Original Article

Thyroid hormone receptor expression in the obligatory paedomorphic salamander *Necturus maculosus*

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ABSTRACT **Amphibian metamorphosis is under the strict control of thyroid hormones (TH). These hormones induce metamorphosis by controlling gene expression through binding to thyroid hormone receptors (TRs). *Necturus maculosus* is considered to be an obligatory paedomorphic Amphibian since metamorphosis never occurs spontaneously and cannot be induced by pharmacological means. Since metamorphosis depends on the acquisition of response of tadpole tissues to thyroid hormone, we aimed to determine *TR* gene expression patterns in *Necturus maculosus* as well as the expression of two TH-related genes: *Cytosolic Thyroid Hormone-Binding Protein (CTHBP)-M2-pyruvate kinase*, a gene encoding a cytosolic TH binding protein and *stromelysin 3*, a direct TH target gene in *Xenopus laevis*. Tissue samples were obtained from specimens of *Necturus maculosus*. We performed *in situ* hybridization using non-cross-hybridizing RNA probes obtained from the cloned *Necturus TRα* and *TRβ* genes. We found clear expression of *Necturus TRα* gene in several tissues including the central nervous system, epithelial cells of digestive and urinary organs, as well as myocardium and skeletal muscle. *TRβ* was also expressed in the brain. In other tissues, hybridization signals were too low to draw reliable conclusions about their precise distribution. In addition, we observed that the expression of *CTHBP* and *ST3* is largely distinct from that of *TRs*. The fact that we observed a clear expression of *TRα* and *TRβ* which are evolutionary conserved, suggests that *Necturus* tissues express TRs. Our results thus indicate that, in contrast to previously held hypotheses, *Necturus* tissues are TH responsive.**

KEY WORDS: *amphibian, metamorphosis, paedomorphosis, thyroid hormone*

Introduction

The development of Amphibians is characterized by metamorphosis, an important postembryonic process by which non-reproductive structures of the larva change drastically to an adult form during a brief discrete period (see Shi, 2000) for a review). Despite the extensive and dramatic changes, Amphibian metamorphosis proved to be largely under the control of a single hormone, i.e. thyroid hormone (TH) (Leloup and Buscaglia 1977). As thyroid gland grows under the control of hypothalamus and pituitary and as levels of circulating TH rise, the sequential changes suggestive of metamorphosis occur (Leloup and Buscaglia 1977). TH controls metamorphosis through gene expression regulation by TH receptors (TRs). TR expression proved to correlate with metamorphosis since high levels of their mRNAs and proteins are present in a given organ when it undergoes metamorphosis, while low levels are generally observed in the same organs before or after metamorphosis (Baker and Tata, 1990, Yaoita and Brown, 1990, Banker *et al.*, 1991, Kawahara *et al.*, 1991, Eliceiri and Brown, 1994). In particular, in *Xenopus laevis*, TRβ mRNA and protein are low during premetamorphosis (larval growth without obvious organs/tissue transformation) and increase dramatically just before the prometamorphosis (proper beginning of metamorphosis with hindlimb development). Their levels reach their maximum during metamorphosis climax. To facilitate dramatic changes needed within this very short developmental period, liganded-TRβ autoregulates its own expression (Tata *et al.*, 1993), through at least one TRE in its promoter (Ranjan *et al.*, 1994, Machuca *et al.*, 1995, Wong *et al.*, 1998).

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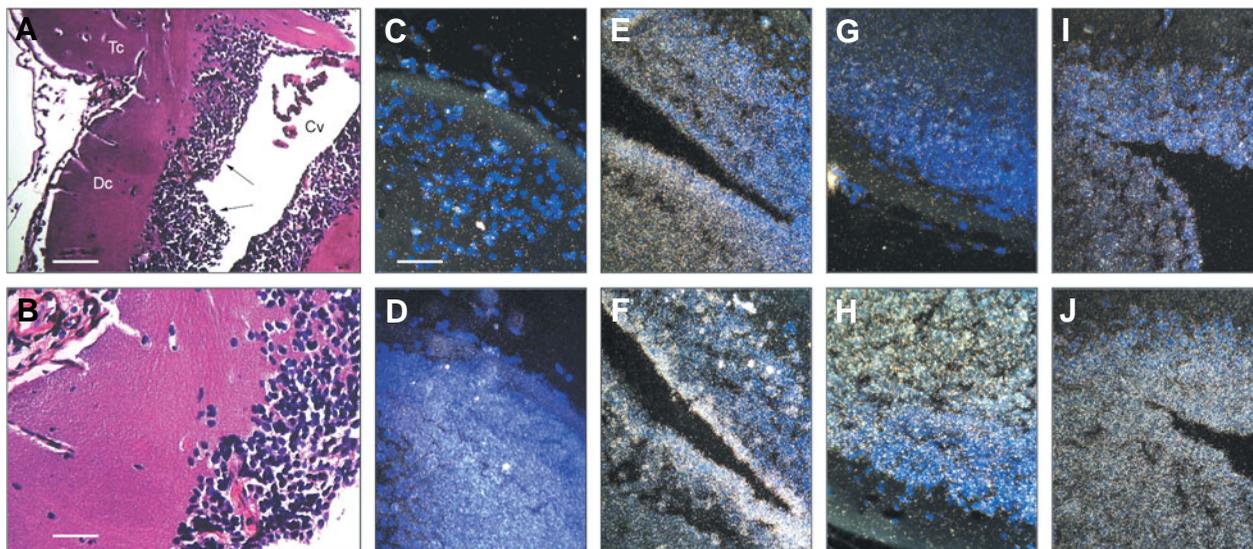
Fig. 1. Expression of *CTHBP, stromelysin 3, TRα* and *TRβ* genes in *Necturus maculosus* brain. (A,B) *Longitudinal sections of the brain were stained with HES (bars in A,B represent 5.7 μm and 2.4 μm respectively). The larval state is suggested by the neural tube-like appearance of the brain, i.e. juxtaposition of primitive cerebral vesicles, such as telencephalic (Tc) and diencephalic (Dc) vesicles shaped as a tube digged by a cavity (Cv) lined by neuroepithelial cells (arrows). Adjacent sections were hybridized with either* CTHBP *sense* **(C)**, CTHBP *antisense* **(D)**, *stromelysin 3 sense* **(E)**, *stromelysin 3 antisense* **(F)**, TRα *sense* **(G)**, TRα *antisense* **(H)**, TRβ *sense* **(I)**, *or* TRβ *antisense* **(J)** *probes (bar, 130 μm). Expression was restricted to neuronal periventricular area of telencephalic vesicles and diencephalons (arrows).*

