

Regionally and hormonally regulated expression of genes of collagen and collagenase in the anuran larval skin

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ABSTRACT The skin of an anuran tadpole undergoes region-dependent metamorphic changes: the body (head and body trunk) transforms into the adult type, while the tail falls into programmed cell death. The present study was undertaken to investigate the regional specificity of metamorphosis at a molecular level, focusing on genes of collagen and collagenase that are known to be activated in their synthesis at metamorphosis. A cDNA probe utilized for collagen was Hf677 (a clone of human type I collagen α_1 chain). A probe for collagenase gene was cloned in the present study from a cDNA library of bullfrog tadpole skin, characterized and named Tc1. Tc1 contained the consensus sequence of zinc-metalloproteinases and showed a high homology to mammalian collagenases. Using these recombinant DNAs as probes, RNA blot analyses were performed for the body and tail skin of tadpoles that had been in spontaneous metamorphosis, induced to metamorphosis by the injection of thyroid hormone, or had been induced to grow by prolactin treatment. Collagenase gene was activated irrespective of regions of the skin, body or tail at the early metamorphic climax stage, although the extent of activation was region-dependent. In contrast, metamorphic changes of collagen gene expression showed a clear regional dependency. The transcription level in body skin was enhanced at the onset of metamorphosis while that in tail skin was markedly suppressed. Thyroid hormone was shown to be responsible for this region-dependent expression of collagen genes at metamorphosis. Prolactin, a suppressor hormone of amphibian metamorphosis, enhanced the transcription of collagen genes and suppressed that of collagenase. The action of prolactin on these gene expressions was region-independent.

KEY WORDS: *anuran metamorphosis, collagenase, collagen, thyroid hormone, prolactin*

Introduction

Attempts to clarify the action mechanism of hormones at the molecular level have progressively increased in studies on anuran metamorphosis. Especially, investigators have focused intensively on the two metamorphosis-associated hormones: thyroid hormone (TH), the trigger and stimulator of metamorphosis (Kollros, 1961), and prolactin (PRL), the suppressor of metamorphosis (Etkin and Gona, 1967). The cDNAs of 3,3',5-triiodo-L-thyronine (T_3) receptors (TRs) were isolated from african clawed frog, *Xenopus laevis* (Yaoita *et al.*, 1990) and bullfrog *Rana catesbeiana* (Helbing *et al.*, 1992). Expression of TR genes was shown to be regulated by TH (Kawahara *et al.*, 1991). TR β , ornithine transcarbamylase, and carbamyl phosphate synthetase mRNAs are up-regulated in the liver of spontaneously metamorphosing or TH-treated bullfrog tadpoles (Helbing *et al.*, 1992). TH increases mRNA of 63 kDa keratin and prolactin prevents this up-regulation (Baker and Tata, 1992).

During anuran metamorphosis, organs of the larval body undergo region-dependent changes: organs such as the tail degrade

and others like the liver are transformed into adult organs (Yoshizato, 1989). However, the molecular mechanism of the region-dependent effect of TH and PRL on tadpole organs has been poorly understood. The present study aimed at understanding these region- and hormone-dependent changes at the gene expression level, focusing on genes involved in the metabolism of collagens of the skin.

Collagens are the most abundant proteins of the extracellular matrix in vertebrates. Though the turnover rate of this protein is very low in normal tissues, its synthesis and degradation are accelerated in tissues undergoing remodeling in processes such as regeneration (Dresden and Gross, 1970) and metamorphosis (Gross and Lapiere, 1962).

Animal collagenase (EC.3.4.24.7, MMP I) catalyzes the cleavage of native triple helices at a unique point in each α chain of interstitial collagen molecules and generates the two fragments (Gross *et al.*, 1974). These fragments spontaneously denature to

Abbreviations used in this paper: p.f.u., plaque forming unit.

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GGCGGAACCCCAATATACATCAGGAACACTGCTAACCTCAATCA	44
ATGTTGTCAGGGTTATGGAGCTCTATCCTGGCACTATTGGGGGTATTCCCTCAAAGCGTCGGGGAGTCCGGGGCGAAACA	125
M L S G L W S S I L A L L G V F L Q S V G E F R A E T	
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Q E Q D V E I V Q K Y L K N Y Y N S D K R N S G L V V	
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E I L K Q F F G L K V T G K P D A E T L V M K Q S T C	
GGAGTTCCTGACGTGGGTGAGTAGTTCTCCTCCTCAGGGAACCCCTCGGTGGGAGAACACACACCTGACCTACAGGATTGAA	368
G V P D V G E Y V L T P G N P R W E N T H L T Y R I E	
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N Y T P D L V S P L T F T K V S E G Q A D I M I S F V	
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R G D H R D K Y P F D G P G G N L A H A S Q P G P G I	
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E V E L G L Q A A Y E M A D R D E V R F F K G N K Y W	
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A V S G Q D V L Y G Y P K D I H S S F G F P T G V A H	
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E C W S Y D E Y K Q S M D T G Y A D E F P G D A V F Q	
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K F F H G T R Q Y Q F D L K T K R I L T L Q K A N S W	
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F N C R K N	
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GTCCTTAACTGGAATATT	1521

