibody mAB TnM1 have been already reported [5,6]. Purified tenascin from chicken embryo fibroblasts was obtained as described [6].

2.3. *Purification of* Xenopus *tenascin and preparation of the antibodies*

Tenascin was purified by immunoaffinity chromatography on a column of immobilized mAb TnM1 as described [6]. The cross-reactivity of mAb TnM1 with *Xenopus* tenascin was tested by immunoprecipitation experiments (not shown). Coupling of mAb TnM1 with CNBr-activated Sepharose CL4B (Pharmacia) was performed according to the manufacturer's instructions. Five ml of mAb TnM1-conjugated Sepharose were mixed with 300 ml of XTC-conditioned medium and incubated for 18 h at 4°C with gentle shaking. After incubation, Sepharose beads were poured in a 10 ml syringe plugged with glass wool and were washed by passing 10 ml of 0.05% Triton X-100, 0.5 M NaCl, 20 mM Tris-HCl, pH 8.4, followed by 10 ml of 0.5 M NaCl, 20 mM Tris-HCl, pH 8.4. Tenascin was eluted with 5 ml of 0.1 M triethylamine. Eluted fractions (0.25 ml) were collected and analyzed by SDS-PAGE. Fractions containing tenascin were pooled and incubated with gelatin-Sepharose in order to eliminate any contaminating fibronectin which could have co-purified with tenascin. After sedimentation of gelatin-Sepharose, protein concentration of the supernatant was determined by measuring OD_{280}. Pooled fractions were stored at −20°C.

Anti-*Xenopus* tenascin antibodies were prepared by injecting the purified protein into rabbits in the presence of Freund's adjuvant. The primary injection was performed with 150 μg of tenascin emulsified with complete Freund's adjuvant. Rabbits were boosted twice with 75 μg of tenascin emulsified with incomplete Freund's adjuvant. They were bled 2 weeks after the last injection and then every 2 weeks during 2 months. Sera were prepared and titrated by ELISA. IgGs were purified by ion-exchange chromatography on DEAE trisacryl.

2.4. *Rotary shadowing electron microscopy*

Rotary shadowing and electron microscopy were carried out as described [25].

2.5. *Electrophoresis; Western blots*

Proteins were analyzed by SDS-PAGE according to [46]. *Xenopus* adult brain proteins were solubilized according to the high pH method of [8]. Samples were either prepared in the presence of 5% 2-mercaptoethanol to disrupt disulfide bridges (reducing conditions) or without 2-mercaptoethanol (non-reducing conditions). Coomassie blue staining of gels as well as electrotransfer and immunodetection were performed as described [47].

2.6. *Immunohistochemistry*

Paraffin sections of metamorphic *Xenopus* were prepared for staining and processed as previously described [48]. XTC cells were cultured on fibronectin-coated glass coverslips and stained as described [49].

3. RESULTS AND DISCUSSION

3.1. *Isolation and characterization of* Xenopus *tenascin from XTC culture supernatants*

In order to find a suitable source of soluble tenascin, we probed the conditioned media of four *Xenopus* cell lines, XTC, XL, XL177 and KR in Western blots with anti-chicken tenascin antibodies. Fig. 1A shows that only XTC cells secrete a 210 kDa polypeptide which reacts with anti-tenascin. XTC-conditioned medium was then incubated with mAb TnM1 coupled to Sepharose. We choose to use this monoclonal antibody to isolate *Xenopus* tenascin since in preliminary experiments we could establish its ability to immunoprecipitate the 210 kDa *Xenopus* component (not shown) and since the conditions to elute tenascin bound to this antibody are well established [6]. SDS-PAGE analysis of the eluted fraction shows that the 210 kDa protein is indeed retained by the column (Fig. 1B, lane 1). Western blots of the eluted fractions and of purified chicken tenascin were reacted with anti-chicken tenascin antiserum. When proteins were run under reducing conditions (Fig. 1B, lanes 2, 3), the antiserum recognized the 220 kDa and 190–200 kDa bands of chicken tenascin as well as the 210 kDa *Xenopus* component. The presence of a unique band is similar to the results obtained with embryos of another amphibian species, *Pleurodeles waltl*, where anti-chicken tenascin antiserum immunoprecipitates only one 220 kDa polypeptide [27]. When blots were performed after protein electrophoresis under non-reducing conditions, the *Xenopus* component behaved very similar to chicken tenascin (Fig. 1C). Both proteins barely entered a 4% polyacrylamide gel showing that the *Xenopus* component isolated by affinity for mAb TnM1 is actually a disulfide-linked oligomer formed of 210 kDa polypeptides. Two distinct bands can be observed for the purified *Xenopus* protein indicating that different oligomeric forms are present.

