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Thyroxine-dependent modulations of the expression of the neural cell adhesion molecule N-CAM during *Xenopus laevis* metamorphosis

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Summary

During amphibian metamorphosis, a complete remodeling of the phenotype takes place under complex hormonal control whose final effectors are thyroid hormones. This process implies the activation of coordinated programs of cell death, proliferation, migration, adhesion and differentiation. Inasmuch as the neural cell adhesion molecule N-CAM is thought to play a central role in the control of morphogenetic processes, we have studied by immunohistofluorescence and immunoblots the patterns of expression of N-CAM at different stages of *Xenopus laevis* metamorphosis. A scan was made of all major organs and appendages.

Before the metamorphic climax, all neuronal cell bodies and processes express high levels of N-CAM. During the metamorphic climax, N-CAM expression decreases sharply on the cell bodies and processes of the peripheral nervous system (PNS) but remains high in the central nervous system (CNS). Towards the end of metamorphosis, the PNS and spinal nerves are virtually negative for N-CAM while the CNS is still positive. The optic and olfactory nerves, although myelinated, are still strongly positive for N-CAM. The lens and olfactory epithelia express N-CAM throughout metamorphosis. In the brain, N-CAM is present at all times as three polypeptides of 180, 140, and 120×10^3 M_r; before metamorphosis some of the N-CAM is in its polysialylated form. During metamorphosis and the subsequent growth of the animal, the amount of N-CAM decreases gradually. In all polypeptides, the polysialylated form is the first to disappear.

Cardiac muscle expresses high level of N-CAM from its first formation throughout metamorphosis; in contrast, the level of N-CAM in skeletal muscle is high in newly formed muscles, but decreases rapidly after myoblast fusion.

The liver of adult *Xenopus* contains large amounts of a 160×10^3 polypeptide that is recognized by polyclonal and monoclonal antibodies against N-CAM. cDNA probes of *Xenopus* brain N-CAM recognize major transcripts of 9.2, 3.8 and 3.3 kb in *Xenopus* liver mRNA; these bands are different in size from those recognized in brain mRNA (9.5, 4.2 and 2.2 kb).

Premetamorphic liver does not express the 160×10^3 form of N-CAM, which can be first detected at stage 59 and persists then through all the life of the animal. Expression of N-CAM in the liver can be induced in premetamorphic animals (stage 51–52) by a 48 h treatment with thyroxine. All hepatocytes are responsive.

The thyroxine-dependent induction of the expression of N-CAM in the liver provides the first example of hormonally induced expression of a cell adhesion molecule. These results are discussed in terms of the potential morphogenetic roles of the various forms of N-CAM in development and metamorphosis.

Key words: cell adhesion, metamorphosis, thyroxine.

Introduction

Numerous studies suggest that cell surface glycoproteins involved in the formation of cell–cell contacts play a critical role in determining cell patterning, movement and differentiation during embryonic development as well as in the control of the cellular events maintaining tissue integrity and repair throughout the life of the animal (Edelman, 1986*a*,*b*; Takeichi, 1988; Thiery, 1989). Using specific monoclonal antibodies that perturb the aggregation or the adhesion of cells *in vitro*, it has been possible to isolate and characterize a number of these molecules known as cell adhesion molecules or CAMs (Thiery *et al.* 1977; Gallin *et al.* 1983; Grumet and Edelman, 1984; Hatta *et al.* 1985; Volk and Geiger, 1986).

N-CAM is a cell surface glycoprotein that mediates Ca^{2+}-independent aggregation of cells by a homophilic mechanism (Hoffman *et al.* 1982; Hoffman and Edelman, 1983). N-CAM is the prototype member of adhesion molecules belonging to the IgG superfamily (Edelman, 1987); it contains five Ig-like constant

domains in the extracellular portion of all its polypeptide variants (Cunningham *et al.* 1987). In the chicken and in the mouse, the polypeptides differ in their cell-associated domains as a result of alternative splicing of RNA (Murray *et al.* 1986*a*,*b*; Owens *et al.* 1987; Hemperly *et al.* 1986*a*,*b*; Cunningham *et al.* 1987; Barbas *et al.* 1988; Santoni *et al.* 1989) transcribed from a single N-CAM gene (D'Eustacchio *et al.* 1985). The relative amount of each polypeptide varies in different tissues and at different epochs of development; a $180\times10^3 M_r$ (K) form of the molecule (the ld polypeptide), for example, is synthesized exclusively in the nervous system and is first produced at relatively late stages of development while a 140K form (the sd polypeptide) is already present in the early blastula and then persists in many different tissues (Murray *et al.* 1986*b*; Levi *et al.* 1987). These changes, most probably, reflect modulations of the capacity of N-CAM to interact with the cytoskeleton and/or with other molecules in the membrane, events that should ultimately affect the adhesive and morphogenetic function of the molecule.

During embryonic development, N-CAM is present transiently on derivatives of all three germ layers; its expression is strongly modulated in areas undergoing embryonic induction such as the neural plate and the placodes and in the somites (Thiery *et al.* 1982; Crossin *et al.* 1985; Levi *et al.* 1987). During later development, the expression of N-CAM becomes progressively restricted to neurons and astrocytes in the CNS and to cell bodies, unmyelinated axons and non-myelinating Schwann cells of the PNS (Chuong and Edelman, 1984; Daniloff *et al.* 1986*a*). In skeletal muscle, the molecule is transiently expressed before and during the formation of neuromuscular contacts and persists mainly in neuromuscular junctions in older animals (Rieger *et al.* 1985; Covault and Sanes, 1986). In denervated muscles and in regenerating nerves, however, the expression of N-CAM can be reinduced (Daniloff *et al.* 1986*b*; Covault and Sanes, 1985).

Despite the volume of descriptive information about the modulation of N-CAM expression at different stages of development, not much is yet known about the molecular signals and modes of regulation of the expression of the molecule in its various forms. So far, the only molecule known to influence N-CAM expression is the nerve growth factor NGF (Prentice *et al.* 1987; Doherty *et al.* 1988).

To establish a system to study other possible molecular controls of N-CAM expression, we have chosen to investigate the modulations of N-CAM expression occurring during *Xenopus laevis* metamorphosis, inasmuch as metamorphosis offers one of the most striking cases of morphogenesis and it occurs under direct hormonal control.

The phenomenon of metamorphosis in amphibia involves morphological, histological and physiological changes associated with the shift from aquatic to terrestrial life and from herbivorous to a carnivorous diet. Virtually all organs and tissues in the animal undergo profound remodeling. For example, the tail is resorbed; limbs develop; the nervous system becomes adapted to control limb movement with a *de novo* formation of the cerebellum, motor neurons and nerves, and profound changes in the ganglia; the digestive tract is redesigned by means of a complex process of simultaneous degeneration and regeneration; muscles are formed; bones and cartilages are reshaped and the skin restructured.