Some Amphibian species that do not undergo metamorphosis are depicted as paedomorphic (Rosenkilde and Ussing, 1996, Stebbins and Cohen, 1997). Extensive among Urodela, paedomorphosis is defined as the retention of larval characters in reproducing adults. Paedomorphic Amphibian species can be grouped in three types according to the degree of metamorphic failure: accidental, facultative or obligatory. Accidental or geographic paedomorphosis is expressed by only some populations of the species (e.g. Alpine lakes salamanders), facultative paedomorphosis by some individuals of a single population (e.g. axolotl *Ambystoma mexicanum* which can undergo metamorphosis after TH treatment; the situation being quite complex in *Ambystoma* (see Voss and Smith, 2005) and references therein) and obligatory paedomorphosis by all individuals of the species. Although geographic paedomorphosis is thought to result from hypothalamic blockade due to unfavorable environmental conditions, the mechanisms underlying facultative and obligatory neotenies remain to be determined (Stebbins and Cohen, 1997, Voss and Smith, 2005).

The so-called mudpuppy, *Necturus maculosus*, is an obligatory paedomorphic Urodela. It is permanently paedomorphic since metamorphosis never occurs spontaneously and cannot be induced by pharmacological means (Harris, 1956, Larsen, 1968). Thyroid glands of *Necturus* are however TSH-responsive and capable of function. Implants of *Necturus* thyroid tissue have been shown to bring about accelerated metamorphosis in frog larvae (Swingle, 1922) and exogenous TSH administration induces hormone secretion by thyroid glands (Kerkof *et al.*, 1963). In addition, thyroid glands of *Necturus* can respond to implants of anterior pituitaries from *Rana pipiens* (Grant, 1930). Likewise, larvae of *Rana clamitans* implanted with a piece of an adult *Necturus* anterior pituitary showed metamorphic features (Charipper and Corey, 1930), suggest-

ing that the *Necturus* pituitary gland is functional. In contrast, *Necturus* displays no evidence of metamorphosis when given T4, whole thyroid substance or thyroid implants (Swingle, 1922). In addition, when skin of a species capable of undergoing metamorphosis is transplanted to *Necturus* and the latter is given T4, the donor skin undergoes metamorphic changes, while that of the host is unaffected (Noble and Richards, 1931). This suggests that *Necturus* tissues are insensitive to T4, but the precise underlying mechanism remains unknown.

It has been suggested from an isotopic study that low receptor number may contribute to paedomorphosis in *Necturus* (Galton, 1985). Likewise, previous studies from our group, using RT-PCR failed to detect *TRβ* mRNA in gill, muscle or liver tissues of *Necturus* (Safi *et al.*, 1997). Since we obtained *Necturus TRα* and *TRβ* specific probes (Safi *et al.*, 2006), we therefore undertook *in situ* hybridization experiments to assess *TR* expression in several *Necturus* tissues. The expression of two TH-related genes was also studied. Cytosolic Thyroid Hormone-Binding Protein (CTHBP)-M2-pyruvate kinase encodes a cytosolic TH binding protein and stromelysin 3 is a direct TH target gene in *Xenopus laevis*. Stromelysin 3 is a particularly interesting case since this gene has been shown to be required for cell migration and apoptosis during tissue remodelling in *Xenopus* (Ishizuya-Oka *et al.*, 2000, Fu *et al.*, 2005). Close examination of all organs examined showed that *TR* genes were expressed in many tissues like the brain, intestine or urinary bladder mucosa and muscle. In addition, we observed that expression patterns of CTHBP and ST3 are largely distinct from those of the TRs. The findings reported here as well as the fact that *in vitro* functional studies show *Necturus* TRs to be functional (Safi *et al.*, 2006), suggest that peripheral organs should be sensitive to TH. These

findings thus contradict previously held concepts as to tissue insensitivity to TH in this species.