In order to ascertain that the purified *Xenopus* protein is *Xenopus* tenascin, we have observed its structure by electron microscopy. Upon rotary shadowing, the molecules appear to be formed of pairs or triplets of arms attached to a central globular domain at opposite sites (Fig. 2). The arms of the molecule contain a thin region attached to the central core followed by a thicker part which is terminated by a globular domain as observed for tenascin isolated from other species [1,2,9,12–14]. However, molecules with 3, 4 or 5 arms were present but we failed to see the typical six-armed oligomers. This might be due to some proteolytic activity present during the cell culture or the purification

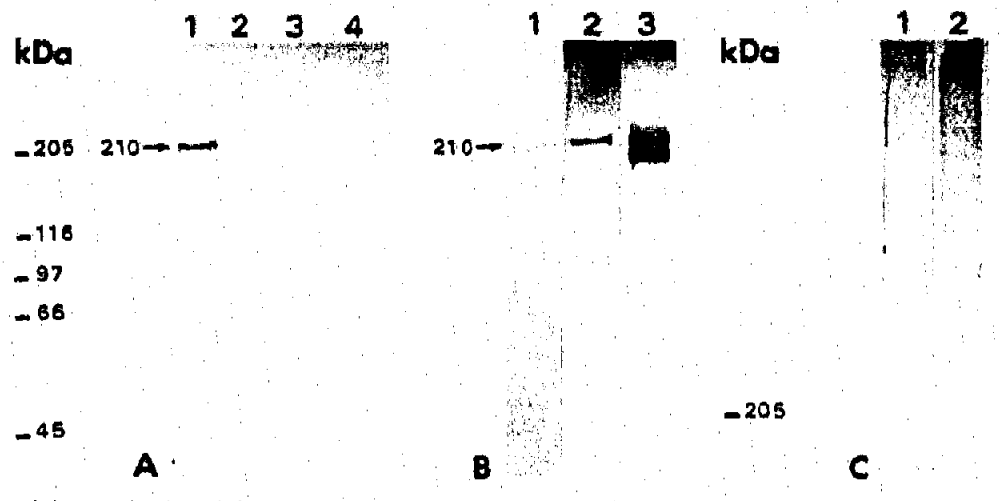

Fig. 1. Purification and characterization of *Xenopus* tenascin. (A) Identification of tenascin in the medium of XTC cells. Immunoblotting of conditioned media with anti-chicken tenascin antiserum (1/100) and peroxidase-conjugated goat anti-rabbit IgG antibody (1/200). (1) XTC; (2) XL; (3) XL 177; (4) KR. (B,C) Characterization of the *Xenopus* 210 kDa component. (B) Reducing conditions. (1) Coomassie blue staining of the eluted protein; (2, 3) Western blots (same conditions as in A), (2) *Xenopus* 210 kDa protein, (3) purified chicken tenascin. (C) Non-reducing conditions. Western blots (same conditions as in (A). (1) *Xenopus* protein, (2) Purified chicken tenascin.

steps rather than indicating a difference in the oligomeric form of the *Xenopus* molecule.

3.2. *Production and specificity of anti*-Xenopus *tenascin antibodies*

Purified *Xenopus* tenascin was injected into rabbits to produce polyclonal antibodies. The specificity of these antibodies was determined by Western blotting. When purified anti-*Xenopus* tenascin igGs were incubated with blots of XTC-conditioned medium they reacted only with the 210 kDa component (Fig. 3A). Anti-*Xenopus* tenascin antibodies were also tested on adult tissue extracts. We chose adult brain since cross-reactivity of anti-chicken tenascin antiserum with *Pleurodeles* tenascin had been studied on this tissue [27]. Fig. 3B shows that the antibody reveals three bands of 200 kDa, 210 kDa and 300 kDa. The observation that additional forms of tenascin polypeptides are expressed in the adult brain is not surprising since 190 kDa, 200 kDa, 220 kDa and 240 kDa compounds were found to react with anti-chicken tenascin polypeptides in the adult *Pleurodeles* brain. On the other hand, high molecular mass forms of tenascin polypeptides are present in the human (320 kDa) and the mouse (260 kDa) [2,50].

Finally, we have determined the distribution of tenascin by immunohistochemistry both on cultured XTC cell lines and on developing *Xenopus* tissues.