All these morphological changes take place in a few days under the direct control of thyroid hormones. That thyroxine, and no other systemic factor, is the essential stimulus for metamorphosis has been shown by treating various tissues *in vitro* with the hormone and observing their subsequent modification (For reviews see: Deuchar, 1975; Fox, 1981; White and Nicoll, 1981). One of the most interesting features of metamorphosis is reflected by the fact that several organs and tissues of the larva respond in such different ways to only one hormone. It has been shown that the nuclear receptor for thyroxine is the cellular equivalent of the oncogene v-erbA (Weinberger *et al.* 1986; Koening *et al.* 1988); it is conceivable that different variants of this molecule might activate totally different developmental programs leading either to histolysis or to further tissue differentiation (Miyajima *et al.* 1989).

We reasoned that, if N-CAM is involved in the control of morphogenetic events, its expression should be modulated by thyroid hormones at sites of tissue remodeling in metamorphosing animals. The identification of such modulation events might then provide a basis for the development of an *in vitro* model system in which to study the regulation of expression of a morphogenetic molecule. Such a system would be ideal for further experimental investigation inasmuch as the molecular mechanisms underlying the thyroxine-dependent control of gene expression are now beginning to be understood (Evans, 1988; Glass *et al.* 1988; Damm *et al.* 1989; Koenig *et al.* 1989).

Materials and methods

Animals, hormonal treatment

Sexually mature *Xenopus laevis* were obtained from the Service d'Elevage de Xenopus of the Centre Nationale de la Recherche Scientifique (Montpellier). Animals at different stages through metamorphosis were purchased from Nasco (Fort Atkinson, Wisconsin). Animals were either used immediately upon delivery or maintained at 24°C and fed twice weekly with Nasco *Xenopus* brittle. Stages of development were determined according to Nieuwkoop and Faber (1967). Treatment of animals with thyroid hormones (TH) was achieved by rearing the animals in water containing 3×10^{-7} M thyroxine (T_4) (Atkinson, 1981). The rearing solution was obtained by diluting a commercial aqueous solution of L-thyroxine (0.2 M, Roche) and was changed daily.

Antibodies

The preparation and the characterization of the polyclonal and monoclonal antibodies directed against *Xenopus* N-CAM used in this study has been previously described (Fraser *et al.* 1984; Levi *et al.* 1987). Characterization of NC-1 antibody and

of its reactivity on early *Xenopus* nervous system has been previously described (Tucker *et al.* 1984, 1988).

Immunohistochemistry

Paraffin sections were prepared for staining using a previously published procedure (Levi *et al.* 1987; Gurdon *et al.* 1976) with some modifications required for the size of metamorphic animals. Whole animals or dissected organs were frozen in isopentane cooled in liquid nitrogen and immediately immersed in methanol at −80°C. The samples were then maintained in methanol at −80°C for periods ranging from one to four months depending on the size of the animal with weekly changes of cold methanol. The tissues were serially transferred to methanol equilibrated at −20°, 4°, and 20°C for at least one day at each step. The samples were then immersed twice in xylene until completely clarified for a total period not greater than 30 min and then transferred to a solution of 50% Paraplast (Monoject Scientific, St. Louis, MO) in xylene at 56°C for 20 min in a vacuum oven, infiltrated three times with Paraplast at 56°C under vacuum for 45 min, and embedded in Paraplast. Sections 10 mm thick were cut using a microtome (Lemardeley, Paris, France), floated on distilled water at 45°C, collected on washed glass slides and dried on a heating plate at 45°C for at least one hour.

For immunofluorescent staining, the deparaffinized sections were incubated sequentially with the primary antibody ($10\,\mu g\,ml^{-1}$ in PBS, 5% foetal calf serum (FCS); overnight) and a rhodamine-conjugated goat anti-rabbit IgG secondary antibody ($10\,\mu g\,ml^{-1}$ in PBS, 5% FCS. Nordic Immunology, Tilburg, The Netherlands) for 2 h. For double labeling, the sections were treated with a mixture of the two primary antibodies ($10\,\mu g\,ml^{-1}$ each in PBS, 5% FCS; overnight) followed by a mixture of rhodamine-conjugated goat anti-rabbit IgG and biotinylated goat anti-mouse secondary antibodies ($10\,\mu g\,ml^{-1}$ each in PBS, 5% FCS; 2 h) and FITC-conjugated streptavidin $5\,\mu g\,ml^{-1}$ in PBS, 5% FCS; 30 min). The sections were observed with a Leitz epifluorescence microscope.

Western blots

Tissues were dissected from animals at different stages of development, homogenized in 10 volumes of PBS containing 0.5% NP40, the protein concentration of the extract was measured, and SDS sample buffer was added to yield a final protein concentration of $2\,mg\,ml^{-1}$. Samples were resolved by SDS–PAGE (Laemmli, 1970) on 7% polyacrylamide gels followed by electrophoretic transfer of the proteins to nitrocellulose paper (Towbin *et al.* 1979). The presence of N-CAM was revealed by incubation with 50 μg of antibodies followed by [^{125}I]protein A and autoradiography. Autoradiograms were scanned and analyzed with an LKB Ultrascan XL computerized gel scanner.

Northern blots

Total RNA from organs was prepared by a modified guanidinium/cesium chloride centrifugation method (Mac Donald *et al.* 1987). Tissues were homogenized in ten volumes of 4 M guanidinium thiocyanate, 0.05 M sodium acetate, 2 mM EDTA and 1 M β-mercaptoethanol. Solid CsCl was further added to reach a concentration of 4.5 M. Homogenates were laid onto a cushion of 6.7 M CsCl ($\rho=1.82\,g\,ml^{-1}$), 0.05 sodium acetate, 1 mM EDTA. After an overnight centrifugation at 40 000 revs min^{-1} at 20°C in a 70.1 Ti rotor (Beckman), RNA appeared as a band in the CsCl gradient and was recovered with a syringe and ethanol precipitated.

About 10 μg of RNA from liver and brain was heated at 60°C in 50% formamide in 4-morpholinopropane sulfonic acid buffer and then electrophoresed in a 1% agarose gel containing 6% formaldehyde in the running buffer. RNAs were blotted onto nylon membrane (Hybond N, Amersham). Recombinant plasmid including a 1.8 kb insert corresponding to an *Eco*RI fragment of the 5′ end of *Xenopus* was a gift from Dr Kintner; the probe was labeled by random priming with α^{32}P-dCTP. Filters were hybridized with the random primed probe at 42°C in buffer containing 50% formamide, 5×SSPE, 2×Denhardt (0.2% polyvinylpyrrolidone/0.2% Ficoll/0.2% bovine serum albumin), 5% dextran sulfate, 0.1% SDS and 20 μg of salmon sperm DNA per ml. Blots were washed in 0.1×SSC, 0.1% SDS at 55°C and autoradiography performed on Kodak X-OMAT film for 1–3 days.