Results

Histological assessment of Necturus maculosus *tissues*

Precise dissection of three specimens of *Necturus maculosus* allowed the isolation of several tissues samples, which were used for *in situ* hybridization experiments. Tissue samples taken for study included the central nervous system (CNS) with certain sensory organs (eye and otic capsule), circulatory and respiratory systems (external gill, lung, heart), digestive system and accessory glands (stomach, intestine, liver, pancreas and spleen), urogenital system (kidney, urinary bladder, testis, ovary, oviduct, homologue of oviduct), as well as parts of the locomotor system (hindlimb, tail) including skeletal and muscular tissues.

Some sections were not hybridized and stained by routine trichrome staining (hematoxylin-eosin-safran) for histological assessment. Histological analysis revealed that some tissues obtained from reproductively competent adults showed characteristics of paedomorphic «larval» stages. For example, *Necturus* brains consist of a simple neural tube with primitive cerebral vesicles (namely diencephalon and telencephalic vesicles) and periventricular neuronal blastema (Fig. 1A-B). Skin, examined in hindlimb and tail sections, exhibited typical features of Urodela larval skin such as a four-layered structure (epidermidis, basement membrane, basement lamella and hypodermidis) and a relative paucity of melanophores. Intestine was seen to be as a weakly villous uni-stratified epithelium (Fig. 2A). Liver was characteristic of a granulopoietic larval organ (Delsol, 1995) (Fig. 3A). As usually described in Urodela larvae, endocrine islets were rare in the *Necturus* pancreas (Fig. 4A) (Delsol, 1995). Lung tissue showed a unistratified epithelium, devoid of septa and suggestive of a larval hydrostatic structure, consistent with the entirely aquatic life of *Necturus* (Delsol, 1995). Pronephrotic kidney of

male specimens comprised the two characteristic parts of the Urodela order, i.e. the cranial «genital» part (connecting to testicular efferent ducts and including ducts for spermatozoids) and the caudal «urinary» part (including the proper pronephretic ducts) (Fig. 5A) (Delsol, 1995). Epiphyses of the hindlimb disclosed mild enchondral ossification, whereas diaphyses were formed by avascular lamellar periostic bone.

CTHBP and stromelysin 3 gene expression in Necturus maculosus

The genes encoding CTHBP (M2-pyruvate kinase) and stromelysin 3 are under the control of TH in many species and their expression has been succinctly described in some metamorphosing Amphibians such as *Xenopus laevis* and *Rana catesbeiana* (Shi *et al.*, 1994). We studied their expression, as representative T3-regulated genes, in *Necturus maculosus*. Except for the gills and otic capsule, all *Necturus* tissues exhibited strong hybridization signals when CTHBP antisense probes were used. Diffuse expression was observed in skeletal muscles (hindlimb and tail) (Fig. 6D-E) and myocardium as well as liver (Fig. 3B), spleen and lung parenchyma. In the digestive system (stomach and intestine), expression was restricted to epithelial cells (Fig. 2B-C) as it was in the urinary bladder and the excretory ducts of testis, oviduct and homologue of oviduct. In the kidney, CTHBP gene was found to be weakly expressed in tubular structures suggestive of pronephretic ducts (Fig. 5B-C). Neuronal areas in telencephalon, diencephalon, mesencephalon (Fig. 1C-D) and eye also expressed CTHBP.

