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[REVIEW]

Heterochrony and Neotenic Salamanders: Possible Clues for Understanding the Animal Development and Evolution

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ABSTRACT—A synthesis of developmental genetics with evolutionary genetics is now making possible to understand significant evolutionary changes in multicellular organisms. The key concept for unifying the two must be heterochrony. Heterochrony causes evolutionary modifications due to changes in timing and/or rate of development. The heterochrony is conventionally categorized into three patterns as neoteny (retardation in somatic development), progenesis (acceleration in gonadal development), and direct development (acceleration in somatic development, resulting in lack of larval or tadpole stages). A lot of species showing neoteny are known in urodeles, but not in anurans. Neotenic urodeles are also divided into three categories; permanent or obligate, "inducible" obligate, and facultative neotenies. *Hynobius retardatus*, a specific population of which had been reported to show neoteny but is believed to be extinct at present, has become to be used for experimental analysis of heterochronic expression of several adult characters during its ontogeny. Gonadal maturation and a transition of globin subunits from larval to adult types have been shown to occur independently on the morphological metamorphosis in *H. retardatus*. Mechanisms underlying the heterochrony, including morphogenetic clock, heterochronic genes in *Drosophila* and *C. elegans*, temporal colinearity in *Hox* gene complex in mice, and atavistic transformation induced by altered expression of *Hox* genes are discussed in terms of current molecular biology.

1. INTRODUCTION

In the last chapter of "Developmental Biology (4th ed.)" by S. F. Gilbert (1994), one of the most reliable and world-famous text book on current developmental biology, Gilbert has stated that "we are at a remarkable point in our understanding of nature, for a synthesis of developmental genetics with evolutionary biology may transform our appreciation of the mechanisms underlying evolutionary change and animal diversity." In other words, current progress in molecular biology has led us to the verge of a merger of the developmental genetics (i.e., synthesis of molecular biology with developmental biology) and the evolutionary genetics (i.e., synthesis of population genetics with evolutionary biology). The synthesis of these long-separated strands of biology may produce a developmental genetic theory capable of explaining the ontogeny and phylogeny, one of the biggest, unsolved problems in biology (Gould, 1977). Recent progress in this field has been reviewed by Akam *et al.* (1994) and Gilbert *et al.* (1996).

Developmental genetics has intensively analyzed the molecular basis for a construction of gross body plan, pattern formation, morphogenesis, cell differentiation, metamorphosis and aging in multicellular organisms. On the other hand, evolutionary genetics will discuss the molecular basis for macroevolution, microevolution, speciation, atavistic transformation and animal diversity (biodiversity) on our earth (Akam *et al.*, 1994; Holland and Garcia-Fernàndez, 1996; Valentine *et al.*, 1996). What is the common factor or parameter involved in these two phenomena, developmental and evolutionary changes ? Because the developmental changes must be considered as a function of a relatively shorter period of the ontogeny and the evolutionary changes have occurred during an enormously longer period of history of the earth (Gilbert *et al.*, 1996), the common parameter between developmental and evolutionary biology must be time, whatever shorter or longer it is. Thus, the key concept for unifying the developmental and evolutionary genetics must be heterochrony. In this review, I will discuss what is the heterochrony, how is gene expression chronologically regulated, and what kind of animals should be studied as a model system for elucidating the heterochrony involved in ontogeny and phylogeny.

2. HETEROCHRONY

Changes in developmental timing (heterochrony) are considered to be important in producing morphological changes during evolution (Gould, 1977; Akam *et al.*, 1994;

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Richardson, 1995). Heterochrony causes evolutionary modifications due to changes in timing and/or rate of development. During evolution, ancestral juvenile characters may be retained by later ontogenetic stages of descendents due to a reduced rate of morphological development and/or a delayed onset of growth in the embryos, as well as precocious sexual maturation. According to classical concepts on the ontogeny and phylogeny (e.g., acceleration, retardation, reiteration and so on, see Gould, 1977), heterochrony which is important for unifying the developmental biology and evolutionary biology is conventionally categorized into (1) neoteny (retardation in somatic development), (2) progenesis (acceleration in germ cell development), and (3) direct development (acceleration in somatic development, and resulting in lack of larval stages).

2–1. Neoteny

In neoteny, somatic development is retarded while gonadal development including germ cell maturation normally occurs. In urodeles, a lot of species to show neoteny have been known (Lynn, 1961; Dent, 1968; Gould, 1977; Armstrong and Malacinski, 1989). In anurans, however, neotenic species have never been reported (Lynn, 1961), except for experimental induction of gonadal maturation by goitrogen treatment (Hanaoka, 1954; Jurand, 1955).

There has been an old hypothesis that neotenic development involves an appearance of novel groups of animals (or phyla) with different body plans during evolution (see Gould, 1977). For instances, the larvae of certain tunicates could have evolved into chordates such as amphioxus, and a myriapod-like ancestor into insects by neotenic development. Molecular basis of the origin and evolution of chordates has recently been reviewed (Satoh and Jeffery, 1995; Satoh, 1995; Holland and Garcia-Fernàndez, 1996).

A lot of neotenic characters of human beings have sufficiently been accumulated and possible involvement of neoteny in the evolution of us has been stressed by Montagu (1981). Extraordinary similarity in the morphological characters between infants but dissimilarity in adults of human and chimpanzees convincingly provides an idea that heterochronic modifications brought about the phenotypic differences between the two.

2–2. Progenesis

In progenesis, gonadal maturation is accelerated while the rest of the body develops normally to a certain stage. The most conspicuous progenesis has been known in insects which propagate enormously during shorter period of their lives (Gould, 1977).

