Thyroid Hormone-dependent Differential Regulation of Multiple Arginase Genes during Amphibian Metamorphosis*

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We have cloned three nonhepatic arginase genes in Xenopus laevis. The deduced amino acid sequences of the three arginases are almost identical and share about 80% identity with mammalian as well as Xenopus liver arginase. Both the liver and nonhepatic arginase genes are activated early during embryogenesis. The liver arginase gene is strongly expressed in tadpole liver, but weakly in other tissues. In contrast, the nonhepatic arginase genes have the strongest expression in the tadpole tail. During metamorphosis, the liver and nonhepatic arginase genes show distinct regulation patterns. In the intestine, both types of arginase genes are activated during the remodeling period. In the tail, the liver arginase gene is activated during tail resorption, whereas the nonhepatic ones are highly expressed throughout all stages examined. Finally, in the hindlimb, the liver arginase is up-regulated slightly during development, whereas the nonhepatic ones have low levels of expression until the end of metamorphosis. During 3,3',5'-triiodothyronine (T3)-induced metamorphosis, the nonhepatic arginase genes are activated very quickly, whereas the liver arginase gene is a late T3 response gene. These differential regulation patterns during normal and T3-induced metamorphosis suggest potential functions for the arginases during tissue remodeling.

Thyroid hormone plays a causative role in the regulation of amphibian metamorphosis and is believed to control metamorphosis by regulating gene expression through its nuclear receptors (Dodd and Dodd, 1976). Both thyroid hormone receptor (TR) α and β genes have been cloned in amphibians and found to be expressed during metamorphosis (Yaoita et al., 1990; Yaoita and Brown, 1990; Kawahara et al., 1991; Schneider and Galton, 1991; Helbing et al., 1992). In the presence of thyroid hormone, these receptors likely activate and/or repress a set of early response genes at the transcriptional level. The products of these early response genes in turn regulate the genes further downstream, leading to the final transformations in different tissues.

Many genes have been found to be regulated either directly or indirectly by thyroid hormone during metamorphosis (Shi, 1994). Among the earliest and best studied are the genes encoding the five urea cycle enzymes: carbomyl phosphate synthetase, ornithine transcarbamylase, argininosuccinate synthetase, argininosuccinate lyase, and arginase. As aquatic tadpoles are transformed into terrestrial frogs, the animals change from ammonia excretion to mostly urea excretion, and the activities of these urea cycle enzymes are dramatically elevated in the liver (Cohen 1970; Weber, 1967). In Xenopus laevis the liver arginase (argL) gene has been shown to be up-regulated in the trunk region, which includes the liver, after prolonged treatment with thyroid hormone (Xu et al., 1993).

Like the other urea cycle enzymes, arginase has been detected in many tissues other than liver (Jackson et al., 1986; Glass and Knox, 1977; Herzfeld and Raper, 1976; Stewart and Caron, 1977; Spector et al., 1983; Skrzypek-Osiecka et al., 1983). The expression of argL in these tissues could contribute in part to the wide distribution of the enzyme. More importantly, however, biochemical and immunological evidence from these and related studies in mammals suggests the existence of nonhepatic arginases. Up to now, none of the nonhepatic arginase genes have been cloned and their regulation and function remain unclear.

We report here for the first time the cloning of full-length cDNAs for three closely related nonhepatic arginase genes (arg1, arg2, and arg3). The cDNAs were isolated from the intestine of X. laevis tadpoles induced to metamorphose by thyroid hormone. We show that like the liver arginase gene, the nonhepatic arginase genes are expressed in many frog tissues. However, compared with the argL gene, the arg1–3 genes have distinct developmental, tissue, and thyroid hormone-dependent regulation patterns, suggesting different roles for arg1–3 and argL in X. laevis, especially during metamorphosis.