Although less intense than CTHBP, stromelysin 3 gene expression roughly overlapped with that of CTHBP gene. Diffuse hybridization signals were seen in myocardium, lung and spleen and as an epithelium-restricted signal in digestive system, urinary bladder, testis, oviduct and homologue of oviduct. Stronger expression was evident in neuronal areas of telencephalon and diencephalons (Fig. 1E-F). No signal was observed in skeletal
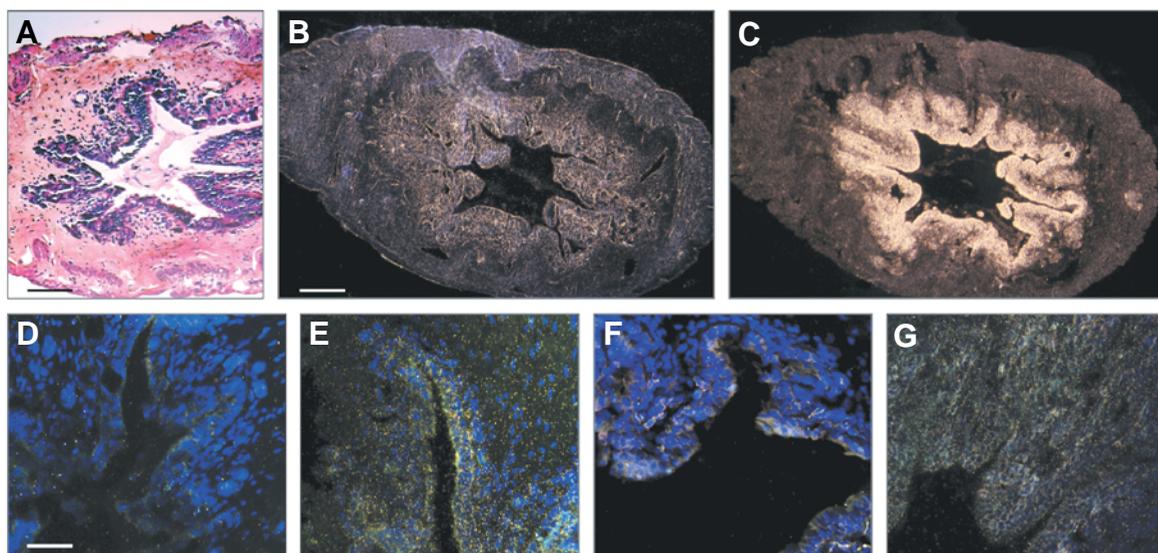
Fig. 2. Expression of *CTHBP, TRα* and *TRβ* genes in *Necturus maculosus* intestine. *Adjacent transversal sections of the intestine were stained with HES **(A)** (bar = 360 μm) or hybridized with either* CTHBP *sense **(B)** (bar for B,C represents 300 μm),* CTHBP *antisense **(C)**,* TRα *sense **(D)**,* TRα *antisense **(E)**,* TRβ *sense **(F)**, or* TRβ *antisense **(G)** probes (bar in D for D-G represents 250 μm). Expression was restricted to mucosa.*
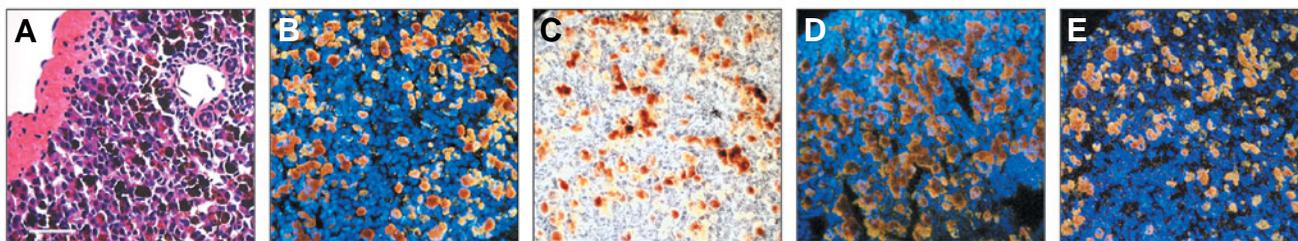
Fig. 3. Expression of *CTHBP*, *TRα* and *TRβ* genes in *Necturus maculosus* liver. *Adjacent transversal sections of the liver were stained with HES* **(A)** *or hybridized with either* CTHBP *sense* **(B)**, CTHBP *antisense* **(C)**, TRα *antisense* **(D)**, *or* TRβ *antisense* **(E)** *probes (bar represents 230 μm).* CTHBP *expression was diffuse in the whole gland, whereas hybridization signal using* TR *probes was similar to background labeling.*

muscle, nor was it in the liver (Fig. 3C), eye and otic capsule. In the kidney, expression was specific to round structures suggestive of glomerules (Fig. 5D-E), whereas it was restricted to acini in the pancreas (Fig. 4D).

Thyroid hormone receptor gene expression in Necturus maculosus

Expression of *TR* genes was studied on adjacent sections of *Necturus* tissues. This expression proved to be restricted to certain tissues such as the brain, muscle, digestive system, urinary bladder and pancreas. No signal was observed in either of the sensory organs examined (eye and otic capsule), nor in the kidney, genital system (testis, ovary, oviduct, homologue of oviduct), liver (Fig. 3D-E), or spleen.

In the brain, *TRα* gene expression was specifically found in the neuronal areas of telencephalon and diencephalon (Fig. 1G-H). In the muscle, expression was diffuse, both in skeletal muscle (hindlimb and tail) and myocardium (Fig. 6B-C). It was also diffuse in the uni-stratified epithelium of stomach, intestine (Fig. 2D-E) and urinary bladder. Only pancreas acini were labeled when *TRα* antisense probes were hybridized (Fig. 4E). The *TRβ* gene was also expressed in the periventricular neuronal areas of the brain (Fig. 1I-J). Although less intense, an expression similar to that of *TRα* was also noticed in the pancreas (Fig. 4F). In intestine and stomach, *TRβ* gene expression was considered as too weak to be ascertained (Fig. 2F-G). It was not observed in the liver (Fig. 3F). In all tissues where both genes were expressed, hybridization signal was more marked for *TRα* probe than the *TRβ* one (see for example expression in the intestine, Fig. 2D-G).

Discussion

TR expression

Thyroid hormone receptors are thought to be the main mediators of TH action during Amphibian metamorphosis. A correlation between their expression and the morphological changes observed during metamorphosis is therefore expected. Several studies have reported dynamical TR mRNA expression in the metamorphosing Anuran *Xenopus laevis* (Baker and Tata, 1990, Yaoita and Brown, 1990, Banker *et al.*, 1991, Kawahara *et al.*, 1991). This expression pattern was latter confirmed by quantitative studies of TR proteins (Eliceiri and Brown, 1994). In whole tadpoles of this species, *TRα* genes have been found to be expressed as early as the immediate post-hatching stage (Baker and Tata, 1990, Yaoita and Brown, 1990, Kawahara *et al.*, 1991). Their expression then increases throughout premetamorphosis, reaching a peak by early prometamorphosis (Baker and Tata,