Fig.1. Nucleotide and deduced amino acid sequence of bullfrog tadpole collagenase (Tc1). The sequence was determined and confirmed on both strands. The upper rows indicate nucleotide sequence and the lower indicate that of deduced amino acids. Base numbers are taken from 5' end to 3' end of Tc1. The putative active center of metalloproteinases is boxed. The underline in the 3' flanking region indicates the potential polyadenylation signal sequence. The putative site of the signal peptide cleavage is shown by the arrow. The putative amino terminus of the active form of the enzyme is indicated by the arrow-head.

gelatin that is then susceptible to further degradation by less specific proteinases. Collagenase is responsible for the first step of the catabolism of collagen fibers (Gross *et al.*, 1974). Genes and cDNAs for collagenase were isolated from several mammalian species such as human (Goldberg *et al.*, 1986; Collier *et al.*, 1988), porcine (Krebs *et al.*, 1990) and rabbit (Fini *et al.*, 1987), but not from non-mammalian vertebrates.

The metamorphic change of tadpole skin cells shows regional specificity; cells in tail tissues fall into programmed cell death and cells in the body proliferate and differentiate (Yoshizato, 1989). We have recently reported that TH increases the amounts of collagenase in the skin of metamorphosing bullfrog tadpoles (Oofusa and Yoshizato, 1991). In the present study, to investigate the region-dependent metamorphic changes of tadpole skin cells at the molecular level, cDNA cloning and RNA blot analyses of both collagens and collagenase were performed in relation to actions of metamorphosis-associated hormones (TH and PRL).

Results

Isolation and characterization of cDNA of tadpole collagenase

A cDNA library of tadpole back skin was screened

immunologically for clones of collagenase using anti-bullfrog tadpole collagenase antisera. Seven positive clones were obtained from 2×10^5 p.f.u. (plaque forming unit) of recombinant phages. All clones were found to contain the same 1.5 kilo base pair inserts, suggesting that they were identical clones. One of them was named Tc1 and was subject to DNA sequencing and further analysis. The nucleotide and deduced amino acid sequences of Tc1 are shown in Fig. 1. The size of bullfrog tadpole collagenase mRNA was estimated as about 1.5 kilo bases (kb) from RNA blot analyses (Fig. 2), suggesting that Tc1 carries the full length of the molecule. The polyadenylation signal was found in its 3' flanking region. However, Tc1 lacks poly (A)-sequence in its 3'-end region. Four cysteine residues were present in the deduced amino acid sequence of Tc1, which contrasts with human fibroblast collagenase in that they contain 3 cysteine residues.

Homologies between the deduced primary structures of bullfrog, porcine and human collagenase are shown in Table 1. Bullfrog collagenase shows about 60% homology to those of human and porcine in the nucleotide sequence of cDNA and about 70% in the deduced amino acid sequence. The amino acid sequence of the putative active center of MMPs (McKerrow, 1987) was found in Tc1 (Fig. 1). This consensus sequence in MMPs

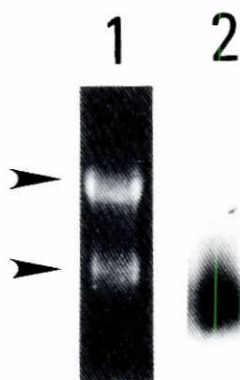


Fig. 2. RNA blot analyses of the size of bullfrog tadpole collagenase mRNA. RNA was extracted from the cultured back skin of bullfrog tadpoles. Ten micro grams of RNA was subject to electrophoresis and RNA blot analyses with ^{32}P -labeled Tc1. (Lane 1) Stained by ethidium bromide; (lane 2) hybridized by Tc1. Arrowheads indicate the position of 28S RNA (above) and 18S RNA (below).

active center was VAAHELGHSLGLSHS and was completely conserved in bullfrog. We isolated a human fibroblast collagenase cDNA clone (Hfc1) from a cDNA library of human foreskin cells. Tc1 and Hfc1 cross-hybridized each other (data not shown). These results indicate that Tc1 is highly homologous to mammalian collagenases.