MATERIALS AND METHODS

Tadpole Treatment and RNA Isolation—Tadpoles of different stages (Nieuwkoop and Faber, 1956) were treated with 5 ng thyroid hormone T3 (3,3',5'-triiodothyronine) as described (Shi and Brown, 1993). When indicated, the protein synthesis inhibitors cycloheximide and anisomycin were added at 20 and 25 μg/ml, respectively, for 13 h beginning 1 h before the addition of T3. This treatment inhibits protein synthesis in tadpole tissues by 99% (Kanamori and Brown, 1992). Total RNA was isolated from dissected organs/tissues or whole animals as described (Shi and Brown, 1990). The synchronized embryos were produced by in vitro fertilization (Dimitrov et al., 1993).

cDNA Cloning—A cDNA fragment of about 300 base pairs in size was isolated previously which hybridized to a few mRNA species that were up-regulated by T3 in the intestine (clone IU22 in Shi and Brown (1993)). This cDNA fragment was used to screen a Lambda Uni-Zap (Stratagene) cDNA library made of intestinal RNA from stage 52–54 tadpoles treated with 5 ng T3 for 13 h. Several cDNA clones were isolated. Three clones had different sizes of cDNA inserts that are close to the sizes of the mRNA species and were found to encode three highly related arginases. The sequences have been deposited into GenBank with the accession numbers of U08406, U08407, and U08408 for arg1, arg2, and arg3, respectively (Fig. 1).

Northern Blot Hybridization—Total RNA was electrophoresed on a 1% agarose formaldehyde gel and Northern blot hybridization carried out with the GenBank accession numbers.
results

Cloning and Sequence Analysis of Three Arginase cDNAs in X. laevis—To isolate genes that are involved in the remodeling of amphibian intestine during metamorphosis, we previously constructed polymerase chain reaction subtractive cDNA libraries using intestinal RNAs from control and thyroid hormone-treated tadpoles. A fragment of about 300 base pairs was found to hybridized to at least two different genes in X. laevis genome and several mRNA species that are highly expressed in the intestine at the climax of metamorphosis and regulated by T3 (Shi and Brown, 1990). To determine the identities and study the function and regulation of the corresponding genes, the polymerase chain reaction fragment was used to screen a cDNA library (Shi and Liang, 1994).

A number of arginase genes have been cloned from yeast to human. All the cloned vertebrate arginase genes encode liver-type arginases. arg1–3 are clearly not the liver-type arginases as they are more homologous to the mammalian liver arginases than to the Xenopus liver arginase (about 61% versus 57% identity, respectively) (Fig. 2 and data not shown). As expected, the Xenopus liver arginase is slightly more homologous (~63–66% identity) than arg1–3 (61%) to the mammalian proteins. The classification of the Xenopus argL as a liver arginase is also supported by the fact that it is strongly expressed in liver of adult frogs, but very weakly in other tissues (Xu et al., 1993). All these vertebrate proteins share about 40% identity with the yeast arginase. Although the structure and functional domains are unknown for any arginase, several regions (underlined with hatched bars in Fig. 2) are conserved from yeast to human, suggesting their involvement in the catalytic activity and/or maintenance of the structure of the enzymes.

Tissue-specific Expression of Arginase Genes—The identification of two types of liver versus nonhepatic arginases prompted us to investigate their expression in detail. First we compared their expression in different tissues in tadpoles with or without 18-h T3 treatment. As expected from our early study (Shi and Brown, 1993), arg1–3 were expressed in the intestine of stage 25–54 tadpoles, and their expression was up-regulated by T3 treatment (Fig. 3A) (due to the high homology among arg1–3, the cDNA probes used hybridized to all three mRNAs). In the tadpole liver, however, an extremely weak signal was observed in control tadpoles. T3 treatment slightly up-regulated the expression of these genes in the liver, but their expression was again much weaker than that in the intestine. In stark contrast, the Xenopus liver arginase gene (argL) was strongly expressed in the liver and was not affected by the T3 treatment (Fig. 3A). No signal was detected in the intestine of either treated or control tadpoles at this stage.

At stage 60, the onset of metamorphic climax, arg1–3 and argL again displayed very different tissue-specific expression patterns (Fig. 3B). arg1–3 were strongly expressed in the tail, but weakly, especially arg3, in the intestine, hindlimb, or the trunk region. In contrast, argL was not detectable in the tail, but strongly expressed in the trunk region, which included the tadpole liver. These results indicate that argL and the nonhepatic arginase genes arg1–3 have different tissue specificities in their expression and that the expression of argL, as expected, is strong in the liver.