1990, Yaoita and Brown, 1990, Kawahara *et al.*, 1991, Eliceiri and Brown, 1994). In contrast, *TRβ* genes have low expression levels during premetamorphosis, but their mRNA and protein levels increase dramatically during metamorphosis (Yaoita and Brown, 1990, Kawahara *et al.*, 1991, Eliceiri and Brown, 1994), in strong correlation with increases in plasma TH levels (Leloup and
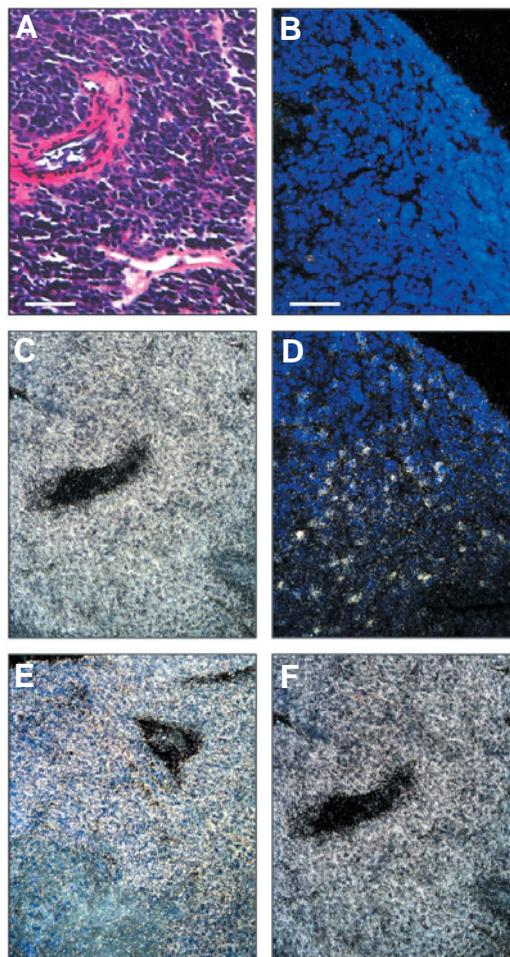
Fig. 4. Expression of *CTHBP*, *stromelysin 3*, *TRα* and *TRβ* genes in *Necturus maculosus* pancreas. *Adjacent transversal sections of the pancreas were stained with HES* **(A)** *(bar represents 210 μm) or hybridized with either* CTHBP *sense* **(B)**, CTHBP *antisense* **(C)**, *stromelysin 3 antisense* **(D)**, TRα *antisense* **(E)**, *or* TRβ *antisense* **(F)** *probes (bar represents 180 μm). The four genes were diffusely expressed in the whole gland.*

Buscaglia 1977). *TRβ* genes are indeed among the early TH response genes isolated from subtractive differential screens for TH target-genes (Kanamori and Brown, 1996). This upregulation is believed to result from TH-mediated induction of the promoters of both *TRβ* genes. These inducible promoters contain at least a strong TRE by which TRs upregulate *TRβ* gene expression (Ranjan *et al.*, 1994, Machuca *et al.*, 1995, Wong *et al.*, 1998). Although clearly less marked than that of TRβ mRNAs, upregulation of TRα levels has also been reported at metamorphic climax (Yaoita and Brown, 1990). In the facultative paedomorphic *Ambystoma mexicanum* (Mexican axolotl), similar results showing dynamic expression patterns during premetamorphosis and TH-induced metamorphosis were obtained using T3-binding studies (Galton, 1992) as well as by using specific RNA probes (Safi *et al.*, 2004).

A strong correlation between TR expression levels and metamorphic change was also observed when *TR* gene expression was analyzed in individual tissues (Yaoita and Brown, 1990, Wang and Brown, 1993, Shi *et al.*, 1994, Wong and Shi, 1995). In general, TR gene expression has been found to be high in a given organ when metamorphosis takes place and low before and after metamorphosis. In the tail for example, *TR* genes are expressed at low levels until climax when they are up-regulated, concurrent with rapid tail resorption. TRα and TRβ mRNA levels remains relatively low in *Xenopus* intestine throughout premetamorphosis but then TRβs mRNA increases during the drastic remodeling of epithelial tissue (Shi *et al.*, 1994, Wong and Shi, 1995). Likewise, TR mRNA levels are high in the hindlimb when limb morphogenesis takes place (Wang and Brown, 1993, Shi *et al.*, 1994, Wong and Shi, 1995). Immunocytochemical analysis in liver, intestinal epithelium and hindlimb bud of *Xenopus* gave similar results when morphological changes were induced during natural and T3-induced metamorphosis (Fairclough and Tata, 1997). In all these studies, TRα mRNA and protein were generally more abundant than TRβ products.