Expression of genes of collagen and collagenase during spontaneous metamorphosis

RNAs were extracted from the body and tail skin of tadpoles at stages X, XV, XX and XXIV defined by Taylor and Kollros (1946), and were subject to RNA blot analyses using Tc1 or Hf677 as a probe (Fig. 3). Expression levels of both collagenase and collagen genes progressively increased in the body as metamorphosis proceeded. Expression of the two genes in the tail was noteworthy. Transcripts of collagenase genes increased in quantity with the



Fig. 3. Expression of genes of collagen and collagenase in spontaneously metamorphosing tadpoles. Five micro grams of RNA was electrophoresed for RNA blot analyses with ^{32}P -labeled Hf677 in the upper row or ^{32}P -labeled Tc1 in the lower one. The left column shows RNA extracted from the body skin and the right column shows that of the tail skin. Roman numerals on each lane represent the developmental stage of tadpoles examined.

progression of metamorphosis, as in the body skin. However, those of collagen genes showed the metamorphic change which was opposite to that in the body. The expression level was markedly decreased at the onset of metamorphosis (stage XX) and kept decreasing during climax stages of metamorphosis. Thus, collagen gene expression shows region-dependent metamorphic changes.

Collagen gene expression in TH- or PRL-treated animals

Results shown in Fig. 3 suggest that genes of collagen and collagenase are regulated in their expression by metamorphosis-associated hormones. This suggestion was experimentally tested for the collagen gene. Tadpoles at stage X were treated with T_3 for 3 days or PRL for 5 days. The northern blot analysis was performed for RNA extracted from the tail skin (Fig. 4). As expected, TH, a stimulator of metamorphosis, greatly reduced the gene expression and PRL, a suppressor of metamorphosis, enhanced it.

Region-specific regulation of gene expression by TH and PRL

The metamorphic northern blot analysis for genes of collagen and collagenase shown in Fig. 3 also suggests that transcription of these genes is hormonally regulated in a region-specific fashion. This suggestion was tested in a series of experiments shown in Fig. 5. RNAs were extracted from both the body and the tail skin of animals treated with TH for 3 days or PRL for 5 days and

TABLE 1

HOMOLOGIES BETWEEN NUCLEOTIDE AND DEDUCED AMINO ACID SEQUENCES OF BULLFROG, PORCINE AND HUMAN COLLAGENASE cDNAs

	Nucleotide	Amino acid
Bullfrog/Porcine	62%	70%
Bullfrog/Human	58%	67%

The nucleotide sequence and its deduced amino acid sequence of bullfrog collagenase cDNA described in Fig. 1 are compared with those of porcine (Krebs *et al.*, 1990) and human (Goldberg *et al.*, 1986).

electrophoresed for the RNA blot analysis. A single band estimated 1.5 kb was reproductively detected by Tc1 and a band larger than 6 kb was detected by Hf677. PRL stimulated the expression of collagen genes in both the body and the tail. In contrast and as expected, the effect of TH was region-dependent: stimulation in the body and the suppression in the tail.

No significant collagenase signals were obtained from the body skin of either control and PRL-treated animals and very weak signals were observed from the tail skin of PRL-treated tadpoles. Figure 5 clearly demonstrates that the expression of collagenase gene is hormonally regulated (up-regulation by TH and down-regulation by PRL), but does not show a region dependency, which is also expected from the result of Fig. 3.

Discussion

The larval skin undergoes region specific transformation in the process of metamorphosis: the body skin transforms into the adult skin while the tail skin falls into programmed cell death (Yoshizato,

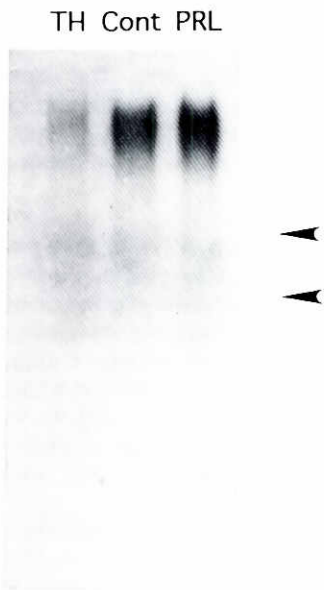


Fig. 4. Changes in mRNA of type I collagen in the tail of TH- and PRL-treated tadpoles. Tadpoles were treated with T_3 (300 pmoles/g body weight) for 3 days or PRL (0.4 units/g body weight) for 5 days. RNAs were extracted from the tail skin and subject to RNA blot analyses with ^{32}P -labeled Hf677. TH: thyroid hormone-treated animals, Cont: control animals, PRL: prolactin-treated animals. Each lane contained 10 mg of total RNA. Arrowheads indicate the position of 28S RNA (above) and 18S RNA (below).