Developmental Regulation of Xenopus Arginase Genes—The expression of arginase genes throughout embryonic and tadpole development was analyzed using total RNA from the entire embryo or tadpoles from stage 9 (midblastula transition) to stage 62 (the climax of metamorphosis) (Fig. 4). arg1 and arg2 mRNAs were clearly detectable as early as stage 16/17 (neurula) and arg3 mRNA by stage 23/24 (early tailbud).
Thyroid Hormone Regulation of Arginase Genes

It should be pointed out that in this and many other Northern blots, arg1 and arg2 were not well resolved. It is, therefore, uncertain whether they have the same expression profile. However, in the instances where they were resolved, the two mRNA species were clearly regulated identically (see Fig. 3 and data not shown). Highest expression of arg1-3 in whole tadpoles was found around stage 47/48. On the other hand, arg1 mRNA was present by stage 10/11 at gastrulation and throughout tadpole development. Higher levels of arg1 expression were found at stages 23/24 and 60-62. Similarly, Xu et al. (1993) reported the expression of arg1 mRNA in tadpoles at stage 46/47 or later, although they did not detect any mRNA at stages
positions of the 28 and 18 S rRNA are indicated. argl-3 could be up-regulated by 18 h T₃ treatment in both tissues, whereas ArgL could not. Five pg of total RNA from each tissue was used per lane. The same or duplicated blots were probed with an argl-3 probe, argL or rpL8, which served as a loading control. B, expression of Xenopus arginase genes in different tissues/regions of stage 60 metamorphosing tadpoles. Ten pg of total RNA was used per lane. The positions of the 28 and 18 S rRNA are indicated.

FIG. 3. The Xenopus liver (ArgL) and nonhepatic arginase (Argl-3) have different tissue specific expression patterns. A, the expression of argl-3 was stronger in the intestine than liver of stage 52–54 premetamorphic tadpoles, opposite to that for argL. In addition, argl-3 could be up-regulated by 18 h T₃ treatment in both tissues, whereas ArgL could not. Five pg of total RNA from each tissue was used per lane. The same or duplicated blots were probed with an argl-3 probe, argL or rpL8, which served as a loading control. B, expression of Xenopus arginase genes in different tissues/regions of stage 60 metamorphosing tadpoles. Ten pg of total RNA was used per lane. The positions of the 28 and 18 S rRNA are indicated.

FIG. 4. Northern blots showing that Xenopus argl-3 and argL are activated early during embryogenesis. Each lane contained 10 pg of total RNA from whole embryos or tadpoles at different developmental stages. argL mRNA was detected by stage 10/11 (gastrula) and argl-2 by stage 16/17 (neurula). Under the hybridization conditions, arg3 was not detectable until stage 33/34 (tailbud). Equal loading was confirmed by staining the membrane for total RNA with methylene blue (not shown, Herrin and Schmidt, 1988).

28–44. It is possible that this discrepancy was simply due to different exposure times or specific activities of the probes used as neither they nor we have determined the absolute expression levels for argL. In this regard, it is also worth pointing out that argl-3 could also be expressed as early as stage 10/11, but the signal was too weak under our hybridization conditions. Thus both argL and argl-3 are expressed very early during embryogenesis, well before liver differentiation, suggesting a role other than that in the urea cycle.

Differential Regulation in Different Tissues during Metamorphosis—The expression of arginase genes in the liver could suggest their participation in the urea cycle. However, both argL and nonhepatic argl-3 are also expressed in other tissues. We were interested in the roles of arginases in tissue remodeling during metamorphosis. Therefore, we analyzed the arginase expression during metamorphosis in three very different tissues, the intestine, hindlimb, and tail (Fig. 5), which undergoes a dramatic remodeling, de novo development, and total resorption, respectively (Dodd and Dodd, 1976; Smith-Gill and Carver, 1981; Yoshizato, 1989). The expression of argl and argl-3 was found to be regulated differently in all three tissues.