Two studies have provided a more accurate picture of tissue distribution of TR mRNAs (Kawahara *et al.*, 1991) and proteins (Fairclough and Tata, 1997) in Amphibians. The first showed TR distribution in *Xenopus laevis* at various larval stages and revealed expression of TRα transcripts in the CNS, epithelial tissues of several organs (intestine, pharynx, liver, kidney and skin), muscular tissues (heart, tail, intestine and skeletal muscles) as well as with lower levels in connective tissues (Kawahara *et al.*, 1991). During development, tissue distribution was not modified, but the spread and the intensity of the hybridization signal increased (Kawahara *et al.*, 1991). No clear signal was observed when the *Xenopus* TRβ probe was hybridized to tissue samples (Kawahara *et al.*, 1991). The second study provided evidence for TRα protein in liver, intestinal epithelium and hindlimb of *Xenopus* (Fairclough and Tata, 1997). To our knowledge, our present study is the first to properly address *TR* gene expression in an obligatory paedomorphic Amphibian. It shows that *TRα* gene is unequivocally expressed in *Necturus maculosus*. This expression pattern is strongly similar to that observed in *Xenopus laevis*, i.e. wide expression in tissues of various origins such as ectoderm-derived central nervous system, endoderm-derived epithelia of digestive and urinary organs, as well as mesoderm-derived muscular tissues (Kawahara *et al.*, 1991). The expression of *TRβ* gene is more questionable and, excepting the brain and the pancreas
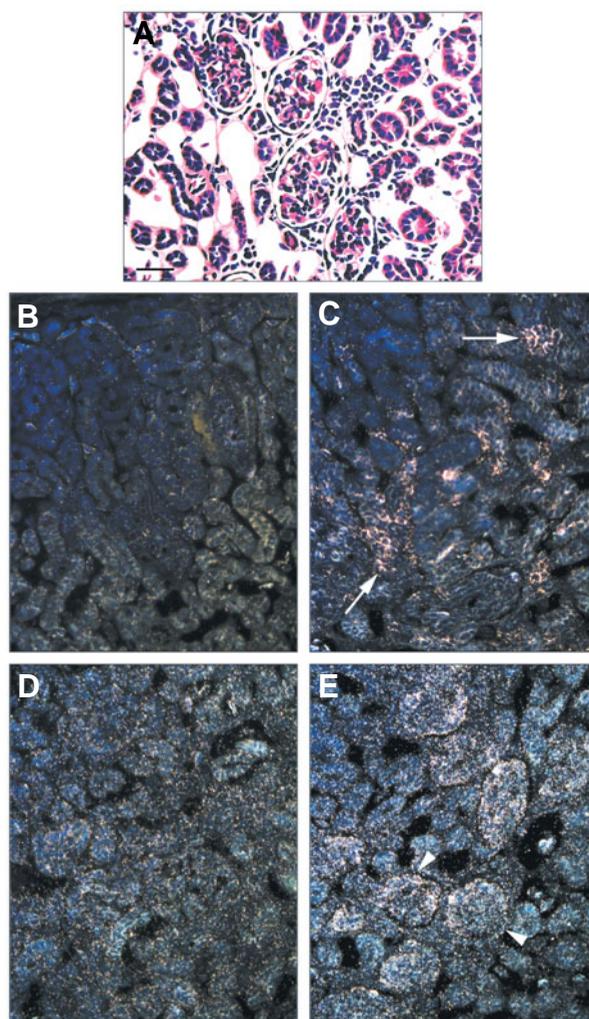
Fig. 5. Expression of *CTHBP* and *stromelysin 3* genes in *Necturus maculosus* kidney. *Adjacent transversal sections of the kidney were stained with HES* **(A)** *or hybridized with either* CTHBP *sense* **(B)**, CTHBP *antisense* **(C)**, stromelysin 3 *sense* **(D)**, *or* stromelysin 3 *antisense* **(F)** *probes (bar represents 230 μm).* CTHBP *expression was restricted to tubular structures suggestive of pronephretic ducts (arrows), whereas* stromelysin 3 *expression was observed in glomerules (arrowheads).*

where clear hybridization signals were observed, the signals obtained with the TRβ probe, for example in the intestinal epithelium, were too weak to draw firm conclusions about tissue distribution. This is reminiscent of the observations cited in *Xenopus laevis* using *in situ* hybridization (Kawahara *et al.*, 1991). These findings may reflect an insufficient sensitivity of the technique and thus, *TRβ* gene expression cannot be excluded in the tissues where we did not find it. Indeed, we have recently observed *TRβ* gene expression in *Necturus* tissues using RT-PCR (Safi *et al.*, 2006). However, as observed in premetamorphotic *Xenopus laevis* (Baker and Tata, 1990, Yaoita and Brown, 1990, Banker *et al.*, 1991, Kawahara *et al.*, 1991), as well as in red blood cells of *Rana catesbeiana* tadpoles (Schneider *et al.*, 1993), *TRβ* gene expression is constantly lower than that of the *TRα* gene.