In XTC cell lines, tenascin appeared to be predominantly localized in secretory vesicles (Fig. 4A) and could be found uniformly deposited in the ECM in more advanced cultures. This subcellular distribution is in line with the presence of tenascin in the medium conditioned by this cell line. During further development

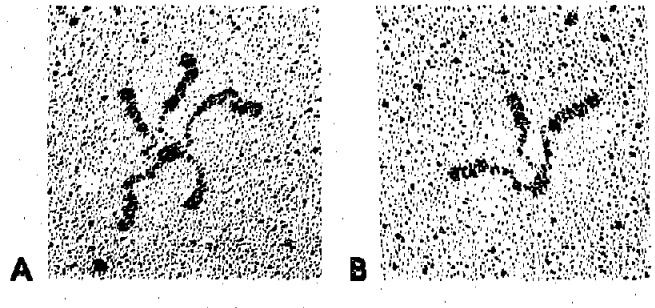

Fig. 2. Rotary shadowed electron micrograph of the purified *Xenopus* protein. (A) Oligomeric form with five arms. (B) Trimeric form. Magnification: 200 000 ×.

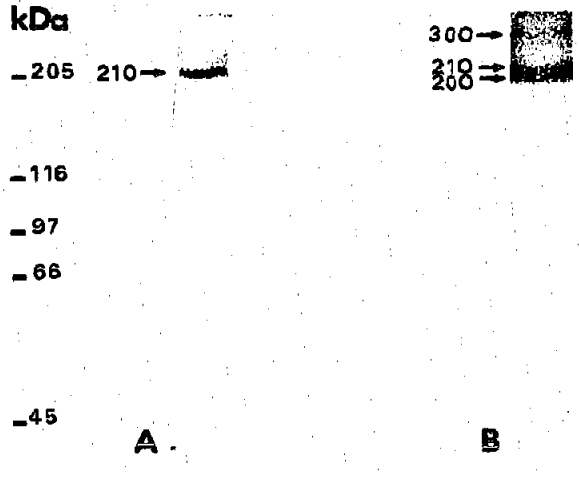

Fig. 3. Specificity of anti-*Xenopus* tenascin IgG. Immunoblotting with anti-*Xenopus* tenascin IgG (20 μg/ml) and peroxidase-conjugated goat anti-rabbit IgG antibody (1/200). (A) XTC-conditioned medium. (B) Adult brain.

