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Original Article

Expression and distribution of regeneration-responsive molecule during normal development of the newt, *Cynops pyrrhogaster*

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ABSTRACT We have investigated the expression and distribution of the regeneration-responsive molecule, 2NI-36, the loss of which is responsible for initiation of dedifferentiation of dorsal marginal iris pigmented epithelial cells to regenerate a lens. In the process of the normal development of the newt, the expression of 2NI-36 could not be detected in embryos at the early developmental stages, i.e., cleavages, gastrulation and neurulation, nor through later developmental stages to tail-bud, even though organogenesis was occurring. 2NI-36 was not detectable in any tissues until embryos reached developmental stage 40 (before hatching). In hatched larvae around developmental stage 46, strong expression of 2NI-36 was observed in several tissues including the vascular endothelium, the pigmented epithelium and the inner layer of skin epidermis. Moreover, 2NI-36 was present on the cell surface of these tissue cells. In conclusion, when the embryos hatch out to become swimming larvae that can feed by themselves, 2NI-36 begins to be expressed in some kinds of differentiated tissues. These results suggest that the function of 2NI-36 might be related to the completion of morphogenesis in development and also to the stabilization of the differentiated state of newly formed tissue cells.

KEY WORDS: *regeneration-responsive molecule, regeneration, organogenesis, eye development, limb development*

Introduction

Every animal possesses, to some degree, the ability to regenerate lost parts of the body. The newt particularly has enormous regenerative ability. Limbs, tails and even lenses can be regenerated in this animal. In a search for molecules responsible for the regulation of regeneration, we found a cell surface glycoprotein designated 2NI-36, which is widely distributed in various tissues and decayed temporarily in the mid-dorsal margin of the iris just before onset of dedifferentiation of iris pigmented epithelial cells (PECs) after lentectomy (Eguchi *et al.*, 1987; Eguchi, 1988; Imokawa *et al.*, 1990, 1992). This finding suggests that 2NI-36 is closely related to the stabilization of the differentiated state of iris PECs at the site of lens regeneration, and that temporary decay of 2NI-36 might be essential to dedifferentiation of iris PECs to produce a lens rudiment in Wolffian lens regeneration.

The process of regeneration can be thought to be repetitive development in lost parts. Thus both regeneration and development involve processes of cellular differentiation and stabilization. To clarify the role of 2NI-36 in the control of cell differentiation, comparison of its expression in regeneration and development must give valuable suggestions. Based on these assumptions and findings in the previous study, we investigated the pattern of expression of 2NI-36 in the development of the newt, *Cynops pyrrhogaster*.

Results

Expression and distribution during gill formation

In this study, the stages of developing embryos were determined according to the normal table for the Japanese newt, *Cynops (Triturus) pyrrhogaster* described by Okada and Ichikawa (1974). Some morphological features of embryos at each stage are described in Materials and Methods. Each stage in the normal table essentially corresponds to that for *Pleurodeles waltl* described by Gallien and Durocher (1957).

The expression of 2NI-36 was not observed in the embryos at the early developmental stages, i.e., cleavage and gastrulation, nor

Abbreviations used in this paper: 2NI-36mAb, 2NI-36 monoclonal antibody; FCS, fetal calf serum; PBS, Ca^{2+}- and Mg^{2+}-free phosphate buffered saline; PECs, pigmented epithelial cells.