In amphibians, species showing progenesis are rather few. *Bolistoglossa occidentalis* is a tropical salamander that, unlike other members of its genus, lives in trees. This salamander has webbed feet and small body size that suit it for arboreal existence, the webbed feet producing suction for climbing and the small body size making such traction efficient. Alberch and Alberch (1981) have shown that *B. occidentalis* resembles juveniles of the related species *B. subpalmata* and *B. rostrata* (whose young are small, with digits that have not yet grown past their webbing). It is thought that *B. occidentalis* became a sexually mature size at a much smaller size than its predecessors. This gave it a phenotype that made tree-dwelling a possibility. Progenesis, thus, has enabled some salamander species to find new ecological nitches.

2–3. Direct development

In ordinary frogs, their lives are divided into aquatic larval (tadpole) stages and terrestrial adulthood (Hourdry, 1993a, b). Morphological and biochemical alterations from larval to adult forms are induced by thyroid hormones (Weber, 1967; Frieden, 1981; Rosenkilde, 1985). The direct development means lack of the tadpole stages in frogs.

Eleutherodactylus nubicola of Jamaica (Lynn, 1961) is a representative of frogs showing the direct development in which the aquatic larval period and most of the larval characters are eliminated (Dent, 1968). Frogs of the genus *Cornufer* also have essentially direct development (Fig. 1A). The eggs of *Eleutherodactylus* are large (3.0-4.0 mm in diameter) and covered by several dense jelly layers. Despite the large amount of stored yolk, cleavage is holoblastic, gastrulation is initiated at a subequatorial position, and the neural folds became elevated from the surface. However, shortly after the neural tube closes, limb buds appear on the surface. This early emergence of limb buds is the first indication that development is direct and will not pass through a limbless tadpole stage. Moreover, the emergence of the limbs is not dependent upon thyroid hormones (Lynn and Peadon, 1955). What emerges from the egg jelly three weeks after fertilization is not a tadpole but a little frog (Fig. 1B). Such direct-developing frogs do not need ponds from their larval stages and can therefore colonize new regions inaccessible to other frogs.

In sea urchins, several species are known to show direct development in which the pluteus larval stages are suppressed while the development of the adult rudiment is accelerating (Raff and Wray, 1989; Raff, 1992).

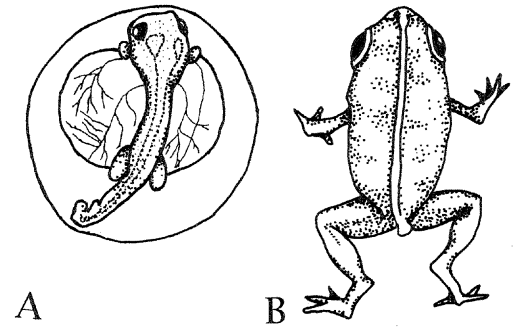

Fig. 1. Dorsal views of a "neurula" embryo and a "froglet" just after hatching, of frogs which show a direct development. (A) *Cornufer pelewensis*, showing four limb buds and large yolk mass. Redrawn from Dent (1968). (B) *Eleutherodactylus nubicola*, eight hours after hatching. Redrawn from Lynn (1961).

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3. NEOTENIC URODELES

In certain urodeles, sexual maturity occurs in what is usually considered a larval state. The reproductive system (and germ cells) mature, while the rest of the body retains its juvenile form throughout its life. The neotenic urodeles have been divided into three categories according to their ability of metamorphosis; permanent or obligate neoteny which cannot metamorphose at all in both natural and experimental conditions, "inducible" obligate neoteny which cannot metamorphose in nature but can metamorphose after treatment with thyroid hormones, and facultative neoteny which metamorphose depending on the environmental conditions (Frieden, 1981). Table 1 shows a summary of degrees of neoteny among the urodeles.

3–1. Permament (or obligate) neoteny

Most striking are the members of the family Proteidae, the blind European cave salamander *Proteus* and the American mudpuppy *Necturus*. These two salamanders retain larval characteristics as adults with nothing that can be designated as a metamorphosis. Although simple lungs develop, the external gills remain large and functional, the eyes are lidless, the tailfins are not resorbed, the skull shows little ossification, no maxillary bones ever appear, and the visceral arches and skin are larval in character (Lynn, 1961). Members of the Sirenidae, the genera *Siren* and *Pseudobranchus*, may be considered permanent neoteny with just a few adult characters. Larval gills and tailfins are retained, neither maxillary bones nor eyelids are ever formed. However, the skin undergoes a typical metamorphosis to attain the usual adult type of structure. This indicates that degrees of the retardation of somatic developments could be tissue- or organ-specific.

The permanent neotenic species of *Necturus* and *Siren* remain unresponsive to thyroid hormones, though their

Table 1. Degree of neoteny among urodeles[1]

Categories	Family	Genus	Species	Region
Permanent (obligate)	Sirenidae	*Siren*	*lacertina*	Southwestern USA
		Pseudobranchus	*striatus*	Southeastern USA
	Proteidae	*Necturus*	*maculosus*	Eastern USA
			punctatus	Southeastern USA
		Proteus	*anguinus*	Central Europe
	Crypto-branchidae	*Cryptobranchus*[2]	*alleganiensis*	Eastern USA
		Megalobatrachus[3]	*japonicus*	Japan
			davidianus	China
	Amphiumidae	*Amphiuma*[3]	*means*	Southeastern USA
			tridactylium	Southeastern USA
"Inducible" obligate	Prethodon-tidae	*Typhlomolge*	*rathbuni*	Texas
		Hydeotriton	*wallacei*	Southeastern USA
		Eurycea	*tynerensis*	Oklahoma
			neotenes	Texas
			nana	Texas
			troglodytes	Texas
		Gyrinophilus	*palleucus*	Tennessee
	Ambysto-matidae	*Ambystoma*	*mexicanum*[4]	Mexico
Facultative	Ambysto-matidae	*Dicampton*	*ensatus*	Wast coast of USA
		Ambystoma	*tigrinum*	USA
			talpoideum	Southeastern USA
			gracile	West coast of USA
			rosaceum	Mexico
			lermaensis	Mexico
	Salaman-dridae	*Notophthalmus*	*perstriatus*	Eastern USA
			viridescens	Eastern USA
		Triturus	*alpestris*	Central Europe
			cristatus	Western Europe
			helveticus	Western Europe
			taeniatus	Central Europe
	Hynobidae	*Hynobius*	*retardatus*[5]	Japan