1989). This region-dependent metamorphic change has been well understood at the cell level (Robinson and Heintzelman, 1987; Yoshizato, 1989; Izutsu *et al.*, 1994). The present study aimed at understanding region-dependent metamorphic changes of the tadpole skin at a molecular level, focusing on genes of collagen and collagenase. For this aim, we first tried to clone a cDNA of tadpole collagenase. A human cDNA clone of type I collagen $\alpha 1$ chain was utilized as a probe of collagen genes.

From an expression library, a cDNA clone for bullfrog tadpole collagenase (Tc1) was successfully screened by its specific antisera. To our knowledge, this is the first cDNA clone of vertebrate collagenase of non-mammalian origin. The deduced amino acid sequence of Tc1 revealed that collagenase was highly conservative from bullfrog through human. The deduced amino acid sequence suggests that bullfrog collagenase has a prepropeptide as the mammalian enzyme. In addition, some unique properties of the tadpole enzyme were revealed. The human fibroblast collagenase contains 2 cysteine residues in its active form (Goldberg *et al.*, 1986). However, Tc1 has 3 cysteine residues in its putative matured form. Windsor *et al.* reported that the cysteine residues in human fibroblast collagenase may be important for maintaining latency (Windsor *et al.*, 1991). Therefore, it is considered that the tadpole collagenase might be activated by a mechanism different from the human enzyme.

To correlate the gene expression of collagen and collagenase with the region-dependent metamorphic change of larval skin, we investigated the metamorphic northern blot analysis for these genes of tadpoles that were in spontaneous metamorphosis. Collagenase gene does not show the regional specificity for their expression. Both the body and the tail enhance the expression

level at the early metamorphic climax. This seems reasonable because the body skin is known to undergo the drastic and rapid remodeling which requires the breakdown of larval connective tissues (Gross, 1982) and the tail is to be totally broken down (Usuku and Gross, 1965).

Gene expression of collagen makes a clear contrast with that of collagenase, because collagen genes are shown to be regionally regulated in their expression. The expression of collagen genes becomes promoted in the body skin at the onset of metamorphosis (stage XX), contrasting with the tail skin in which expression begins to be sharply suppressed. The present study shows that TH is responsible for this region-dependent metamorphic expression of collagen genes. TH stimulates expression in the body skin but represses it in the tail skin. As mentioned above, a marked reduction in the gene expression level is observed in the tail at stage XX of spontaneous metamorphosis, where no apparent degenerative changes occur, indicating that TH reduces the transcription rate of collagen genes before histolysis starts. Evidently, the enhancement of collagen gene expression in the body skin by TH correlates with the remodeling of the skin at metamorphosis. At present the mechanism is not known by which TH exerts dual actions that are opposite each other. It is assumed that region-specific transcription factors are involved in this mechanism.

PRL does not show region-dependent action on either collagen or collagenase genes. Transcription of collagen genes is accelerated in both the body and tail skin and that of collagenase gene is depressed also in the two tissues. It has been shown that PRL markedly stimulates collagen synthesis and suppresses collagenolysis in the tadpole tail (Yoshizato and Yasumasu, 1970, 1972a,b). It is generally accepted that PRL antagonizes the action of TH. Kawahara *et al.* (1991) showed that TH up-regulates the expression of TR genes in *Xenopus* tissues. PRL is shown to prevent this autoinduction of TR mRNAs by TH (Baker and Tata, 1992). The molecular mechanism of antagonistic actions of PRL on TH effects remains to be elucidated.