Results

Changes in the nervous system during metamorphosis

Using double labeling, we compared the patterns of staining of antibodies against *Xenopus* N-CAM to those of the monoclonal antibody NC-1. This monoclonal antibody recognizes a carbohydrate epitope that in *Xenopus laevis* is present on most neurons and neural processes; in this species, it is therefore a useful marker of neural cells (Tucker *et al.* 1988).

In premetamorphic and prometamorphic animals, all neuronal cell bodies and processes of the CNS and the PNS and the neuroepithelial cells lining the ependymal canal were intensely stained by anti-N-CAM antibodies (Figs. 1A, 2A). The NC-1 epitope was present in neural processes of the CNS and PNS (Figs. 1B, 2B) and on cell bodies in the PNS but was only weakly expressed by CNS neural cell bodies and was not present in the neural epithelium (Fig. 1B). Reactivity to both antibodies was limited to the cell surface. This pattern of staining persisted unchanged between stages 51 and 61 NF. During the metamorphic climax, between stages 62 and 66 NF, the staining of N-CAM, while persisting in the CNS, decreased dramatically and rapidly in the PNS, so that by stage 64, the dorsal root ganglia (DRG) (Fig. 1C) and peripheral nerves (Figs. 1C, 2C) were virtually negative for the molecule, which persisted only in few unmyelinated fibers. In contrast, reactivity to NC-1 persisted unchanged on the cell bodies of both DRG (Fig. 1D) and peripheral nerve (Figs. 1D, 2D). Both the optic nerve (Fig. 2E,F) and the olfactory nerve (Fig. 8D) maintained a high level of N-CAM expression; embryologically and cytologically both these nerves constitute an integral part of the CNS.

Treatment of stage 56 animals with 3×10^{-7} M thyroxine for 4 days, although inducing profound morphological changes in the nervous system (e.g. the formation of the lateral motor columns) did not induce a rapid disappearence of N-CAM from the PNS. The disappearance of N-CAM was induced only by a much longer treatment (at least 10 days), suggesting that the down-regulation of N-CAM in the nervous system is not under the direct control of the hormone, but depends on a more complex cascade of events.

Throughout metamorphosis, N-CAM is expressed in the nervous system as three principal components of relative molecular mass 180, 140, and 120×10^3. In

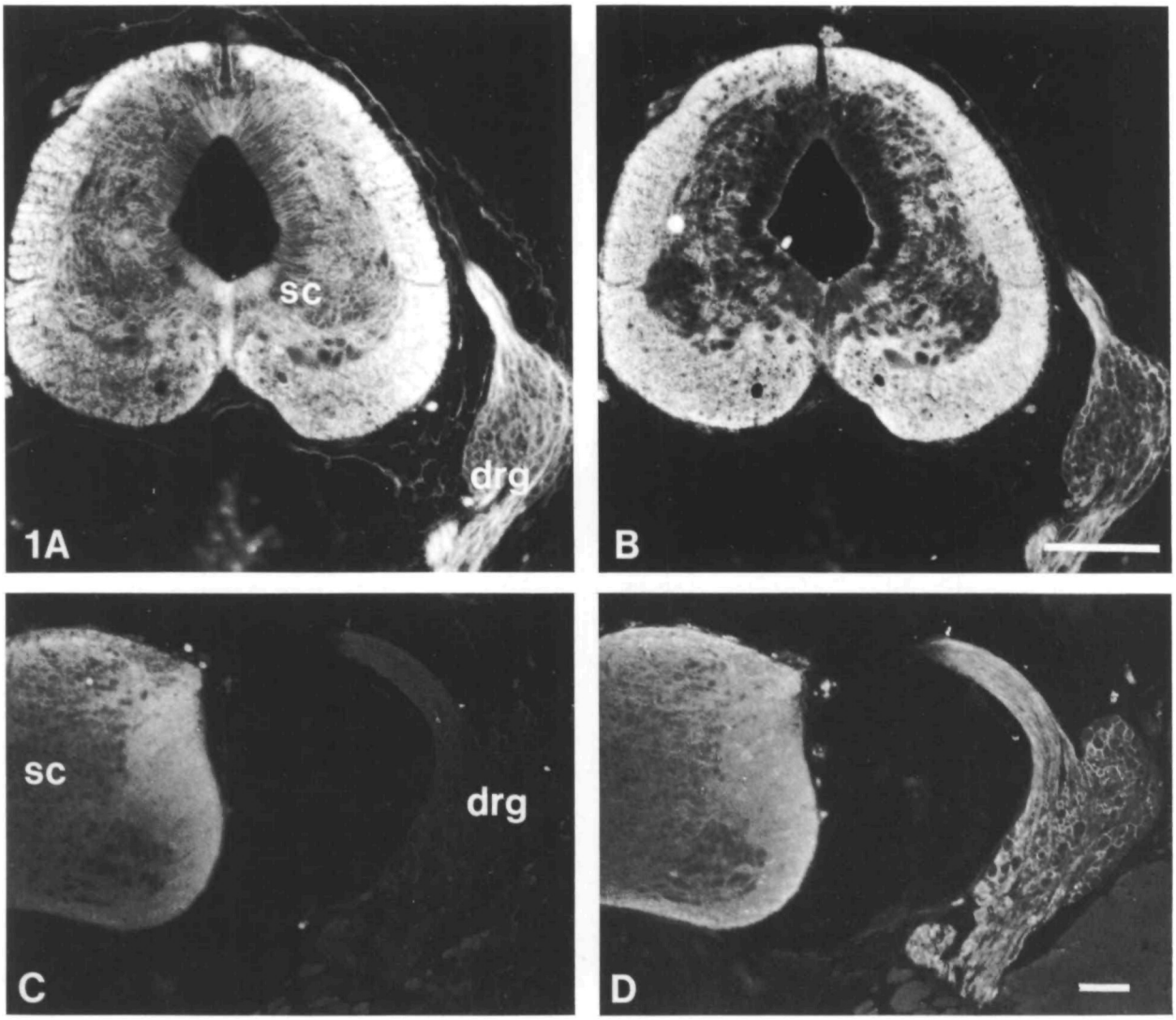

Fig. 1. Distribution of N-CAM and NC-1 immunoreactivity in the spinal cord and dorsal root ganglia. Section of premetamorphic *Xenopus* tadpoles (stage 54 NF) (A,B) and of animals toward the end of the metamorphic climax (stage 64 NF) (C,D) were double stained with polyclonal antibodies against *Xenopus* N-CAM (A,C) and with NC-1 monoclonal antibody (B,D). Before metamorphosis all neuronal cell bodies and processes of the CNS and peripheral ganglia including the neural epithelium lining the ependymal canal were labeled by anti-N-CAM antibodies; NC-1 recognized mostly the fibers in the CNS and cell bodies and fibers in the PNS. After metamorphosis the staining of anti-N-CAM antibodies persisted in the CNS but disappeared on the cell bodies and fibers of the PNS; intense staining with anti-NC-1 persisted both in the CNS and in the PNS. drg, dorsal root ganglia; sc, spinal cord. Bar: 100 μm.

premetamorphic stages, some of the molecule appears as a polydisperse region corresponding to the highly polysialylated E (embryonic) form that has been described in the early stages of development of all species examined so far (Hoffman *et al.* 1982; Friedlander *et al.* 1985; Levi *et al.* 1987). During metamorphosis and later development, the E form of N-CAM disappears and the relative amount of the molecule present in the nervous system diminishes gradually as normalized to total membrane protein (Fig. 5 lanes 5, 7, 8). Quantitative analysis of different autoradiograms showed that the relative amount of the three major polypeptides did not change considerably during development. Moreover, treatment of stage 56 tadpoles with thyroxine for 4 days does not produce any evident change in the forms of the molecule (Fig. 5 lane 5, 6).