[1] Combined and modified from Lynn (1961), Dent (1968) and Brandon (1989).
[2] In *Cryptobranchus*, its metamorphosis is a little proceeded.
[3] In *Megalobatrachus* and *Amphiuma*, their metamorphoses are nearly completed.
[4] Formerly named as *Siredon pisciformis*.
[5] Neotenic population in this species is believed to be extinct.

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hypophyseal-thyroidal system seems to be normal (Frieden, 1981). In other words, these animals never metamorphose even though they have thyroid hormones in their blood stream. In this respect, Yaoita and Brown (1990) reported that the mRNA for thyroid hormone receptor β is absent in *Necturus* and thus cannot be induced by T_3.

3–2. "Inducible" obligate neoteny

The most famous "inducible" obligate neoteny is of *Ambystoma mexicanum*, whose neotenic form is called axolotl, originally from a Spanish word *ajolote* which means "water slave" or preferably "water dog" (Smith, 1989). In the axolotl which has originally been found in Lake Xochimilco in Mexico, a lot of mutants have been isolated and provided to investigators (Malacinski and Brothers, 1974; Malacinski, 1978), and have long been employed for experimental embryology and developmental genetics.

Neoteny in the axolotl is caused by homozygosity for a single recessive gene. Dominant allele causing physical metamorphosis is found in the closely related species, *Ambystoma tigrinum*, with which it can hybridize (Tompkins, 1978). Despite the failure of axolotls to undergo morphological metamorphosis, they do undergo a cryptic or "biochemical" metamorphosis: transition of globin subunits (Ducibella, 1974) and some serum protein changes (Nussbaum, 1976) from larval to adult types occur on almost the same time schedule as *A. tigrinum* does, indicating a possibility of a separation of morphological and biochemical metamorphoses in the axolotl.

Although the axolotl never metamorphose in its native habitat in Mexico or under laboratory conditions, morphological metamorphosis can be induced by a single injection of thyroxine or by immersion of thyroxine solution (Prahlad and DeLanney, 1965; Tompkins and Townsend, 1977), and by a daily injection of thryoid stimulating hormone (TSH) (Taurog, 1974). Similarly to the axolotl, *Eurycea tynerensis*, *E. neotenes*, and *Gyrinophilus palleucus* cannot metamorphose in nature, but are able to induce metamorphosis by treating with thyroid hormones or TSH.

3–3. Facultative neoteny

It has long been known that specimens of *Ambystoma tigrinum* living in some of high lakes of the Rocky Mountains may fail to metamorphose, generation after generation, passing without the appearance of a typical adult. On the other hand, members of the same species living in other localities, exhibit normal metamorphosis (Lynn, 1961; Dent, 1968; Sprules, 1974a). Thus, this type of neoteny is called facultative. Their failure to metamorphose under normal conditions could be explained either by some deficiency in the animal's production of thyroid hormone, or by a failure of the thyroid gland to become active and release its hormone into the blood stream (Dent, 1968; Norris, 1980). Indeed, the thyroid glands in *Hynobius retardatus* reared at 4°C are reported to be completely inactive (Moriya, 1983a), and thyroid hormones are believed to be insensitive at the lower temperature (Moriya, 1983b). European species, *Triturus taeniatus* and *T. cristatus*, which had become world-famous newts by Spemann's monumental works on organizer (Spemann, 1936), belong to this type of neoteny.

A particular population of *Hynobius retardatus* has been reported to show neoteny in a specific environment of Lake Kuttara, a small volcanous lake in Hokkaido, Japan (Sasaki, 1924; Makino, 1934; Sasaki and Nakamura, 1937). Unfortunately, however, the neotenic population in Lake Kuttara is believed to be extinct at present. Since it was reported that the neotenic individuals of *H. retardatus* which had been captured at Lake Kuttara metamorphosed under the laboratory condition (Sasaki, 1924), it is reasonable to assume that the neoteny in *Hynobius* must be a facultative neoteny, in which animals metamorphose depending on the environments. The adaptive and evolutionary ecological significance of facultative neoteny in salamanders has been discussed in many species (Sprules, 1974a, b; Tilley, 1973; Wilber, 1971; Neish, 1971).

4. *HYNOBIUS RETARDATUS* AS A MODEL ANIMAL

We have recently demonstrated that *H. retardatus* can produce morphologically mature spermatozoa even in larval forms with well developed gills and tailfins when the metamorphosis is arrested by goitrogens (Wakahara, 1994; Yamaguchi *et al.*, 1996), and that a transition of globin subunits from larval to adult types occurs even in the metamorphosis-arrested larvae (Arai and Wakahara, 1993; Wakahara *et al.*, 1994). These findings suggest that the gonadal development and certain biochemical alterations from larval to adult types will be independent on the morphological metamorphosis in this species. In other words, it seems possible to induce neoteny in *H. retardatus* under certain experimental conditions.