By unequivocally demonstrating the presence of TRs in *Necturus maculosus*, our study suggests that the markedly low
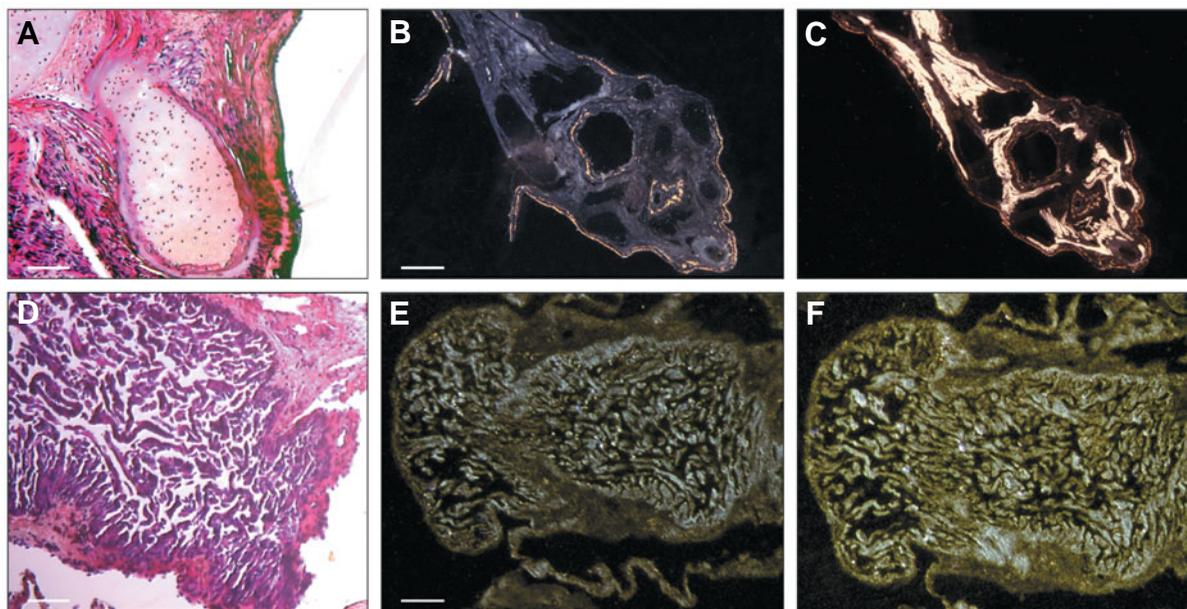
Fig. 6. Expression of *CTHBP* and *TRα* genes in *Necturus maculosus* muscle and myocardium. *Adjacent longitudinal sections of the caudal limb were stained with HES* **(A)** *(bar represents 6.7 mm) or hybridized with either* CTHBP *sense* **(B)**, *or* CTHBP *antisense* **(C)** *probes (bar represents 800 μm).* CTHBP *expression was intense in skeletal muscle. Adjacent longitudinal sections of the heart were stained with HES* **(D)** *(bar represents 390 μm), hybridized with either* TRα *sense* **(E)** *or* TRα *antisense* **(F)** *probes (bar represents 470 μm).* TRα *expression was weak and restricted to the myocardium.*

sensitivity of *Necturus* tissues to TH is not due to a total lack of TR mRNAs as previously suggested (Yaoita and Brown, 1990).

CTHBP and stromelysin 3

The results obtained with *CTHBP* and *stromelysin 3* genes are consistent with those obtained in the *Xenopus* tadpole (Shi *et al.*, 1994, Patterton *et al.*, 1995, Ishizuya-Oka *et al.*, 1996, Berry *et al.*, 1998a, Berry *et al.*, 1998b). Stromelysin 3 is weakly expressed in the intestine and totally absent from the tail of the premetamorphotic *Xenopus* (Patterton *et al.*, 1995), a pattern we report here in *Necturus*. Likewise, as in *Necturus*, CTHBP mRNA levels are high in the *Xenopus* pre-metamorphic tail and intestine (Shi *et al.*, 1994). In addition to the regulation of TR levels, a way for controlling metamorphosis is modulation of intracellular TH levels. CTHBP is a cytosolic protein that binds T3 and is likely to serve several roles including that of a buffer to modulate intracellular free T3 levels (reviewed in Shi, 2000)). In the present study, we showed that CTHBP is widely expressed in *Necturus* tissues, notably in all tissues where *TR* genes are expressed. Whether increased expression of CTHBP could account for metamorphic inhibition cannot be excluded from our non-quantitative study but the observation that its expression pattern is similar to that obtained in *Xenopus* argues against this hypothesis.

Metamorphosis in Necturus?

It is quite surprising to find measurable expression of *TR* genes in an animal that has been described as TH-resistant. This is even more striking since our recent results also suggest that, at least TRα protein is expressed i*n vivo* for example in the intestine. In addition, functional studies carried out in our laboratory, suggest that both TRα and TRβ proteins behave *in vitro* as efficient T3-inducible transcription factors (Safi *et al.*, 2006). Indeed we found

Necturus TRα and *TRβ* genes to encode fully functional TRs that bind as heterodimers with RXR to DNA configured in various types of TREs. *Necturus* TRs also bind T3 and T4 with a high affinity (Kd in the nano molar range) and transactivates target genes in response to TH with EC50 values very similar to the ones found for *Xenopus* TRs (Safi *et al.*, 2006). The fact that TR genes are expressed and functional led us to question the insensitivity of *Necturus* tissues treated with extremely high amounts of exogenous TH (Swingle, 1922). In fact we recently demonstrated that, when observed at the gene expression level *Necturus* tissues are sensitive to TH treatment since we observed an upregulation of TRα, TRβ and stromelysin 3 in gills. Our data which show TRα levels comparable to those observed in adult mammals, but very low TRβ levels, lead to the suggestion that *Necturus* may have a relative resistance to TH due to the low amount of expressed receptor, but not a generalized resistance to the hormone. This suggests that detailed molecular analysis should detect a clear TH response in terms of gene activation patterns in tissues that express high levels of receptors, such as intestine, brain or pancreas.