Thyroxine-dependent induction of N-CAM in the liver

We have previously reported that adult *Xenopus* hepatocytes express high levels of a particular variant of N-CAM of relative molecular mass 160×10^3. This molecule can be immunoprecipitated and recognized in immunoblots by polyclonal and monoclonal antibodies against *Xenopus* N-CAM as well as by cross-species polyclonal antibodies against chicken brain N-CAM (Levi *et al.* 1987). It is unusual to observe N-CAM in adult liver and other species examined so far do not show it.

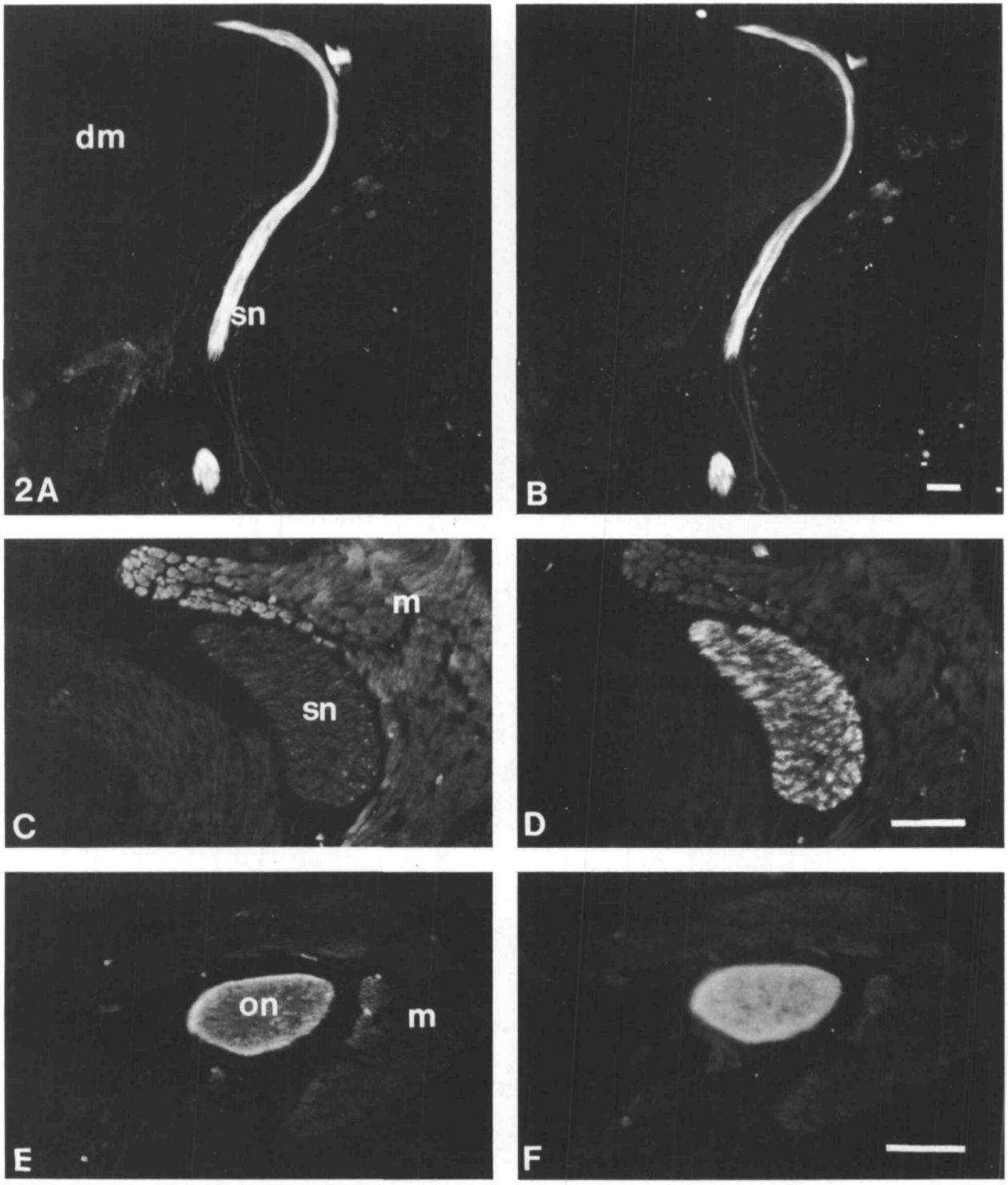

Fig. 2. Distribution of N-CAM and NC-1 immunoreactivity in the nerves. Section of premetamorphic *Xenopus* tadpoles (stage 52 NF) (A,B) and of animals toward the end of the metamorphic climax (stage 64 NF) (C–F) were double stained with polyclonal antibodies against *Xenopus* N-CAM (A,C,E) and with NC-1 monoclonal antibody (B,D,F). Before metamorphosis all nerves were intensely stained by anti-N-CAM and NC-1 antibodies. During metamorphosis the staining of anti-N-CAM antibodies was greatly reduced in peripheral nerves (C) but remained high in central nerves such as the optic nerve (E); NC-1 staining persisted in the nerves throughout metamorphosis. dm, dorsal muscle; m, muscle; on, optic nerve; sn, sciatic nerve. Bar: 100 μm.