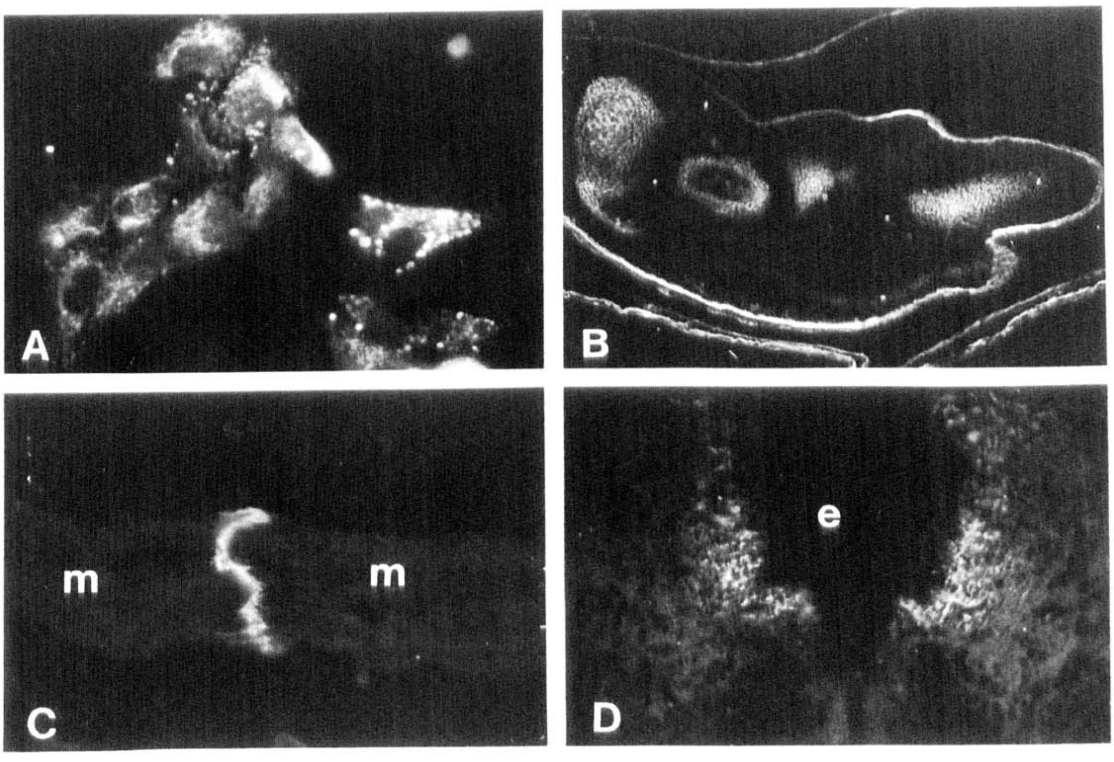

Fig. 4. Immunohistochemical localization of *Xenopus* tenascin. (A) Cultured XTC cells stained by anti-*Xenopus* tenascin antibodies (1/200). The staining is very strong in secretory vesicles and in the Golgi apparatus. (B) Section through the developing limb of a stage 55 *Xenopus* tadpole. Strong tenascin immunoreactivity is present in the basal lamina of the skin and in all the sites of cartilage condensation. (C) Section through the junction of two muscular masses in the ventral aspect of a stage 53 *Xenopus*. Tenascin immunoreactivity is restricted to the perimysium. (D) Section through the tectal area of a stage 55 *Xenopus* central nervous system. Tenascin immunoreactivity is restricted to a small area surrounding the ependyme. m, muscle; e, ependyme. Magnification: A, 500 ×; B, 50 ×; C, D, 200 ×.

the major sites of tenascin expression corresponded with those found in other species such as e.g. the developing cartilage (Fig. 4B), the basal lamina (Fig. 4B), connective tissues such as the myotendinous ligaments, the perimysium (Fig. 4C) and the periosteum and some restricted areas of the central nervous system (Fig. 4D). It must be noted that when sequential sections were stained with anti-*Xenopus* tenascin antibodies and with anti-chicken tenascin antibodies, the latter could not reveal the presence of the molecule in some regions of the nervous system thus indicating the presence of additional variants of the molecule in this tissue which are not recognized by anti-chick tenascin antibodies.

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REFERENCES

[1] Chiquet, M. (1989) Dev. Neurosci. 11, 266–275.
[2] Erickson, H.P. and Bourdon, M.A. (1989) Annu. Rev. Cell Biol. 5, 71–92.
[3] Arsanto, J.P., Dlano, M., Thouveny, Y., Thiery, J.P. and Levi, G. (1990) Development 109, 177–188.
[4] Chiquet-Ehrismann, R. (1990) FASEB J. 4, 2598–2604.
[5] Chiquet, M. and Fambrough, D.M. (1984) J. Cell Biol. 98, 1926–1936.
[6] Chiquet, M. and Fambrough, D.M. (1984) J. Cell Biol. 98, 1937–1946.
[7] Grumet, M., Hoffman, S., Crossin, K.L. and Edelman, G.M. (1985) Proc. Natl. Acad. Sci. USA 82, 8075–8079.
[8] Crossin, K.L., Hoffman, S., Grumet, M., Thiéry, J.P. and Edelman, G.M. (1986) J. Cell Biol. 102, 1917–1930.
[9] Hoffman, S., Crossin, K.L. and Edelman, G.M. (1988) J. Cell Biol. 106, 519–532.
[10] Bourdon, M.A., Matthews, T.J., Pizzo, S.V. and Bigner, D.D. (1985) J. Cell. Biochem. 28, 183–195.
[11] Faissner, A., Kruse, J., Chiquet-Ehrismann, R. and Mackie, E. (1988) Differentiation 37, 104–114.
[12] Erickson, H.P. and Iglesias, J.L. (1984) Nature (Lond.) 311, 267–269.
[13] Vaughan, L., Huber, S., Chiquet, M. and Winterhalter, K.H. (1987) EMBO J. 6, 349–353.
[14] Erickson, H.P. and Taylor, H.C. (1988) J. Cell Biol. 105, 1387–1394.