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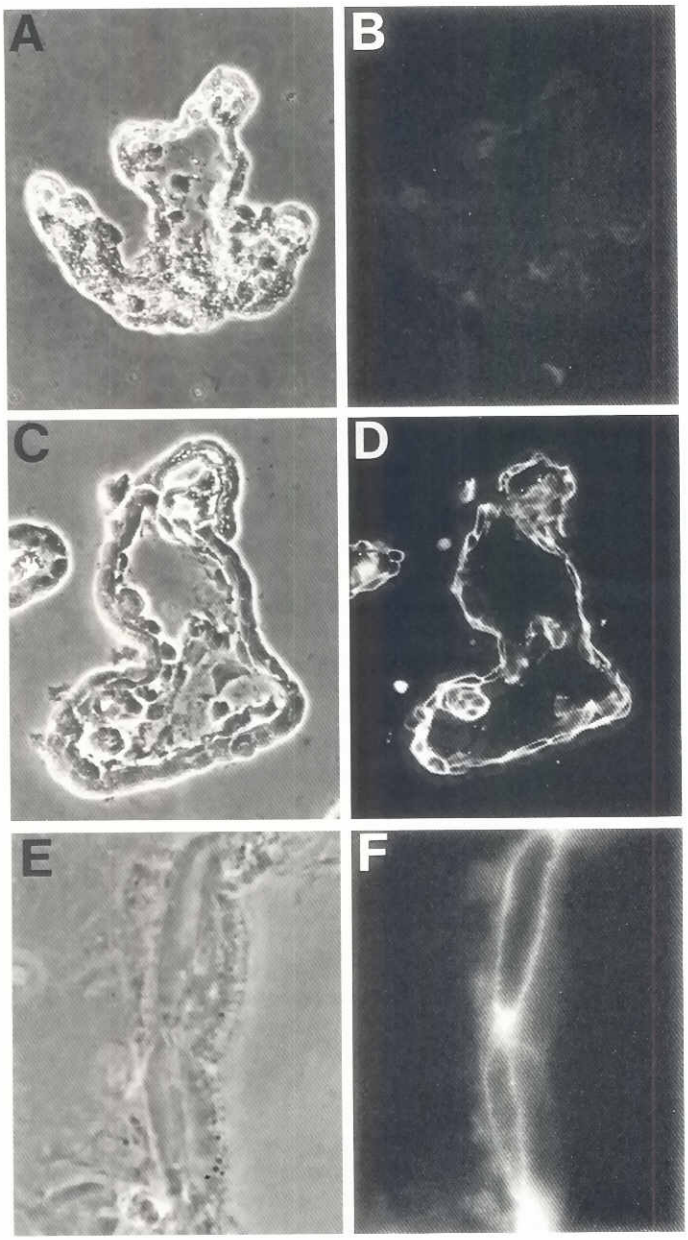


through later developmental stages to tail-bud. The Y-shaped blood vessels and tube-like hearts are developed in the tail-bud stage embryos which did not react to 2NI-36 mAb. Even in embryos developed to stage 40, the differentiated gills and accompanying blood vessels did not express 2NI-36 (Fig. 1A and B), in marked contrast with the findings for adults (Imokawa *et al.*, 1990, 1992). When development reached stage 46 (soon after hatching) the vascular endothelium in gills expressed 2NI-36 strongly (Fig. 1C and D). 2NI-36 existed on the cell surface of the vascular endothelial cells (Fig. 1E and F).