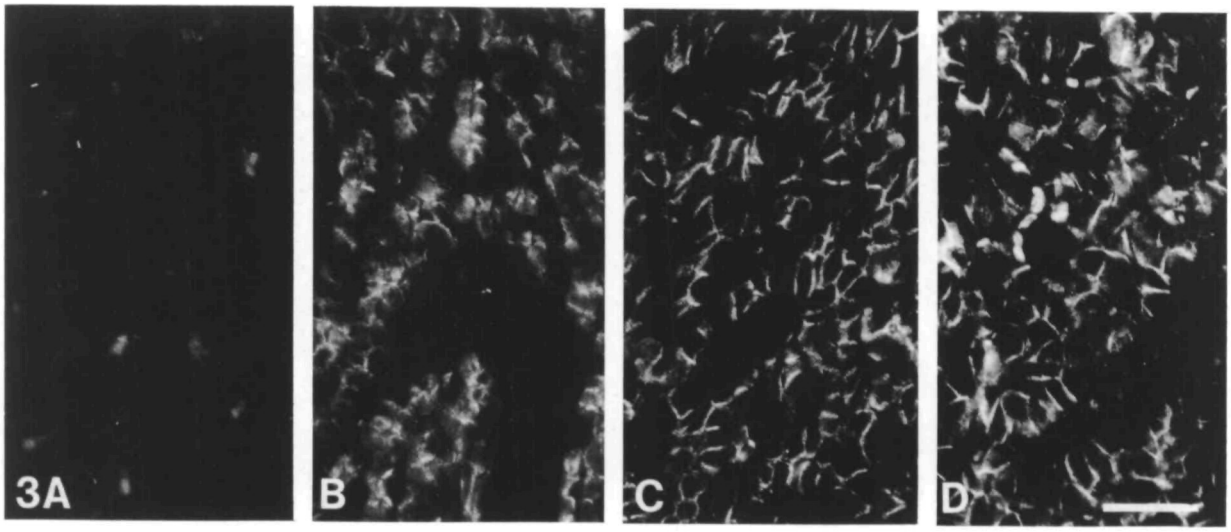

Fig. 3. Staining pattern of anti-N-CAM antibodies on *Xenopus* liver at different stages through metamorphosis. (A) Stage 57 NF; (B) stage 59 NF; (C) stage 61 NF; (D) stage 64 NF. N-CAM reactivity is first detectable on hepatocytes at stage 59 and persists then throughout the life of the animal. Only hepatocytes and no other cell types were labeled by anti-N-CAM antibodies. The staining was clearly a cell surface staining particularly intense in regions of cell–cell contact. Bar: 50 μm.

Premetamorphic hepatocytes do not express N-CAM. The molecule first appears on parenchymal cells of livers at stage 59 and then persists throughout the life of the animal (Fig. 3). Liver N-CAM is present on the cell surface of hepatocytes and is accumulated in regions of cell–cell contact; the luminal aspect of the hepatocytes and other liver cell types such as the melanocytes are not stained by anti-N-CAM antibodies (Figs. 3, 4B).

Treatment of stage 52 animals with 3×10^{-7} M thyroxine for periods as short as 48 h induced the appearance of N-CAM at the surface of hepatocytes (Fig. 4); shorter periods of treatment were not sufficient to induce the response. All hepatocytes responded simultanously to the hormone excluding the possibility of replacement of the population of parenchymal cells by newly produced cells. Hepatocytes were first responsive to thyroxine at stage 51–52; at earlier stages, N-CAM could not be induced in the liver even after prolonged hormonal treatment.

The induction of N-CAM in *Xenopus* liver could be confirmed by Western blot analysis. The major form of the molecule synthesized after hormonal treatment has a relative molecular mass of 160×10^3 similar to that appearing during spontaneous metamorphosis and in adult animals; two minor components at 135 and 125K could also be observed in Fig. 5A (lanes 1–4).

In order to characterize differences between brain N-CAM and *Xenopus* liver N-CAM, we isolated mRNAs from adult *Xenopus* brain and liver and performed a Northern blot analysis using a 1.8 kb fragment corresponding to an *Eco*RI insert from the 5′ end of *Xenopus* N-CAM cDNA. Transcripts were present in both organs. In adult brain, in accord with what has

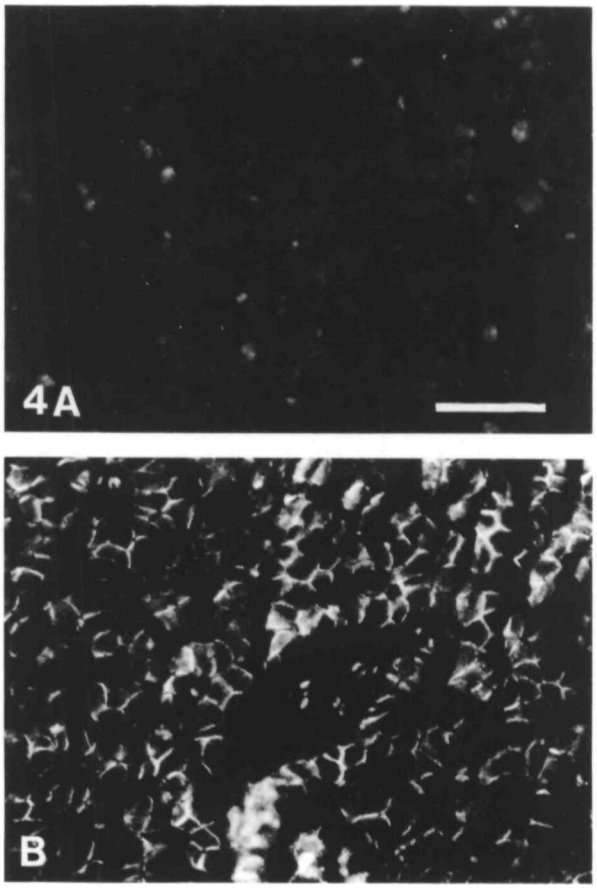

Fig. 4. Induction of N-CAM expression in the liver of premetamorphic *Xenopus*. Premetamorphic tadpoles (stage 54 NF) were treated for 48 h with $3\ 10^{-7}$ M thyroxine added directly in the rearing water. The reactivity of anti-N-CAM antibodies on livers of control (A) and treated animals (B) was then compared. All hepatocytes respond to thyroxine treatment synthesizing N-CAM that is then rapidly incorporated in the membrane and localized in areas of cell–cell contact. Bar: 60 μm.

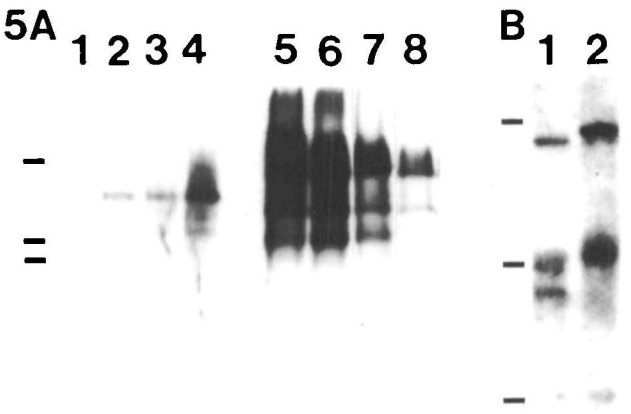

Fig. 5. Western and Northern blot analysis of N-CAM expression in *Xenopus* liver and brain. (A) Western blot analysis of N-CAM expression in *Xenopus* liver and brain. 100 μg of proteins extracted from different tissues were resolved on 7% polyacrylamide gels in the presence of SDS and immunoblotted with anti-N-CAM polyclonal antibodies. Lane 1, premetamorphic liver (stage 54 NF). Lane 2, as lane 1 after 48 h treatment of the animal with 3×10^{-7} M thyroxine. Lane 3, postmetamorphic liver (stage 64 NF). Lane 4, adult liver. Lane 5, premetamorphic brain. Lane 6, as lane 5 after 48 h thyroxine treatment. Lane 7, postmetamorphic brain (stage 64). Lane 8, adult brain. M_r markers are at 205, 116 and 97×10^3, respectively. (B) Northern blot analysis of RNA from adult *Xenopus* liver (lane 1) and adult *Xenopus* brain (lane 2), with a *Xenopus* N-CAM random primed probe. Size markers are at 9.9, 3.8 and 2.2 kb respectively.