In the intestine, both argL and argl-3 mRNA levels were elevated during metamorphosis (stages 58–64) and decreased by the end of metamorphosis (stage 66). However, a noticeable increase in argl-3 expression and its subsequent down-regulation was found to be earlier, at stage 58 and stage 64, respectively, compared with those for argL (stage 60 and stage 66, respectively). In the tail, argl-3 were very highly expressed throughout metamorphosis, although slightly less mRNA was present at stages 58 and 64. In contrast, argL expression was low until stage 62, when it was up-regulated, coinciding with the tail resorption process (Nieuwkoop and Faber, 1956). Finally, in the hindlimb argl-3 were expressed at low levels until stage 66, the end of metamorphosis, when it was dramatically up-regulated. argL, on the other hand, maintained low levels of expression after stage 58 with very little expression at stage 56.

When the expression of the same gene was compared in different tissues, it was found that argl-3 had the highest levels of expression in tail at all stages and in hindlimb at stage 66. Coincident with these high levels of expression, the amount of argL mRNA relative to that for argl-2 was much higher in these tissues at these stages, suggesting the existence of a factor(s) in maintaining both the very high levels of argl-3 expression and the high ratio of arg3 to argl-2 mRNA levels. More interesting, argl-3 mRNA levels in the intestine, although much weaker, were dramatically activated during the period of intestinal remodeling. Similarly, argL mRNA levels were high during periods of intestinal remodeling and tail resorption. The mRNA levels of argL were considerably weaker in the hindlimb compared to those in the metamorphosing intestine and tail.

FIG. 5. Northern blots showing differential regulation of the liver and nonhepatic arginase genes in the intestine, tail, and hindlimb during metamorphosis. The most dramatic period of intestinal remodeling is around stages 60–62 and that of tail resorption is stage 62 and later (Nieuwkoop and Faber, 1956; Dodd and Dodd, 1976; Mckovy and Dixon, 1977). The tail is completely resorbed by stage 66, the end of metamorphosis. Hindlimb morphogenesis is completed around stages 56 and undergoes rapid growth in the remaining metamorphic period. Ten pg of total RNA was loaded per lane, except for stage 64 tail and stage 56 hindlimb, which contained only 5 pg. The same or duplicated blots were probed with an argl-3 probe, argL, or rpL8. The upper band detected with the rpL8 probe is likely to be a precursor of the mRNA, as it was not observed in oocytes where no transcription takes place (not shown). Slight variation does occur in the levels of rpL8 mRNA during development, especially for the precursor (Shi and Liang, 1994).
Differential Regulation of argL and arg1-3 by Thyroid Hormone—Thyroid hormone controls amphibian metamorphosis, and the endogenous T3 concentration is known to be high at the climax (stages 60–66) of metamorphosis (Leloup and Buscaglia, 1977). The high levels of argL and arg1-3 mRNAs in the remodeling intestine and resorbing tail suggest that T3 may influence arginase gene expression. Indeed, we have shown previously that arg1-3 expression could be induced in the intestine by treating stage 52–54 tadpoles with exogenous T3 (clone IU22, Shi and Brown, 1993). We have now extended this observation by showing that arg1-3 genes could be similarly activated in the intestine of stage 50 (not shown) and stage 56 (Fig. 6) tadpoles by exogenous T3 (5 nM) when the endogenous T3 concentration was low (Leloup and Buscaglia, 1977). Maximum expression in the intestine was observed after 2–3 day T3 treatment. In the tail exogenous T3 could slightly up-regulate (2–3-fold) the arg1-3 mRNA levels after 1–7 day T3 treatment (Fig. 6 and data not shown).