Expression and distribution during eye development

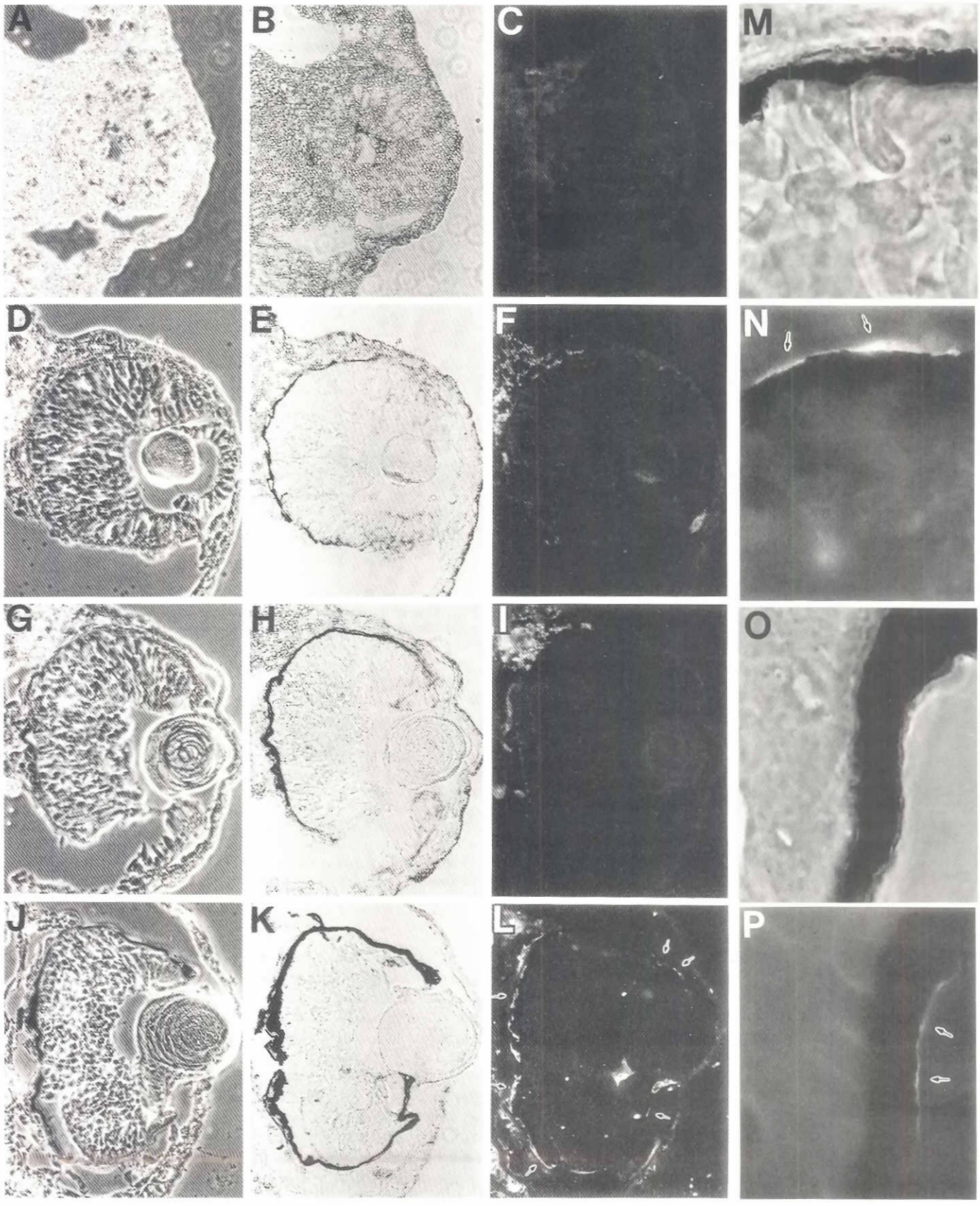
As 2NI-36 was found to decay at the dorsal marginal iris prior to dedifferentiation of PECs, we investigated the pattern of expression of this molecule in eye development. The optic vesicles of tail-bud stage embryos (stage 28) showed no expression of 2NI-36 (Fig. 2A-C). Expression of 2NI-36 was not detected in the developing eye of either stage 36 or 40 embryos (Fig. 2D-I). At both stage 36 and 40, the retinal pigmented epithelium had differentiated from the outer layers of the optic cup and was well pigmented, while the iris pigmented epithelium had few pigment granules (Fig. 2E and H). When the iris pigmented epithelium differentiated and became well-pigmented (stage 46, soon after hatching), strong expression of 2NI-36 was observed on both retinal and iris PECs (Fig. 2J-L). 2NI-36 was present on the cell surface and extracellular matrix (ECM) of both PECs (Fig. 2M-P). Blood vessels distributed around the eye strongly expressed 2NI-36, as shown in Fig. 2L. The neural retina did not express 2NI-36 in any stage, as observed in normal adult eye. The corneal stroma of stage 46 larvae did not show any expression of 2NI-36, in contrast with that of adults (Imokawa *et al.*, 1990, 1992).

Expression and distribution during limb development

Since our preliminary data suggest that 2NI-36 modulation occurs during limb regeneration (Imokawa *et al.*, 1990, 1992), we also examined the expression of this molecule in the developing limb at stages 36, 40 and 46 (Fig. 3). The expression of 2NI-36 could not be detected in either limb bud of stage 36 embryo or developing limb bud of stage 40 embryo (Fig. 3A-D). When the development of embryos progressed to stage 46 (soon after hatching), the fundamental structures of forelimbs had been almost formed and strong expression of 2NI-36 was observed in the epidermis (Fig. 3E and F). Observation at higher magnification showed that 2NI-36 specifically localized on the cell surface of an

Fig. 1 (up). Immunohistochemical staining of the developing gill with 2NI-36 mAb. *(A, C and E) Phase-contrast micrographs. (B, D and F) Fluorescent micrographs.* **(A, B)** *Transverse section of the gill of stage 40 embryo. 2NI-36 could not be detected.* **(C, D)** *Transverse section of the gill of stage 46 larva. 2NI-36 mAb strongly reacted with the vascular endothelium in the gill.* **(E, F)** *Highly magnified micrographs of a similar section as in C and D. Right portions of both Figs. E and F point to the outside of the gill. 2NI-36 was present on the cell surface of vascular endothelial cells. Magnification: A-D, x125; E and F, x800.*