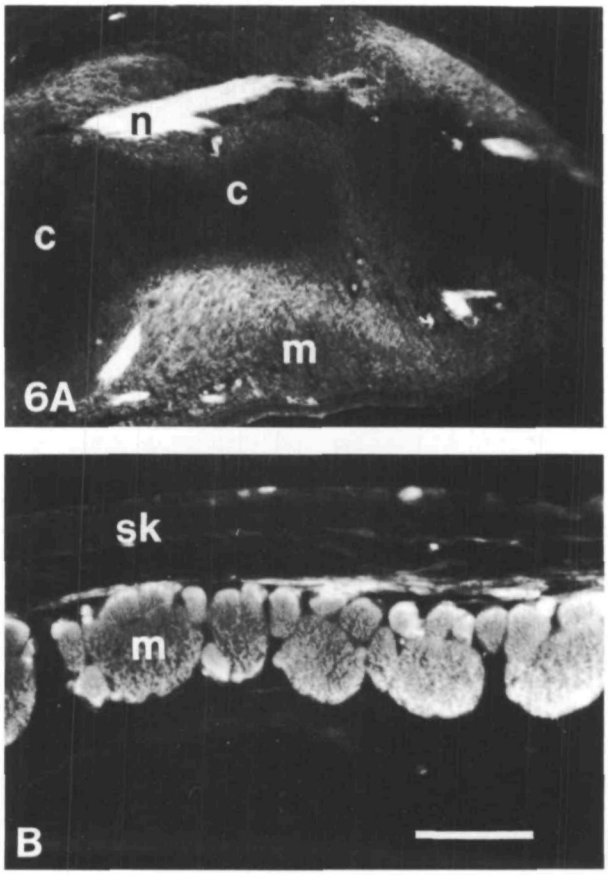

Fig. 6. Staining pattern of anti-N-CAM antibodies in developing muscles during *Xenopus* metamorphosis. (A) Section through a stage 52–53 NF *Xenopus* hindlimb. The nerves and the condensing muscles are strongly N-CAM-positive. Condensing cartilage and skin are negative. (B) Section through the abdominal wall of a stage 54 NF *Xenopus*. Muscle bundles are strongly N-CAM-positive, the skin is negative. c, cartilage; m, muscle; n, nerve; sk, skin. Bar: 100 μm.

been described (Kintner and Melton, 1987), two predominant transcripts at 9.6 and 4.2 kb and a minor component at 2.2 kb were observed (Fig. 5B lane 2). In the liver (Fig. 5B lane 1), a predominant transcript was present at 9.2 kb while other minor bands could be observed at 3.8, 3.3 and 2.2 kb. The two larger mRNAs of liver had a size unequivocally different from larger components found in the brain (Fig. 5B).

Changes in cardiac and skeletal muscle

During prometamorphosis and metamorphosis, many new muscle bundles are formed while pre-existing muscle masses, such as the dorsal muscles, increase in volume due to the addition of new fibers. N-CAM is strongly expressed in all newly formed muscles from their first condensation up to their complete differentiation in contracting muscle bundles; at later stages N-CAM disappears rapidly (see also Kay *et al.* 1988). This type of modulation is particularly evident in the muscles of the developing limb (Figs. 6A, 2C) and in the ventral muscles surrounding the abdominal cavity (*m. rectus abdominis* and *m. obliquus abdominis*, Fig. 6B). During further development, N-CAM is expressed only in newly formed fibers that are added to differentiated muscle masses. This process can be seen in the dorsal muscles of the trunk, in the ventral muscles and in differentiated muscles of the limb (see Fig. 2C).

Treatment of animals with thyroid hormones accelerates greatly the myogenic process and the corresponding modulation in the expression of N-CAM, which is always transiently expressed in condensing and differentiating muscle masses.

In contrast to skeletal muscles, cardiac muscles express N-CAM from their first differentiation throughout metamorphosis. Both the walls and the trabeculae of the ventricle as well as the atrial walls are intensely stained by anti-N-CAM antibodies at all stages of development (Fig. 7A, B).

Other sites of N-CAM expression

It has been reported that, during embryonic development, N-CAM is transiently expressed in certain epithelia particularly in areas where inductive processes are taking place (Crossin *et al.* 1985). During late development of *Xenopus*, two types of epithelia are strongly labeled by anti-N-CAM antibodies: the proliferating epithelium of the lens (Fig. 8A–C) and the sensory part of the olfactory epithelium (Fig. 8D). The

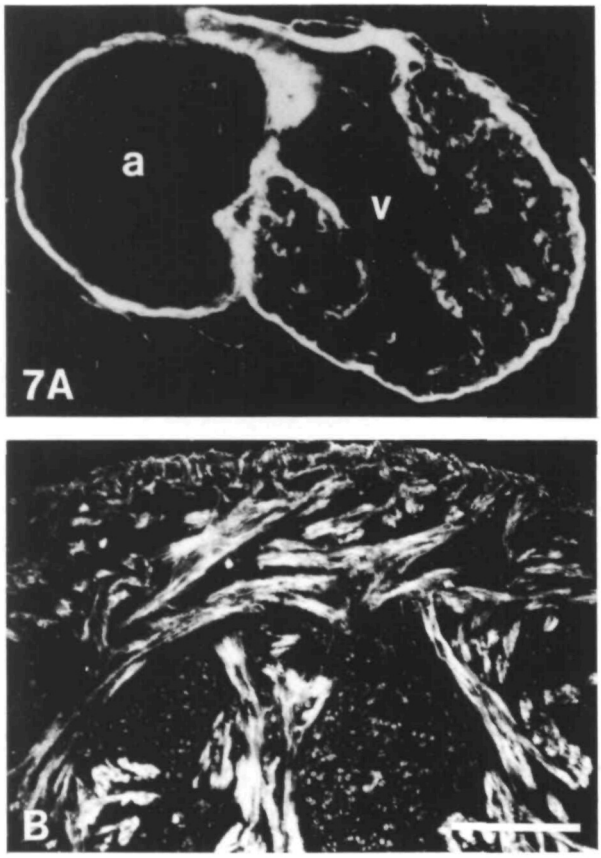

Fig. 7. Expression of N-CAM in *Xenopus* heart during metamorphosis. Cardiac muscle was brightly stained by anti-N-CAM antibodies from its first formation throughout metamorphosis. (A) Stage 48 heart; (B) stage 64 cardiac muscle. a, atrium; v, ventricle. Bar: 100 μm.

staining of these tissues persists throughout metamorphosis. The staining of the lens epithelium is clearly polarized being limited to areas of cell–cell contact and being absent from the apical aspect of the cells (Fig. 8B,C). Lens fiber cells are not stained.