4–1. Chronological separation between gonadal and somatic developments

When the larvae of *H. retardatus* were reared in goitrogens, morphological metamorphosis was substantially inhibited, and the aged larvae still remained in aquatic habitat (Arai and Wakahara, 1993; Wakahara, 1994). Histological observation revealed that the gonadal development was extraordinarily accelerated in the metamorphosis-arrested larvae compared with the normal controls (Fig. 2). The metamorphosis-arrested larvae produced morphologically mature spermatozoa approximately 5 months after hatching, when the gonads in the controls began to differentiate into testes, or to show the premeiotic proliferation of germ cells (Fig. 3). This precocious maturation of gonads induced by goitrogen treatment, however, may correspond to the progenesis (acceleration in germ cell development) rather than the neoteny (retardation of somatic development). Whatever the mechanism of this phenomenon is (Yamaguchi *et al.*, 1996), this observation convincingly demonstrates that the gonadal development including germ cell maturation is controlled by different chronological regulation from the somatic development in this species.

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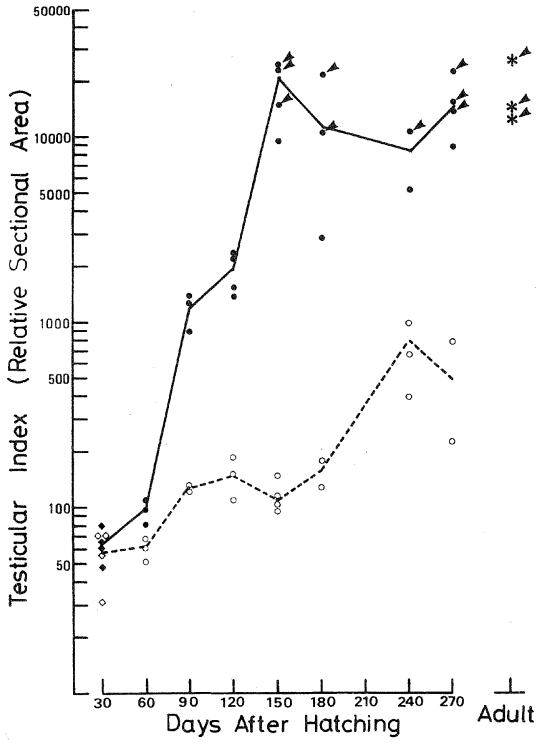

Fig. 2. Testicular development in normally metamorphosing and metamorphosed *Hynobius retardatus* (open circles, broken line) and in goitrogen-treated, metamorphosis-arrested larvae (closed circles, solid line). The ordinate (testicular index) shows relative average sectional area of each testis in a logarythmic scale. Each point shows a value in one individual. Asterisks on the right show the degree of the testicular development in ordinary adult males at the breeding season. Arrowheads indicate the testes with morphologically mature spermatozoa. From Wakahara (1994).

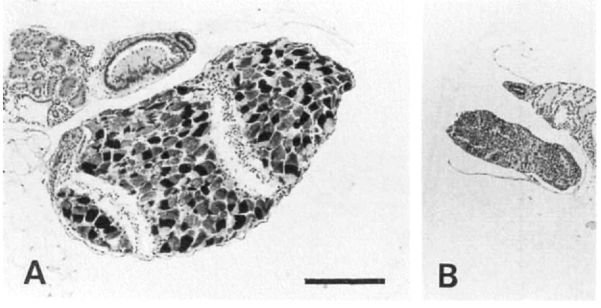

Fig. 3. Histology of developing testes in *H. retardatus*: a goitrogen-treated larva (A) and a control (B) at 220 days after hatching, at the same magnification. A lot of bundles of spermatozoa with densely stained heads and moderately stained tails are observed in the testis of the goitrogen-treated larva but not of the control. Delafield's hematoxylin and eosin stain. Bar, 400 μm.

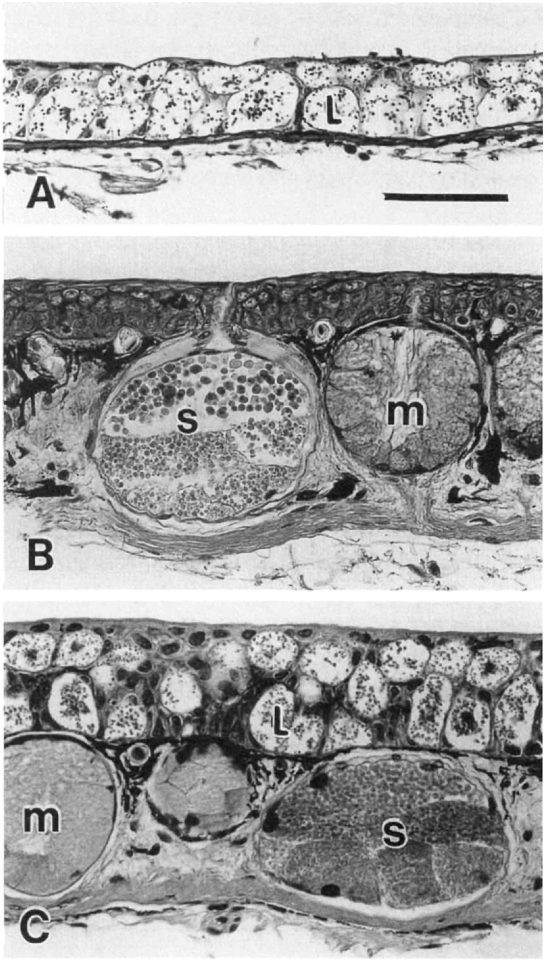

Fig. 4. Histology of head skins of normal controls (A and B) and of goitrogen-treated, metamorphosis-arrested larvae (C) in *H. retardatus*. (A) Typical larva (25 days after hatching). The skin is almost composed of an epidermis which is mainly occupied with Leydig's cells. (B) Typical adult (sexually mature male). The skin is composed of an epidermis which consists of stratified squamous cells and a dermis which is occupied with well developed dermal glands; mucous and serous galnds. (C) Metamorphosis-arrested larvae at 270 days after hatching. Leydig's cells are still main constituents of the epidermis and well developed dermal glands occupy the dermis. L, Leydig's cell; m, mucous gland; s, serous gland. Hematoxylin-eosin stain. Bar indicates 100 μm.