Fig. 2 (next page). Immunohistochemical staining of the developing eye with 2NI-36 mAb. *(A, D, G, J, M and O) Phase-contrast micrographs. (B, E, H and K) Transmission light micrographs. (C, F, I, L, N and P) Fluorescence micrographs.* **(A-C)** *Tail-bud stage. optic vesicle shows no staining with 2NI-36 mAb.* **(D-F)** *Stage 36.* **(G-I)** *Stage 40. 2NI-36 mAb did not react with the eyes of embryo at either stage. Note that the retinal pigmented epithelial cells (PECs) of both stages 36 and 40 embryos are well-pigmented, while the iris PECs have few pigment granules (E and H). Fluorescent spots in the top left corner of (F) and (I) are autofluorescence of granules in the skin, which are present also in the negative controls (not shown).* **(J-L)** *Stage 46. 2NI-36 mAb strongly reacted with both iris and retinal PECs. (K) shows that iris is well-pigmented. Strong staining at the top left and bottom left are blood vessels around the eye.* **(M-P)** *Highly magnified micrographs of a similar section as in (J) and (L). (M) and (N) show the retina pigmented epithelium, top pointing to the outside of the eye. (O) and (P) show the dorsal iris, the right hand side pointing to the anterior. 2NI-36 was present on the basal surface of both retinal and iris PECs. Arrows indicate the expression sites of 2NI-36. Magnifications: A to I x100. J to I x85, M to P x950.*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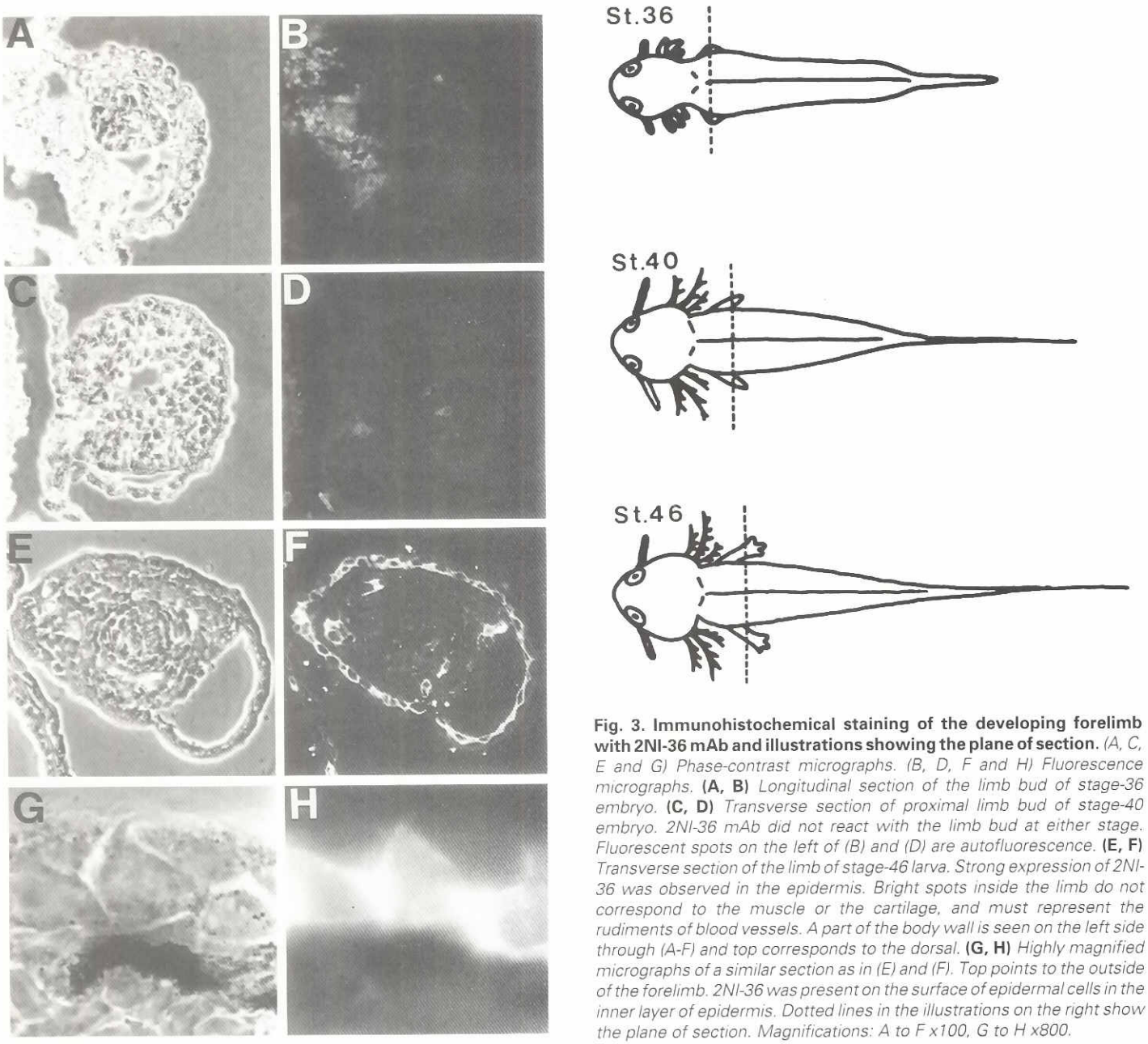