In contrast to arg1-3, the expression of the argL gene was not affected by T3 in the intestine and tail at stage 50 (not shown) or at stage 56 during the first 3 days of treatment (Fig. 6). Only after 5–7 days of T3 treatment was there a fewfold up-regulation in argL mRNA level in both the intestine and tail. This slow response of argL gene to T3 is in agreement with that reported by Xu et al. (1993), where it was shown that argL expression could be up-regulated in the middle portion of stage 51–53 tadpoles by T3 with a latent period of 70 h. Under our treatment conditions, intestinal remodeling, including length reduction and epithelial folding, is known to occur after 2–3 days (Shi and Hayes, 1994). Thus, the expression of both argL and arg1-3 during T3 treatment correlate with that during natural metamorphosis (Fig. 5).

The more dramatic activation and lower basal levels of arg1-3 expression in the intestine enabled us to further investigate this regulation by T3. When stage 52–54 tadpoles were treated with T3 for varying time periods, it was found that activation of the arg1-3 gene was detectable as early as 4 h after T3 addition, and the mRNA level continued to increase up to 48 h (Fig. 7). This fast kinetics of the activation suggest that T3 may directly regulate arg1-3 expression. To test this, tadpoles at stage 52–54 were treated with T3 in the presence or absence of protein synthesis inhibitors, and arg1-3 mRNA levels were determined in the intestine. Although arg1-3 were again found to be activated after 12-h T3 treatment in the absence of protein synthesis inhibitors, the results were less clear when these inhibitors were present due to their dramatic stabilizing effect on the mRNAs (Fig. 8). On a lighter exposure, however, it did appear that the mRNA levels were slightly higher in the intestine from tadpoles treated with both T3 and the inhibitors compared with those from tadpoles treated with the inhibitors alone (Fig. 8). This resistance to protein synthesis inhibitors together with the fast kinetics argues that arg1-3 genes are early T3 response genes.

**DISCUSSION**

**Multiple Arginase Genes With Different Tissue Specificities Exist in X. Laevis**—The three nonhepatic arginases reported here are highly homologous to each other, over 97% identity among them. In addition, they share about 60% identity with liver arginases, including the one from Xenopus. However, the nonhepatic Xenopus arginases are more similar to mammalian liver arginases than to the Xenopus liver arginase. Since there has been evidence that nonhepatic arginases exist in mammals (Jackson et al., 1986), these sequence similarities suggest an order of their emergence during evolution. A gene duplication before the separation of amphibians and mammals likely gave rise to the liver and nonhepatic arginases. arg3 and arg1 or arg2 were likely derived from a gene duplication in Xenopus and still more recently, as most of the Xenopus genome was duplicated to produce a pseudotetraploid organism, arg1 and arg2 evolved.

The liver arginase gene is, as expected, strongly expressed in tadpole liver but much more weakly in other tissues. Similar observations have been made in adult frogs (Xu et al., 1993). In contrast, the nonhepatic arginase (arg1-3) genes are only weakly expressed in the tadpole liver. The highest levels of arg1-3 mRNA are present in the tail and postmetamorphic hindlimb. Furthermore, compared with arg1-2, arg3 expression is much lower except in the tail and postmetamorphic hindlimb. Although it is unknown whether such a regulation has any functional importance, as arg1-3 are more than 97% identical, it is possible that the very high levels of arg3 in these
tissues simply serves to provide sufficient amounts of arginases when needed (see below). It would be interesting, however, to determine the mechanisms of the differential regulation among these highly related genes as well as between liver and nonhepatic arginase genes.

Correlation of Arginase Gene Expression with Normal and T3-induced Metamorphosis—We were initially interested in the arginase genes because of their expression in the intestine at the metamorphic climax and their up-regulation by T3 (Clone IU22, Shi and Brown, 1993). The remodeling of the intestine begins around stage 58 (Marshall and Dixon, 1978; McAvoy and Dixon, 1977; Ishizuya-Oka and Shimozawa, 1987). The first and most dramatic event is the gradual reduction of the length by as much as 10-fold. At the cellular level, massive cell death occurs in the primary epithelium around stage 61. By the end of metamorphosis at stage 66, a complex frog intestine is formed, which consists of a multiply folded secondary epithelium with elaborative connective tissue and muscles, in sharp contrast to the tadpole intestine, which is basically a simple tubular structure of primary epithelium with little connective tissue or muscles.