[15] Jones, F.S., Burgoon, M.P., Hoffman, S., Crossin, K., Cunningham, B.A. and Edelman, G.M. (1988) Proc. Natl. Acad. Sci. USA 85, 2186–2190.
[16] Jones, F.S., Hoffman, S., Cunningham, B.A. and Edelman, G.M. (1989) Proc. Natl. Acad. Sci. USA 86, 1905–1909.
[17] Pearson, C.A., Pearson, D., Shibahara, S., Hofsteenge, J. and Chiquet-Ehrismann, R. (1988) EMBO J. 7, 2677–2681.
[18] Spring, J., Beck, K. and Chiquet-Ehrismann, R. (1989) Cell 59, 325–334.
[19] Gulcher, J.R., Nies, D.E., Marton, L.S. and Stefansson, K. (1989) Proc. Natl. Acad. Sci. USA 86, 1588–1592.
[20] Prieto, A.L., Jones, F.S., Cunningham, B.A., Crossin, K.L. and Edelman, G.M. (1990) J. Cell Biol. 111, 685–698.
[21] Mackie, E.J., Chiquet-Ehrismann, R., Pearson, C.A., Inaguma, Y., Taya, K. and Sakakura, T. (1987) Proc. Natl. Acad. Sci. USA 84, 4621–4625.
[22] Tan Seong Seng, Crossin, K.L., Hoffman, S. and Edelman, G. (1987) Proc. Natl. Acad. Sci. USA 84, 7977–7981.
[23] Friedlander, D.R., Hoffman, S. and Edelman, G.M. (1988) J. Cell Biol. 107, 2329–2340.
[24] Lotz, M.M. Burdsal, C.A., Erickson, H.P. and McClay, D.R. (1989) J. Cell Biol. 109, 1795–1805.
[25] Chiquet-Ehrismann, R., Kalla, P., Pearson, C.A., Beck, K. and Chiquet, M. (1988) Cell 53, 383–390.
[26] Riou, J.F., Shi De Li, Chiquet, M. and Boucaut, J.C. (1990) Dev. Biol. 137, 305–317.
[27] Riou, J.F., Shi De Li, Chiquet, M. and Boucaut, J.C. (1988) Development 104, 511–524.
[28] Mackie, E.J., Thesleff, I. and Chiquet-Ehrismann, R. (1987) J. Cell Biol. 105, 2569–2579.
[29] Kruse, J., Keilhauer, G., Faissner, A., Timpl, R. and Schachner, M. (1985) Nature (Lond.) 316, 146–148.
[30] Sanes, J.R. (1989) Annu. Rev. Neurosci. 12, 431–491.
[31] Aufderheide, E. and Ekblom, P. (1988) J. Cell Biol. 107, 2341–2349.
[32] Aufderheide, E., Chiquet-Ehrismann, R. and Ekblom, P. (1987) J. Cell Biol. 105, 599–608.
[33] Inaguma, Y., Kusakabe, M., Mackie, E.J., Pearson, C.A., Chiquet-Ehrismann, R. and Sakakura, T. (1988) Dev. Biol. 128, 245–255.
[34] Thesleff, I., Mackie, E.J., Vaino, S. and Chiquet-Ehrismann, R. (1987) Development 101, 289–296.
[35] Mackie, E.J., Halfter, W. and Liver[illegible] (1988) J. Cell Biol. 107, 2757–2767.
[36] Daniloff, J.K., Crossin, K.L., Pi[illegible]aymond, M., Murawsky, M., Rieger, F. and Edelman, G.M. (1989) J. Cell Biol. 109, 625–635.
[37] Epperlein, H.H., Halfter, W. and Tucker, R.P. (1988) Development 103, 743–756.
[38] Mackie, E.J., Tucker, R.P., Halfter, W., Chiquet-Ehrismann, R. and Epperlein, H.H. (1988) Development 102, 237–250.
[39] Halfter, W., Chiquet-Ehrismann, R. and Tucker, R.P. (1989) Dev. Biol. 132, 14–25.
[40] Smith, J.C. (1989) Development 105, 665–678.
[41] Boucaut, J.C., Nakasuiji, N. and Johnson, K.E. (1989) In: Cytoplasmic Information Systems (Malacinski, G.M. ed.) Macmillan, New York.
[42] Wallace, H. (1981) J. Wiley and Sons.
[43] Pudney, M., Varma, M.G.R. and Leake, C.J. (1973) Experientia 29, 466–467.
[44] Anizet, M.P., Huwe, B., Pays, A. and Picard, J.J. (1981) In vitro 17, 267–274.
[45] Ellison, T.R., Mathisen, P.M. and Miller, L. (1985) Dev. Biol. 112, 329–337.
[46] Laemmli, U.K. (1970) Nature (Lond.) 227, 680–689.
[47] Riou, J.F., Shi De Li, Darribère, T., Boucaut, J.C. and Charlemagne, J. (1987) Dev. Growth Differ. 29, 443–454.
[48] Levi, G., Broders, F., Dunon, D., Edelman, G.M. and Thiery, J.P. (1990) Development 109, 681–692.
[49] Boyer, B., Tucker, J.C., Valles, A.M., Franke, W.W. and Thiery, J.P. (1989) J. Cell Biol. 109, 1495–1509.
[50] Weller, A., Beck, S. and Ekblom, P. (1989) Cell Diff. Dev. 27 Suppl. S54.