Since we show that a TH response can occur in *Necturus* tissues and since the hypothalamo-pituitary-thyroid axis is functional, all our data are consistent with the following model: in the normal wild condition, a post-embryonic period of TH sensitivity would occur, leading to some modest changes, mainly at the biochemical or gene-regulation level (such as the partial larval to fast isomyosin transition). Other normal TH response pathways such as gill resorption would not occur because the relevant target genes have lost their ability to be regulated by TH. This implies that paedomorphosis is not the absence of metamorphosis, but simply an attenuated and unnoticed response, that may even be diluted over a relatively long period of time. It is clear that this

model should be tested using juvenile *Necturus*, in particular to study *in vitro* and *in vivo* the effect of TH in controlling gene expression. The situation in *Necturus* is quite different from that in other paedomorphic amphibians such as axolotl. In this species it is the «decision» to undergo metamorphosis itself that is affected whereas in *Necturus* is probably the process of metamorphosis itself that has been modified. In the axolotl, our previous work has clearly shown that TRs are indeed functional (Safi *et al.*, 2004), whereas using a genetic approach these genes have been excluded as genes playing important roles in the evolution of paedomorphosis (Voss *et al.*, 2000). The situation in *Necturus* is reminiscent to that which occurs in mammals, such as mice, were there is a post-embryonic period of high TH sensitivity (Fraichard *et al.*, 1997).

Materials and Methods

Preparation of **Necturus maculosus** *tissues*

Samples of *Necturus maculosus* tissues were obtained from 3 wild North-American specimens provided by Dr. Planelles, from the French Museum d'Histoire Naturelle (Paris). They were 2 males and 1 female, which were killed by decapitation after anesthesia. After precise anatomic dissection (Gilbert, 1986), tissues were fixed by immediate immersion *via* phosphate buffer saline in freshly prepared 4% paraformaldehyde. They were fixed at 4°C for 16h, consecutively dehydrated in increasing concentration of ethanol, cleared in xylene, embedded in paraffin and stored at −20°C. Cartilage or bone containing tissues were immersed for 8h before dehydratation in a formic acid-based solution to favor cryotome section. Six to 8 μm-thick sections were obtained from the paraffin blocks and then mounted on silane-treated glass slides (Sigma ®). Slides were incubated at 42°C for 2 days and stored at 4°C until use. For each tissue, sections not treated for hybridization were stained by routine trichrome staining (hematoxylin-eosin-safran) for histological assessment.

Preparation of radiolabeled RNA probes

Antisense and sense ^{35}S-labeled RNA probes were prepared from *Necturus maculosus* c-erbAα and c-erbAβ cDNA (Safi *et al.*, 1997), as well as from CTHBP (M2 pyruvate kinase) and stromelysin 3 cDNAs, which were both cloned by RT-PCR using primers chosen in inter-species conserved regions. These primers are available from the authors upon request. All cDNAs were cloned in the pBluescript II KS plasmid (Stratagene ®). Transcription with T7 polymerase (Stratagene ®) from HindII-linearized plasmids generated radiolabeled antisense probe for c-erbAα and c-erbAβ in presence of α-^{35}S-CTP. Transcription with T3 polymerase (Stratagene ®) from BamHI-linearized plasmids generated sense probe for c-erbAα and c-erbAβ. The c-erbAα and c-erbAβ probes used in this study encompassed the region coding the C, D and E domains of the proteins. Transcription with T3 polymerase from NotI-linearized plasmids generated antisense probe for CTHBP and ST3, whereas sense probe were obtained from XhoI-linearized plasmids by using T7 polymerase. All plasmids were degraded by RNAse-free DNAse (Promega ®) and the phenol-chloroform-extracted probes were precipitated and purified using Quick Spin Columns (G-50 Sephadex fine ®). The average probe size was 300 bp after partial alkaline hydrolysis (Cox *et al.*, 1984, Angerer *et al.*, 1987, Angerer *et al.*, 1989).

In situ *hybridization experiments*

In situ hybridization experiments were performed on serial consecutive sections from the same paraffin blocks. Sections were dewaxed, rehydrated and pretreated with 1μg.ml^{-1} of proteinase K. Then slides were treated with 0.1 M triethanolamine (in PBS, pH 8.0) and acetic anhydre 0.25% for 10 min at room temperature, in order to block non-specific binding of probes to amino residues. After dehydratation in 30%, 70%,

95% and 100% ethanol, the sections were hybridized according to the recommendations of Cox (Cox *et al.*, 1984), with 10^6 dpm of ^{35}S-labeled probe at 60°C for 16 h in hybridization buffer (containing 50% deionized formamide, 20 mM tris-HCl, pH 8.0, 5 mM EDTA, 300 mM NaCl, 10% dextran sulfate, Denhardts' solution 1X, 100 mM dithiothreitol). This was followed by a high stringency wash for 30 min, at 65°C in solution containing 100 mM dithiothreitol. After treatment with RNAse A (20 μg.ml^{-1}) for 1 h at 37°C, sections were washed for 15 min in 2X standard saline citrate (SSC) and for 15min in 0.1X SSC at 65°C, dehydrated in alcohol and dried.

After hybridization, slides were immersed for autoradiographic detection of the hybrids in Kodak ® NTB2 emulsion (diluted 1:1 with 0.6 M ammonium acetate) and stored at 4°C. The slides were then exposed for 3 to 4 weeks (c-erbAα, c-erbAβ and ST3 probes) or 5 to 7 days (CTHBP probe), developed and stained with bisbenzimide (Hoechst 33258® intercaling DNA dye) to visualize the nuclei and mounted in glycergel (Sebia®). Sections were examined under dark field and epifluorescence illumination with Leica® MZ FLIII and Axioskop 50Zeiss® microscopes, respectively. Control hybridization of similar sections with sense probe did not produce any specific detectable signal above background.

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