4–2. Larval and adult skin

Extensive reconstruction of skin occurs during amphibian metamorphosis (Yoshizato, 1989, 1992). Changes in skin (epidermal and dermal) cells have been described in several anuran species (Heady and Kollros, 1964; Fox, 1985; Robinson and Heintzelman, 1987; Izutsu *et al.*, 1993; Amano *et al.*, 1995). On the other hand, the transition of larval to adult types of skin in neotenic urodeles is a little more complicated; e.g., the skin undergoes a typical metamorphosis to attain the usual adult type of structure in some neotenic salamanders, while the external gills and dorsal tailfins still remain larval type (Lynn, 1961; Dent, 1968).

Typical larval skin in *H. retardatus* is entirely composed of an epidermis which is mainly occupied with Leydig's cells (Fig. 4A), epidermal specific cells in urodeles (Kelly, 1966). On the other hand, adult skin is composed of an epidermis which is predominantly occupied by stratified squamous cells, and of dermal tissues which consist of two types of dermal glands, mucous and serous glands (Fig. 4B). The mucous

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gland is composed of several gland cells, a central cavity and a duct opening to body surface. The serous gland is also composed of several gland cells which contain secretory granules of various size. It has recently been reported that the epidermis and dermis are differently regulated during the metamorphosis in *H. retardatus* (Wakahara and Yamaguchi, 1996).

4–3. Heterochrony in epidermal and dermal development

Figure 5 shows developmental changes in Leydig's cells in the epidermis and in dermal glands in the controls and metamorphosis-arrested larvae of *H. retardatus.* The Leydig's cells are main constituents of larval epidermis, and thus already observed at the hatching stage. They develop thereafter during the premetamorphic stage. Leydig's cells in the controls gradually disappear from the epidermis and are replaced by stratified squamous cells during the metamorphosis. On the one hand, they are well developed in the metamorphosis-arrested larvae during the period when the controls are metamorphosing and metamorphosed (Fig. 5A). At the end of the experiment, Leydig's cells are still main constituents of the epidermis in the metamorphosis-arrested larvae (Fig. 4C).

The dermal glands begin to be observed 25 days after hatching, and then develop rapidly during the metamorphic stages in the controls. After the metamorphosis, they are major constituents of the dermal tissues (Fig. 4B). In the metamorphosis-arrested larvae, however, these glands are not observed during the period when the controls are metamorphosing (Fig. 5B). They are firstly observed at 100 days after hatching, when the controls have completely metamorphosed. At the end of the experiment, morphological properties and the degree of development of these glands in the metamorphosis-arrested larvae (Fig. 4C) are identical to those in the controls (Fig. 4B). Thus, the aged metamorphosis-arrested larvae have skin which consists of "larval" epidermis (mainly occupied with Leydig's cells) and "adult" dermis (mainly occupied with two types of dermal glands).

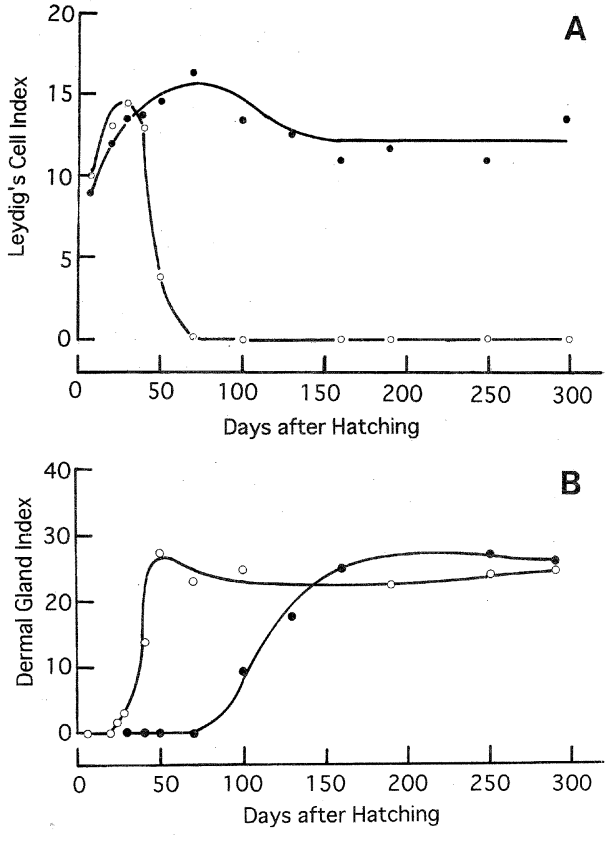

Fig. 5. Developmental fates of Leydig's cells in the epidermis (A) and of mucous glands in the dermis (B) of *H. retardatus*: normal controls (open circles) and metamorphosis-arrested larvae (closed circles). The developmental fates of both cells types are differently regulated even though they constitute the same skin. Redrawn from Wakahara and Yamaguchi (1996).