Fig. 3. Immunohistochemical staining of the developing forelimb with 2NI-36 mAb and illustrations showing the plane of section. *(A, C, E and G) Phase-contrast micrographs. (B, D, F and H) Fluorescence micrographs.* **(A, B)** *Longitudinal section of the limb bud of stage-36 embryo.* **(C, D)** *Transverse section of proximal limb bud of stage-40 embryo. 2NI-36 mAb did not react with the limb bud at either stage. Fluorescent spots on the left of (B) and (D) are autofluorescence.* **(E, F)** *Transverse section of the limb of stage-46 larva. Strong expression of 2NI-36 was observed in the epidermis. Bright spots inside the limb do not correspond to the muscle or the cartilage, and must represent the rudiments of blood vessels. A part of the body wall is seen on the left side through (A-F) and top corresponds to the dorsal.* **(G, H)** *Highly magnified micrographs of a similar section as in (E) and (F). Top points to the outside of the forelimb. 2NI-36 was present on the surface of epidermal cells in the inner layer of epidermis. Dotted lines in the illustrations on the right show the plane of section. Magnifications: A to F x100, G to H x800.*

inner layer of epidermal cells facing the future dermis (Fig. 3 G and H). Although skeletal muscle and cartilage did not express 2NI-36 at stage 46, some expression sites were observed in larval skeletal muscle (Fig. 3E and F). We assumed that the tissues at these sites might correspond to blood vessel rudiments.

Discussion

In this paper, we have reported the pattern of expression of a regeneration-responsive molecule, 2NI-36, in the developing embryo of the newt. This molecule could not be detected by immunohistochemical examinations during the early stages of development through stage 40. It first became detectable in the iris and retinal pigmented epithelium at developmental stages later than stage 46, at which stage embryos hatched and began to swim. Likewise, 2NI-36 could not be detected throughout the earlier stages of limb development. But strong expression was observed only in an inner layer of epidermis at developmental stages later than stage 46, at which stage the fundamental structures of forelimbs have already differentiated. It is also interesting that expression of 2NI-36 in vascular endothelium of developing gills synchronized with that in both developing eyes and limbs. These findings lead us to conclude that 2NI-36 is not required during the development of tissue and organ rudiments and might play highly

important roles in stabilizing and maintaining the differentiated state of cells to organize the tissue architecture.

Although other monoclonal antibodies cross-reacting with regeneration-responsive molecules have been reported (Kintner and Brockes, 1984, 1985; Tassava *et al.*, 1986, 1987), only the 22/18 antigen of these molecules was examined for the pattern of its expression on development. This antigen was expressed on the early blastemal cells of the regenerating limb of the newt. The same antigen was also found to be expressed on the epidermis, glial cells and the lens at the early developmental stages, i.e., stages 33 and 38 embryos of the European newt, *Pleurodeles waltl* (Fekete and Brockes, 1987). In contrast, 2NI-36 became detectable at a later developmental stage (stage 46), after the completion of morphogenesis of fundamental tissue structure. The timing of the expression of 2NI-36 is obviously different from that of 22/18 antigen. This point also suggests that 2NI-36 is different from 22/18 antigen and may be involved in stabilizing the differentiated state of several tissue cells after the completion of morphogenesis.