We have shown that arg1-3 are activated around the onset of intestinal remodeling (stage 58) and relatively high levels are present during the period of epithelial replacement and connective tissue development (stages 60–62). Lower levels of expression persist in post-metamorphic intestine. In addition, the liver arginase gene is also up-regulated during this period, although its peak expression in the intestine is shifted toward a later period of intestinal development (stages 62–64). No strong correlation exists between the regulation of arg1-3 expression and tail resorption or limb development (Nieuwkoop and Faber, 1956), even though very high levels of expression are present in the tail and much lower levels in the hindlimb except at the end of metamorphosis. On the other hand, argL is activated during tail resorption (stages 62–64) and hindlimb development (stages 58–66), although at much lower levels in the limb.

Possible Roles of Arginases in Arginine Metabolism and Amino Acid Biosynthesis during Tissue Remodeling—Arginase is best known as the last enzyme of the urea cycle in the liver. It catalyzes the conversion of arginine to ornithine and urea (Jackson et al., 1986; Cohen, 1970). However, both the liver and nonhepatic arginase genes in X. laevis are expressed in many different tissues, suggesting potential functions outside the urea cycle. In fact, nonhepatic arginases have also been reported in mammals (Jackson et al., 1986), although none of these genes have been cloned. It has been proposed that the nonhepatic arginases are involved in the conversion of arginine to proline and glutamate as well as polyamine biosynthesis (Yip and Knox, 1972; Takiguchi et al., 1988; Tabor and Tabor, 1984). Although the hypothesis remains to be verified, the regulation of arginase genes during metamorphosis is, at least, consistent with it. This stems from the fact that proline and glutamate are abundant in collagens.

Collagens are major components of extracellular matrices such as the basement membrane and interstitial connective tissue (Weiss, 1984). Although type IV collagen is rich in basement membranes and type I in interstitial connective tissues, they share with other collagens some important sequence features. Collagens consist of three chains with (α1), α2 configuration for type I and IV collagens. These polypeptides are mostly α helices. In the α helical region, the protein consists of repeating triplets of Gly-X-Y. Proline is generally present at the X position and hydroxyproline at the Y position. Such a sequence organization results in unusually high proportions of glycine and proline in collagen molecules. In fact, an analysis of eight collagen chains, two for each of α1(I), α2(I), α1(IV), and α2(IV), showed that on the average, glycine represents 27.7% of the proteins and proline, 17.8% (data not shown). In addition, glutamate and glutamine are 4.4 and 3.6% of the total protein, respectively. Taking into account the high abundance of proline and glycine, the average proportion of the remaining amino acids is expected to be 22.7%. Thus, glutamate and glutamine are also over-represented in collagens. It is, therefore, conceivable that the demand for proline and glutamate, and the derivative of the latter, glutamine, may be high in collagen-rich tissues. As arginase can be involved in the biosynthesis of proline and glutamate from arginine, this could account for the high levels of arg1-3 expression in the tail and postmetamorphic hindlimb. Similarly, in the metamorphosing intestine, the activation of arg1-3 and argL could also be required for the biosynthesis of proline and glutamate since the connective tissue develops extensively and the new basement membrane is formed upon secondary epithelial cell differentiation. It is unclear why lower levels of arg1-3 are present in the hindlimb before stage 66. It is possible that other arginases such as argL may be involved. Finally, the delayed expression of argL compared with arg1-3 during intestinal remodeling and its activation during tail resorption suggest that argL could also be involved simply in arginine removal after cell degeneration as both tissues undergo extensive cell death. The cloning of both liver and nonhepatic arginases now makes it possible to study these functions directly.

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REFERENCES


**Fig. 8. The activation of arg1-3 appear to be a direct response to T3 treatment.** Each lane had 10 µg of total intestinal RNA from stage 52–54 tadpoles treated with or without 50 nM T3 in the presence or absence of protein synthesis inhibitors (CHX). Cycloheximide (CHX) stabilized the mRNA, but a short exposure (top panel) showed that a slight up-regulation by T3 was produced even in the presence of CHX.
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