4–4. Changes in globin subunits

A changeover from a larval or foetal form of hemoglobin to an adult form is a frequent phenomenon in vertebrates (MacLean and Jurd, 1972). In anurans, a switch in hemoglobin synthesis is reported to occur at metamorphosis, resulting in the replacement of the larval globin subunits by a set of distinct adult ones (Cardellini and Sala, 1979; Hosbach *et al.*, 1982). This event, therefore provides a useful model for investigating the developmental and/or chronological control of gene expression (Banville and Williams, 1985a, b; Widmer *et al.*, 1981).

In the axolotl, the transition of globin subunits from larval to adult types occurs without any indication of anatomical metamorphosis (Ducibella, 1974; MacLean and Jurd, 1971;

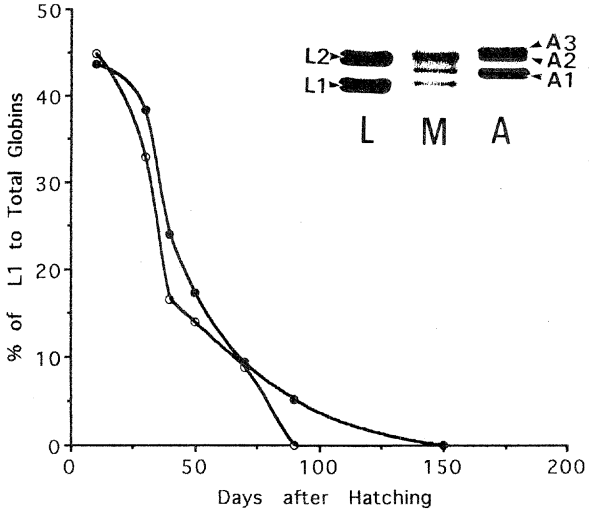

Fig. 6. Transitions of globin subunits from larval to adult types in normally metamorphosing *H. retardatus* (open circles) and metamorphosis-arrested larvae (closed circles). Chronological patterns of the globin transition are almost identical even in the controls and metamorphosis-arrested larvae. Inset: Electrophoregram of globin subunits on SDS-PAGE. L, typical larva; M, metamorphosing larva; A, typical adult. Proportion of L1 to total globins are caliculated using computerized image analizer (NIH-Image). From Wakahara and Yamaguchi (1996).

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Jurd, 1985). Similarly to this, in metamorphosis-arrested larvae of *H. retardatus*, the transition of hemoglobins occurs on the same time schedule as in the controls (Fig. 6), suggesting that the transition is completely independent on the morphological metamorphosis (Arai and Wakahara, 1993; Wakahara *et al.*, 1994; Wakahara and Yamaguchi, 1996).

5. CHRONOLOGICAL REGULATION OF GENE EXPRESSION

Many mechanisms for producing heterochrony have been proposed and reviewed (Raff and Wray, 1989; Satoh, 1990). Work on invertebrates has identified genes which may be involved in the control of developmental timing. The *lin-14* gene of *Caenorhabditis elegans* (Ambros and Horvitz, 1984; Ruvkun and Giusto, 1989) and the *period* gene in *Drosophila* (Konopka, 1987) are good examples of the heterochronic genes. In silkworm embryos, a "timer" enzyme which might determine the duration of diapause has been reported (Kai *et al.*, 1995, 1996).

Little is known about the control of developmental timing in vertebrates, although several cell-autonomous mechanisms have been proposed (Cooke and Smith, 1990; Satoh, 1982, 1985, 1990). Temporal regulation of genes encoded globin subunits in chicken and mammals must be the most excellent example for the control of developmental timing in vertebrates (Choi and Engel, 1988; Engel, 1993). In the mouse, disruption of the *Hoxd-13* gene has effects on limb development which have been interpreted as heterochronic (Dollé *et al.*, 1993).

5–1. Morphogenetic clock

DNA replication cycles and nuclear-cytoplasmic ratio may be responsible for a "developmental" or morphogenetic clock. One example of the developmental clock has been described in early development of ascidian embryos (Satoh, 1982, 1985). The timing of events during the early development of ascidian embryos is not mediated by chronological time or elapsed time since fertilization, but is likely related to the number of cell division cycles (Satoh, 1990).

The onset of midblastula transition (MBT) in amphibian embryos (Newport and Kirschner, 1982) is another example of the morphogenetic clock. It can be manipulated by ligating off a region of egg cytoplasm, thus altering the ratio of nuclear material to inherited store of regulator. Such ligation does not, however, affect numbers of cells, and at different times after the MBT, in experimental and control embryos (Kobayakawa and Kubota, 1981). It seems unlikely that the cell-autonomous timing mechanism is involved in the longer-term changes in ontogeny, and thus unsuitable for later development such as metamorphosis and gonadal maturation. These are more likely to involve cumulative effects of feedback processes at hormonal and tissue levels of organization (Cooke and Smith, 1990).

5–2. Time-keeping enzyme

Another type of the developmental clock which is involved in the longer-term changes in ontogeny must be related to a direct function of protein or enzyme itself. EA4, a DNA-dependent ATPase in silkworm (*Bombyx mori*) embryos, has been reported to act as a timer for determining the duration of diapause in the silkworm (Kai *et al.*, 1995). The period of cold indispensable for the resumption of embryonic development in diapausing eggs coincides with the period of cold storage required for the ATPase activation of EA4 *in vivo* and *in vitro*. EA4 has been reported to possess some sort of time-measuring activity. Recent study by Isobe *et al.* (1995) has shown that the resumption of arrested development in the silkworm at the embryonic stage is parallel to the activation of TIME (Time-Interval-Measuring-Esterase), which initially exists as complex form with PIN peptide. TIME-PIN complex dissociates by exposing to low temperatures, and thier dissociation from it triggers a conformational change which results in the activation of EA4 as a timer (Kai *et al.*, 1996).