Although lens and nervous tissues are well differentiated around developmental stage 46, 2NI-36 was not present in these differentiated tissue cells. We suggest two possibilities for explaining this point. First, these tissue cells may have another molecule similar to 2NI-36. Second, these tissue cells may not need to have the differentiated state stabilized by 2NI-36, because these tissue cells, which are usually terminal-differentiated, cannot grow and transdifferentiate into another type of tissue cell. In contrast with those tissue cells, PECs, epidermal cells and vascular endothelial cells, which continue to express 2NI-36 after stage 46, have the capacity to grow and construct other tissue cells. Therefore, these cells may need 2NI-36 to stabilize their differentiated state. Interestingly, the adult epidermis does not express 2NI-36, but larval epidermis temporarily expressed this molecule after hatching. And the adult muscle and dermis express 2NI-36, but larval skeletal muscle does not express this molecule around stage 46. This difference of the timing in the expression on these tissues may correspond to metamorphosis.

Finally, it must be relevant to the proposed function of 2NI-36 that the larval skeletal muscle does not express this molecule even after the formation of the fundamental structures of forelimbs, even though the adult skeletal muscle strongly expresses the molecule (Imokawa *et al.*, 1990, 1992). This difference of expression of 2NI-36 may be the cause of the faster regeneration of larval limbs. Earlier studies showed that larval limbs can regenerate much faster than those of adults (Tomlinson *et al.*, 1985; Tassava *et al.*, 1987). Moreover, we observed that 2NI-36 temporarily disappeared during adult limb regeneration. We assume that larval limbs can regenerate faster than those of adults because 2NI-36 is not yet expressed in the larval skeletal muscle and larvae do not have to degenerate this molecule. We are now extending our study to molecular analysis and cloning the gene coding 2NI-36 in order to understand the precise function of this molecule and also the regulatory mechanism of stabilization of the differentiated state of tissue cells.

Materials and Methods

Animals, eggs and embryos

Adult newts, *Cynops pyrrhogaster*, were collected from Tagarasu, Obama, Fukui prefecture, and maintained at 10°C. Female newts were injected with 50 units/day of gonadotropin (Sigma Chemical Co.) for three days. The obtained fertilized eggs and embryos were maintained at 20°C during development. The developmental stages of embryos and larvae were determined according to the normal table of Okada and Ichikawa (1947). Stage 28 embryos are late tail-bud embryos which have formed eye-cups, Y-shaped blood vessels and tube-like hearts. Stage 36 embryos have formed conspicuous limb buds. Stage 40 embryos have posteriorly elongated limb buds without digits. Stage 46 larvae, which have hatched and begun to swim, have three digits in forelimb (see Fig. 3).

Preparation of embryos for immunohistochemistry

Embryos and larvae were fixed with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 5 h at 4°C and washed 6 times with the same buffer. The jelly coats and the vitelline membranes were removed from embryos using sharp forceps after fixation. Fixed samples were infiltrated with a graduated series of sucrose solutions in 0.1 M phosphate buffer (pH 7.4) at 4°C and embedded in Tissue-Tek O.C.T. compound (Miles Scientific). They were then rapidly frozen in liquid nitrogen. Serial frozen sections of 10 μm in thickness were cut in a cryostat (Cambridge Instruments GmbH) at -20°C and were collected on albumin-coated glass slides.

Antibody and indirect immunofluorescence

The antibody used as the probe is 2NI-36 monoclonal antibody (2NI-36 mAb), which was generated by immunization of mice with homogenates of the newt iris tissues. This 2NI-36 mAb detects a novel regeneration-responsive molecule, 2NI-36, which is degraded during the initiation of lens regeneration in the newt (Eguchi *et al.*, 1987; Eguchi, 1988).

Cryosections of embryos were treated with fetal calf serum (FCS) diluted 1:10 with Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline (PBS) for 1 h and subsequently treated with 2NI-36 mAb for 12 h at 4°C. After washing in PBS for 30 min, the sections were treated with fluorescein isothiocyanate-conjugated anti-mouse IgG (Cappel Laboratories Inc.) diluted 1:50 with PBS for 4 h. After washing with PBS, the specimens were mounted and observed under a fluorescence microscope.

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