Other sites in the animal did not show major changes in N-CAM expression during metamorphosis.

Discussion

In this study, we have examined the patterns of expression of a primary cell adhesion molecule, N-CAM, during metamorphosis of *Xenopus laevis*. As the morphological transitions that take place during metamorphosis occur under the direct control of thyroid hormones, we wished to detect any thyroxine-induced modulations in the expression of the molecule. Our major findings may be summarized as follows: (a) N-CAM is expressed by all the cell bodies, processes and neural epithelial cells of the CNS throughout metamorphosis. The optic and olfactory nerves, which belong embryologically and cytologically to the CNS, express high levels of the molecule throughout the life of the animal. In the CNS, N-CAM is present as three polypeptides of 180, 140 and 120K. The ratio of the

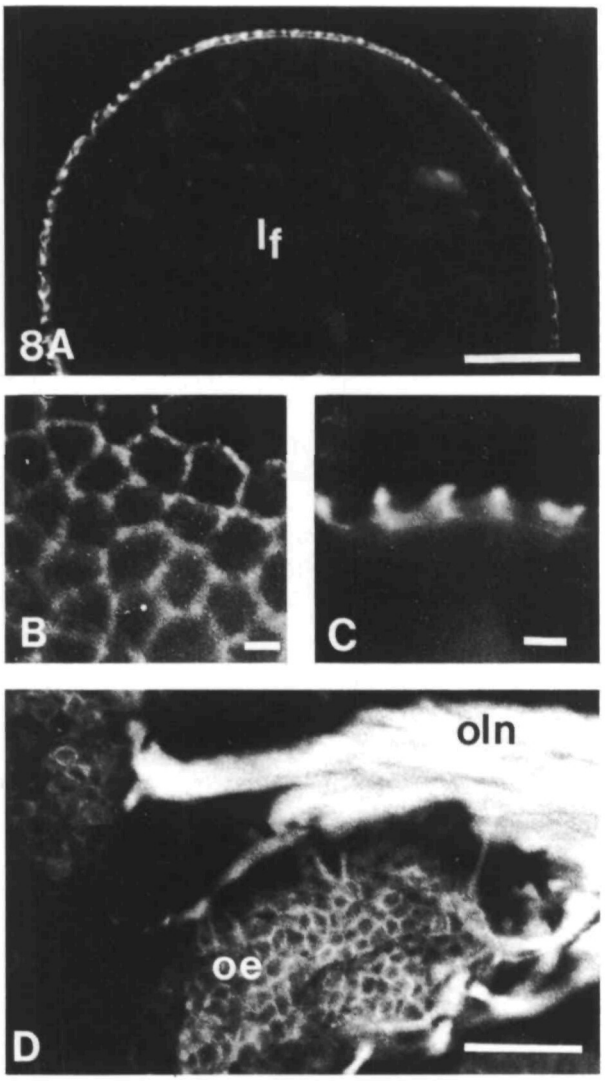

Fig. 8. Staining pattern of anti-N-CAM antibodies on *Xenopus* lens and olfactory epithelium. (A) The lens epithelium of stage 52 *Xenopus* is strongly labeled by anti-N-CAM antibodies; lens fibers are not stained. (B) Tangential section through the same lens; the cell surface of the epithelial cells is strongly stained in areas of cell–cell contact. (C) Transverse section through the same epithelium, the apical aspect of the epithelial cells is not stained. (D) Section through the olfactory organ of a stage 64 NF *Xenopus* tadpole. The olfactory nerve and the sensory part of the olfactory epithelium are strongly stained by anti-N-CAM antibodies. lf, lens fiber; oe, olfactory epithelium; oln, olfactory nerve. Bar=50 μm.

three polypeptides does not change considerably during development, but the amount of N-CAM relative to total protein expressed in the CNS decreases gradually during the growth of the animal as seen by immunoblots. A polysialylated, polydisperse form of the molecule present in the premetamorphic brain diminishes during metamorphosis. (b) In the cell bodies and processes of the PNS, the expression of N-CAM is strongly repressed towards the end of metamorphosis, so that in postmetamorphic animals the peripheral ganglia and nerves are virtually N-CAM-negative. (c)

Premetamorphic liver does not express N-CAM. During metamorphosis a 160K polypeptide that is recognized by both polyclonal and monoclonal antibodies to N-CAM is strongly induced in the liver. It can first be observed at stage 59 and persists then throughout the life of the animal. Liver N-CAM is a cell membrane protein and is predominantly localized in regions of cell–cell contact. A cDNA probe of *Xenopus* brain N-CAM recognizes four transcripts (9.2, 3.8, 3.3 and 2.2 kb) in adult liver RNA, these transcripts are definitely different is size from those recognized in brain RNA (9.5, 4.2 and 2.2 kb). (d) Expression of N-CAM in the liver can be induced by a 48 h treatment of premetamorphic tadpoles with thyroxine. (e) In skeletal muscle, N-CAM is strongly expressed in fusing myoblasts and in extending myofibers, but in later stages of myogenesis N-CAM is no longer expressed. In contrast, cardiac muscle expresses N-CAM throughout the life of the animal. (f) Beside the liver, strong N-CAM expression is maintained in two other epithelia: the sensory olfactory epithelia and the epithelium of the lens. In both cases, N-CAM is present since the first formation of the epithelium and persists then unchanged through the life of the animal.

In Fig. 9, we have summarized the general types of modulation of N-CAM expression that are observed during metamorphosis; three different modes can be distinguished: (1) a sharp thyroxine-dependent induction of expression in the liver; (2) a sharp inhibition of expression in the PNS and in differentiated skeletal muscle and (3) a persistence of expression with a gradual reduction during the growth of the animal in the CNS, heart, lens and olfactory epithelia.