Unfortunately, however, this type of enzyme which acts as a timer has not been found in other organisms.

5–3. Heterochronic genes

It has long been postulated that the temporal control of developmental events may be genetically controlled within embryos. In this respect, several heterochronic mutants which affect only the temporal sequence of cell fates, but not the formation of spatial patterns, have been isolated in *Caenorhabditis elegans* (Ambros and Horvitz, 1984, 1987). Recessive mutations of *lin-14* in *C. elegans*, e.g., *lin-14* (*n536 n540*) cause the precocious expression of specific developmental events.

Other heterochronic genes identified in invertebrates are *period* (*per*) in *Drosophila* (Konopka, 1987), and *frequency* (*frq*) in the fungus *Neurospora* (McClung *et al.*, 1989), both of these affect the circadian biological clocks. Sequence analysis of these genes allowed the identification of a common element between *frq* and *per* which, given the background similarities in their classical genetic characteristics, suggests the possibility of a common element in the clock mechanisms of these two organisms. Similar mutations have been found in mouse (Takahashi *et al.*, 1994; Vitaterna *et al.*, 1994) and hamster (Ralph and Menaker, 1988; Loudon *et al.*, 1994). Unfortunately, however, such single mutations affecting the circadian rhythm in vertebrates have not been proved to affect on the specific developmental events during their ontogeny.

5–4. Temproral colinearity

Temporal colinearity in the expression of *Hox* genes could provide a mechanism for marking time, particularly with respect to limb development and maturation along the primary axis (Duboule, 1994). Figure 7 shows schematic illustration of *Hox* gene complex in amphioxus and mouse. Vertebrate *Hox* genes are sequentially activated during the morphogenesis and pattern formation. The anterior-posterior succession in the topography of the *Hox* gene expression domains may depend on the 3'- to 5'-arrangement of the genes. A delay is observed in the appearance of the transcripts encoded by the more 5'-

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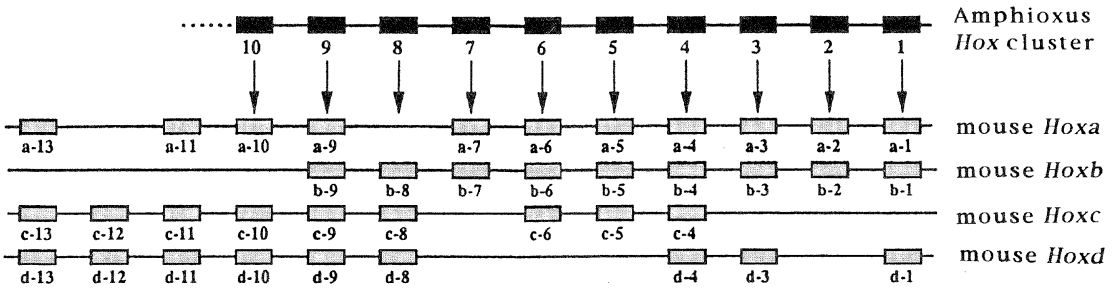

Fig. 7. Suggested evolutionary relationship bewteen mammalian and amphioxus *Hox* gene clusters. The amphioxus *Hox* gene cluster has an archetypal organization in relation to the vertebrate clusters. Modified from Holland and Garcia-Fernàndez (1996). Vertebrate *Hox* genes are sequentially activated during the morphogenesis and pattern formation, and thus, the physical ordering of the genes along their complexes will reflect the temporal sequence of their activation.

located genes; for example, *Hoxd-13* transcripts appear after those encoded by *Hoxd-10*. The rule is that one can never detect transcripts from a given *Hox* gene before transcripts are produced by its 3'-located neighbour in the complex. This implies that physical ordering of the genes along their complexes reflects the temporal sequence of their activation (Dollé *et al.*, 1989; Izpisúa-Belmonte *et al.*, 1991). This phenomenon, referred to as temporal colinearity, suggests that a part of mechanisms of chronological expression of the specific genes may be involved in the evolutionarily cumulative genomic structure itself.

5–5. Atavistic transformation

Atavism, i.e. an accidental expression of ancestral characters in descendents, is oftenly observed in a variety of animals (Gould, 1977). The atavistic transformation is considered to be a result from slight changes in regulatory elements responsible for the chronological gene expression, and thus provides some clues for elucidating the structure of genes involved in heterochrony and possible genomic changes during evolution. Recent progress in molecular biology on the morphogenetic genes including *Hox* gene complex makes possible to induce an atavistic transformation by knock out or overexpression of the specific genes (Duboule, 1994).

In *Hoxd-13* deficient mice, which have been made using the approach of gene disruption via homologous recombination, some phenotypec traits are suggestive of atavism; for example, an increase in the number of carpal bones of the distal row that is thought to be closer to the ancestral tetrapod (Dollé *et al.*, 1993). Another example of atavism in *Hox*-deficient mice is the phenotype observed in mice lacking a functional *Hoxa-2* gene (Rijli *et al.*, 1993). In these animals, homeotic-like transformations of the skeletal elements derived from the second branchial arch are observed and remodelling of bones in the middle ear has led to the appearance of a bone resembling a pterygoquadrate element, a bone that is found in reptiles, but not in mammals. The temporal colinearity in the expression of *Hox* genes and the atavistic transformation induced by an artificial modification of their expression are consistent with the idea that the history of animals is chronologically or sequentially engraved on the genomic structure.