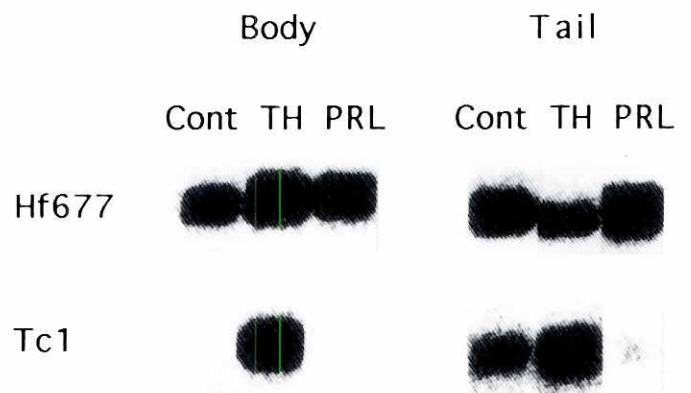


Fig. 5. Collagen and collagenase gene expression of TH- or PRL-treated tadpoles. Animals were injected with T_3 (300 pmoles/g body weight) for 3 days or PRL (0.4 units/g body weight) for 5 days. Ten micrograms of RNA was separated through formaldehyde denatured agarose gels for the RNA blot analysis with ^{32}P -labeled Hf677 (upper row) or ^{32}P -labeled Tc1 (lower row). The left column shows RNA extracted from the body skin and the right column shows that of the tail skin. Cont: control animals, TH: TH-treated animals, PRL: PRL-treated animals.

Materials and Methods

Animals and reagents

Tadpoles of bullfrog, *Rana catesbeiana*, were purchased from a local animal supplier and were staged according to Taylor and Kollros (1946). Hf677, a cDNA clone of human type I collagen $\alpha 1$ chain was a gift from Dr. Ramirez (Chu *et al.*, 1982). A cDNA library of human foreskin cells (Okayama and Berg, 1983) was distributed from Japanese Cancer Research Resources Bank-Gene. Chemicals were purchased as follows; T₃ and PRL from Sigma Chemical Co. (St. Louis, USA); Uni-Zap cDNA synthesis system and pBSK(-) plasmid vector from Stratagene (La Jolla, USA); nylon membrane (Hybond N+) from Amersham (Little Chalfont, UK); Vectastain ABC system from Vector Laboratory (Burlingame, USA); [α^{32} P]-dCTP from Dupont (Wilmington, USA); and enzymes for DNA manipulation from Takara Shuzo (Kyoto, Japan), Toyobo (Osaka, Japan) and Gibco/BRL (Grand Island, USA). All other chemicals were of analytical or of molecular biological grade, if not specified otherwise.

Cloning of cDNA of tadpole collagenase

The back skin of bullfrog tadpoles (stage X) was removed and cultured for 2 days at 37°C as described previously (Oofusa and Yoshizato, 1991). The tissues that had been shown to actively produce collagenase during the period of culture were broken down by a Polytron homogenizer (Kinematica AG, Lucerne, Switzerland) and subjected to RNA extraction with acid guanidinium thiocyanate-phenol-chloroform (Chomoczynski and Sacchi, 1987). The quality of RNAs was checked by formaldehyde denatured agarose gel electrophoresis (Sambrook *et al.*, 1989). A cDNA library was constructed from poly(A)⁺ RNA using Uni-Zap cDNA synthesis system according to the manufacturer's protocols. The library contained 8x10⁵ independent clones and a portion of this library was subjected to immunological screening. Plaques of recombinant phages were transferred to nitrocellulose filters saturated with isopropyl-1-thio- β -D-galactoside (Sambrook *et al.*, 1989). The collagenase-containing fusion proteins on filters were detected by anti-bullfrog tadpole collagenase antisera (Oofusa and Yoshizato, 1991) and the Vectastain ABC system. Positive plaques were isolated and purified by repeated immunological screening.

DNA sequencing

Inserts of cDNA clones were subcloned into the pBSK(-) plasmid vector. Subclones for DNA sequence containing overlapping cDNA fragments were generated by digesting the cloned cDNA with exonuclease III and mung bean nuclease (Sambrook *et al.*, 1989). Nucleotides of cDNA clones were sequenced by the "Dye-deoxy" termination reaction using an automated DNA sequencer (ABI; Foster City, USA, Model 373A) according to the manufacturer's protocol.

RNA blot analysis

RNA was extracted from both the body and the tail skin of hormone-treated and spontaneously metamorphosing tadpoles. Hormones were intraperitoneally given to tadpoles at stage X which were kept at 24°C. T₃ was injected through the tail muscle at 300 pmoles per gram body weight of tadpoles (Oofusa and Yoshizato, 1991) and the RNA extraction was performed on day 3. PRL was injected at 0.4 units per gram body weight every other day during the period of treatment (Yoshizato and Yasumasu, 1970) and RNA was extracted from the skin on day 5.

Solutions of total RNA fractions were subjected to formaldehyde denatured agarose gel electrophoresis in the presence of formaldehyde and formamide, transferred onto nylon membranes and hybridized with ³²P-labeled Tc1 or Hf677 (Chu *et al.*, 1982). Membranes were washed to remove unbound probes and were dried for autoradiography (Sambrook *et al.*, 1989).

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