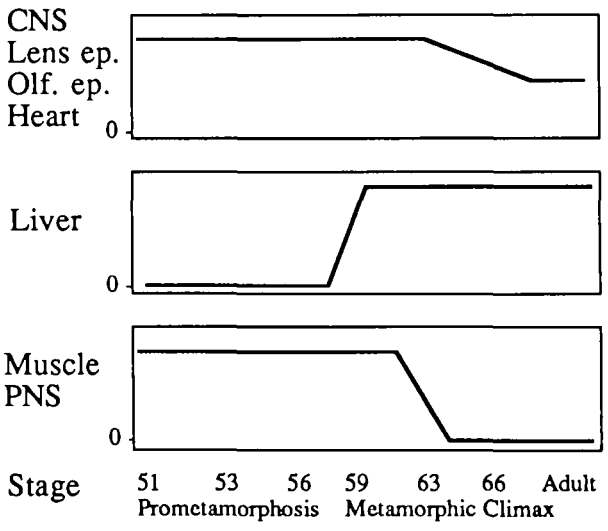

Fig. 9. Summary diagram of the types of N-CAM modulation during metamorphosis of *Xenopus laevis*. In the CNS, heart, lens and olfactory epithelia, the level of N-CAM remains high throughout metamorphosis and decreases during further development. In the liver, N-CAM expression is sharply induced during metamorphosis while in the muscle and in the PNS the expression of N-CAM is suppressed at the end of metamorphosis.

It has been proposed that N-CAM plays an important morphoregulatory role (Edelman, 1985, 1986*a*,*b*, 1988*a*,*b*). To fulfill this hypothesis, it is necessary that the levels of expression and molecular forms of the molecule change in sites where major changes in the morphology of the animal are taking place. Indeed, during embryonic development and in the course of nerve and muscle regeneration, the levels of N-CAM expression are strongly regulated at specific locations. For example, N-CAM is strongly modulated in sites of embryonic induction (Crossin *et al.* 1985; Levi *et al.* 1987), it increases in denervated muscle (Covault and Sanes, 1985; Daniloff *et al.* 1986*b*) and during nerve regeneration (Nieke and Schachner, 1985; Daniloff *et al.* 1986*b*).

So far, the molecular mechanisms by which the levels of N-CAM expression are modulated remain elusive. There are three published examples of factors affecting the expression of N-CAM: NGF on cultures of PC12 cells (Prentice *et al.* 1987; Doherty *et al.* 1988), retinoic acid in embryonal carcinoma cell lines (Husman *et al.* 1989) and laminin in cultures of N2A neuroblastoma cells (Pollerberg *et al.* 1985). In all these cases, however, the changes in N-CAM expression are associated either with cell differentiation into neurons or with changes in the morphology of the culture (e.g. neurite extension) and could be a consequence of these variations.

Amphibian metamorphosis provides a further ideal system to study the regulation of morphoregulatory molecules *in vivo* and possibly *in vitro*. During metamorphosis, the body plan of the animal is profoundly changed as the animal adapts to live in a completely new environment. The whole reshaping of the body plan takes place over a few days and is under the direct control of thyroid hormones; it depends extensively on processes of cell migration, adhesion, proliferation, differentiation and death which are directly affected by the presence of thyroid hormones (Deuchar, 1975; Frieden and Just, 1970; Fox, 1981; White and Nicoll, 1981).

We report here three different modes of regulation of N-CAM expression during *Xenopus* metamorphosis. The modulations that we observe in the CNS, the PNS and muscle could be ascribed to a decrease in the transcription rates of the N-CAM gene. Similar types of modulation are also observed during development of other species (Daniloff *et al.* 1986*a*; Rieger *et al.* 1985; Nieke and Schachner, 1985) and it could well be that the effect of thyroxine in these cases is only an acceleration of a preexisting regulatory mechanism of N-CAM. The diminution of N-CAM expression in the PNS of *Xenopus* is, however, much more abrupt and drastic than in chicken and in mouse; in less than a week at the end of the metamorphic climax (between stage 61 and 66), N-CAM expression is virtually abrogated in the PNS of *Xenopus*, while in other species a N-CAM decreases gradually during the postnatal development of the animal and persists at low levels in adult animals.

The mode of regulation of N-CAM expression that we observe in the liver is novel. In all species studied so

far, the transcription of the single gene encoding the N-CAM molecule is strongly repressed in the parenchymal cells of the liver. In *Xenopus* liver, thyroid hormones induce the abrupt expression of a special form of N-CAM in all hepatocytes; the molecule is not expressed in the absence of the hormone. The fact that N-CAM expression can be induced in all hepatocytes already at early premetamorphic stages (stage 51–52) excludes the possibility that the responsive cells constitute a new cellular population replacing larval hepatocytes as is the case for cells competent for the estrogen-dependent vitellogenin synthesis (Kawahara *et al.* 1987, 1989).

Our observations can be interpreted in several ways. (1) It is possible that in *Xenopus* different N-CAM genes are under the control of different gene regulatory elements and those in the liver may be in part activated by thyroid hormones *via* the proto-oncogene c-*erb*A nuclear receptors. This activation would bypass the mechanism of repression normally acting in the liver in other species. Indeed, due to gene duplication, the *Xenopus* genome contains two N-CAM genes with slightly different sequence (Krieg, personal communication); nothing is known so far about their promotors. (2) The molecule expressed in the liver could represent the product of a completely different gene that would maintain, however, both at the protein and at the nucleic acid levels sufficient homology to N-CAM to be recognized by monoclonal antibodies and cDNA probes. (3) The expression of N-CAM in the liver might be the result of a more complex thyroxine-dependent mechanism implying the activation of a specific form of the molecule resulting from an organ-specific alternative splicing. In this case, the liver form of N-CAM could be induced only in an organ-specific context in which other forms of the molecule are repressed by liver factors.

The analysis of the sequence, gene organization and gene regulation of liver N-CAM (already started in our laboratory) should provide an answer to this aspect of the problem as the mechanisms of action at the transcriptional level of thyroid hormones are now beginning to be elucidated (Evans, 1988; Glass *et al.* 1988; Damm *et al.* 1989; Koenig *et al.* 1989).

A second question raised by our findings regards the function of liver N-CAM. Although the involvement of liver N-CAM in cell adhesion needs still to be proved directly in primary cultures of hepatocytes, its polarized distribution in areas of cell–cell contact in the membrane of hepatocytes and other epithelial cells of the liver is typical of molecules involved in intercellular adhesion. In all species described so far, the parenchymal cells of the liver express high levels of the Ca^{+2}-dependent cell adhesion molecule L-CAM (E-cadherin or uvomorulin) (Gallin *et al.* 1983; Peyrieras *et al.* 1983; Takeichi, 1988). This molecule is also expressed in many other epithelia throughout development and in the adult (Thiery *et al.* 1984). In contrast to other species, it appears that the level of expression of L-CAM (E-cadherin) in *Xenopus* liver is relatively low compared to other epithelia (Levi *et al.* 1987; Choi and Gumbiner, 1989; Gumbiner, personal communication). These molecular differences might be at the origin of other cytological differences such as the massive invasion of melanocytes occurring in *Xenopus* liver.

These observations all suggest, therefore, that it is possible for the same organ in different species to utilize different adhesion systems to maintain its integrity. These types of changes suggest that regulatory genes controlling the expression of CAMs may act differently depending on the morphogenetic context (embryonic development, metamorphosis or regeneration) but nevertheless preserving the general body plan (Edelman, 1988*b*).

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