6. TENTATIVE MODEL FOR NEOTENY

As described above, there are a lot of neotenic species in urodeles but not in anurans. This implies that these two different groups of amphibians will show different heterochrony in the development of their germ cells and somatic cells. Because one of the most conspicuous difference between urodeles and anurans is in their modes of germ cell formation; an early segregation of germ cells from soma in anurans and later induction in urodeles (see Nieuwkoop and Sutasurya, 1979; Wakahara, 1996), the difference in their modes of germ cell formation will reflect their ability of heterochrony. Furthermore, there is another heterochrony between anurans and urodeles; e.g., forelimb development occurs much earlier than hindlimb development in urodeles, while the hindlimbs develop earlier than forelimbs in anurans (Richardson, 1995). These differences in heterochrony will explain the predominance of neoteny in urodeles but not in anurans.

6–1. Germ cell formation in anurans and urodeles

In urodeles, primordial germ cells (PGCs) are morphologically recognized at first in the early tailbud embryos. The PGCs which locate within the lateral plate mesoderm are induced through as part of the regional induction of the mesoderm by the vegetal yolk endoderm (Kotani, 1957, 1958; Sutasurya and Nieuwkoop, 1974; Michael, 1984). Contrary to this, the PGCs in anurans are preformistically established much earlier than in urodeles under the certain influences of the germ plasm (Smith, 1966; Wakahara, 1977, 1978). Because all germ cells, once established, show virtually identical behavior, regardless of whether different modes of germ cell formation are employed, the basic mechanism of the germ cell formation and differentiation in all animals could be similar at the molecular levels, except for the timing of establishment of the germ cell line (see Wakahara, 1990a, b, 1996).

Assuming that germ cell formation in all the animals

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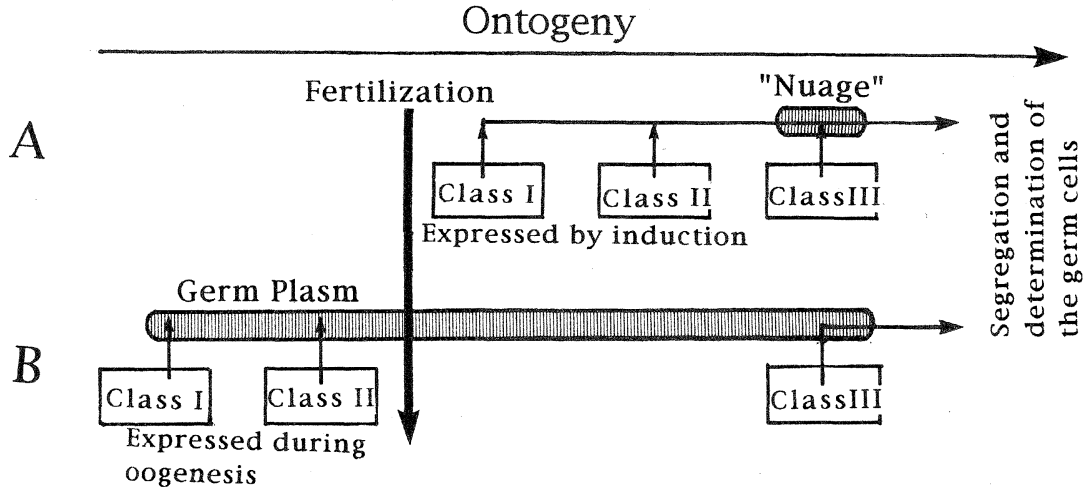

Fig. 8. A model for the temporal gene expression in germ cell formation in urodeles (A) and anurans (B). Class I genes which are corresponding to *cappucino*, *spire* and *staufen* in *Drosophila*, may function in the pattern specification in the germ cell formation. Class II genes which are corresponding to *oskar*, *vasa* and *tudor*, may regulate the assembly of germ plasm components; and Class III genes corresponding to *germ-cell-less* and mitochondrial large-ribosomal RNA gene in *Drosophila*, may control the germ cell segregation from the soma and the migration of the germ cells. In anurans, the Class I and II genes are expressed during the oogenesis, much earlier than in urodeles. The heterochronic expression of these genes will explain the different patterns of the germ cell formation in anurans and urodeles.

requires the expression of a series of genes which constitute a cascade and show complex interactions of those gene products as in *Drosophila* (see Lehmann and Ephrussi, 1994), the different modes of germ cell formation could be explained in terms of spatio-temporal gene expression (Fig. 8). For the convenience of explanation, all the genes that may be involved in germ cell formation could be classified into three categories: class I genes may function in the pattern (or spatial) specification in the germ cell formation (e.g., *cappucino*, *spire*, and *staufen* in *Drosophila*), class II genes may regulate the assembly of germ plasm components (e.g., *oskar*, *vasa* and *tudor* in *Drosophila*), and class III genes may control the germ cell segregation from the soma and the migration of the germ cells (*germ-cell-less* and mitochondrial large ribosomal RNA gene in *Drosophila*).

6–2. Heterochronic genes in amphibians

The preformistic mode in anurans implies a much earlier expression of the genes: possibly all the genes of the class I and II may be maternally expressed. The localization of germ plasm is considered to be a cytoplasmic manifestation of factors preformed during oogenesis as a result of the maternal expression of class I and II genes. In urodeles, neither the class I nor class II genes may be expressed before fertilization. Instead, they are first expressed during early embryogenesis under the influences of certain mesodermal inducer molecules.

Heterochronic genes which could exist on the upstream of the cascade of a series of genes involved in the germ cell formation and govern the expression of the class I and II genes, will explain the difference in the timing of germ cell formation between anurans and urodeles. Although such heterochronic genes in vertebrates have not been found yet, subtraction screening of genes between urodeles and anurans, or more specifically between neotenic urodeles and anurans of direct development will be promising.

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