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Expression of the Epidermis-specific Gene *ep*37 before and after Metamorphosis of the Japanese Newt, *Cynops pyrrhogaster*

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ABSTRACT—We studied expression of the epidermis-specific gene, designated *ep*37, by means of Northern blotting in various tissues and at different developmental stages of the Japanese newt, *Cynops pyrrhogaster*. Using the coding region of *ep*37 cDNA as a probe, we detected five RNA species. Of these, two were found before metamorphosis and the other three were detected in adult tissues. Amounts of the pre-metamorphic products markedly decreased and two of the three adult products promptly appeared at metamorphosis. These observations along with the difference in affinity of each RNA species to the cDNA probe suggest that the *ep*37 gene is a member of a novel epidermis-specific gene family, the expression of which is under metamorphic regulation.

INTRODUCTION

In the early development of vertebrates, epidermis differentiates from the ectoderm, then covers the entire external surface of the embryo [4, 18]. The major cell groups, cutaneous, neural, and neural crest cells derive from the ectoderm [1, 6]. However, when surgically excised and cultured in isolation, the presumptive ectoderm can only form "atypical" epidermis as demonstrated in amphibian embryos [14]. This fact indicates that the autonomous developmental fate of the ectoderm is the epidermis and hence the differentiation of the epidermis is a fundamental process in animal development.

We found a protein specific for epidermal differentiation of the gastrula ectoderm and isolated its cDNA from the Japanese newt, *Cynops pyrrhogaster* [16, 17]. The cloned gene was expressed solely in the epidermis and not in the neural plate of the neurula embryo. The deduced amino acid sequence did not correspond to known protein sequences, therefore it was thought to be a novel epidermis-specific protein. We designated the protein as EP37 (from epidermis-specific protein and its molecular weight deduced from the cDNA sequence of 37 kilodaltons) and the gene as *ep*37.

Several epidermal proteins and genes have been reported and the regulation of their expression has also been studied in detail. Some of them described the changes that occur before and after the amphibian metamorphosis [3, 9, 10]. For example keratins in *Xenopus laevis* were grouped into three classes according to their patterns of expression in the context of metamorphosis [3]. Amphibian metamorphosis is a spectacular event which adapts the pre-metamorphic, submerged larva to life on land. Because the epidermis changes to withstand exposure to air, *ep*37 expression should differ before and after the metamorphosis.

In this study we examined the expression of the *ep*37 gene in various tissues and at different developmental stages by means of Northern blotting. The results demonstrated not only the presence of multiple RNA species homologous to the *ep*37 gene but also changes in their emerging patterns before and after the metamorphosis of *Cynops pyrrhogaster*.

MATERIALS AND METHODS

Biological materials

We collected adult Japanese newts (*Cynops pyrrhogaster*), from the countryside near Nagoya, in central Japan. Fertilized eggs obtained by sequential injection of human chorionic gonadotropin (Gonatropin; Teikoku Zoki, Tokyo, Japan) into adult females, were reared at 18°C until metamorphosis began. Embryos were staged according to Okada and Ichikawa [12].

RNA preparation

Total RNAs were extracted using acid guanidinium thiocyanate-phenol-chloroform [2] as follows. Embryos or tissues up to 100mg of wet weight were homogenized directly in at least 10 volumes of 4 M guanidinium thiocyanate containing 0.5% sarcosyl, 0.1 M 2-mercaptoethanol, 25 mM sodium citrate (pH 7.0). A one tenth volume of 2 M sodium acetate (pH 4.0), one volume of water-saturated phenol, and 0.2 volumes of chloroform were sequentially added to the homogenate with thorough mixing. The final suspension was shaken vigorously and cooled on ice for 15 min. After centrifugation, RNA was precipitated from the aqueous phase with isopropanol.

Northern blots

Total RNA (10 μg) equivalent to the amount from about 10 mg of tissues was electrophoresed through 1.2% agarose gel containing formaldehyde. RNA markers (0.16–1.77 kb and 0.24–9.5 kb ladders; Bethesda Research Laboratories, Maryland, USA) were in-

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cluded and photographed after staining by ethidium bromide. RNAs were then capillary-transferred to a nitrocellulose membrane (Nitroplus 2000; MSI, Massachusetts, USA) [13]. After baking at 80°C for 2 hr the membranes were prehybridized and hybridized with a radioactive cDNA probe at 42°C overnight in the presence of 1x Denhardt's solution, 2x SSC, 100 μg/ml denatured salmon sperm DNA and 50% formamide. A radioactive cDNA probe was prepared by nick translation [13] of the HindIII-SphI fragment of pG3m11N138–48 cDNA [17], which corresponded to amino acids 30–275 of the EP37 protein, as the template in the presence of alpha-^{32}P-dCTP. Radioactive signals were detected using a Fujix BAS2000 analyzing system.

RESULTS

RNAs from various adult tissues such as heart, eye, intestine, stomach, liver, kidney, anal gland, testis, skin and muscle together with RNAs from hatched swimming larvae at stage 42, were Northern blotted. The *ep*37 cDNA probe hybridized to more than two RNA bands at a stringency of 0.4x SSC at 56°C (Fig. 1, upper panel). Five transcripts homologous to *ep*37 cDNA were detected. The sizes of these RNA products estimated by interpolating the electrophoretic mobility of the RNA markers, were 4.0 and 2.9 kb in the eye and in skin, 3.5 kb in the stomach, 2.3 and 1.9 kb in the swimming larva. We tentatively designated these RNA products as A4.0, A2.9, A3.5, L2.3 and L1.9, respectively. When the same membrane was washed at a stringency of 0.2x SSC at 68°C (Fig. 1, lower panel), A4.0 in the eye and in skin disappeared but A2.9 remained. A3.5 in the stomach lane almost completely disappeard. Both L2.3 and L1.9 in the swimming larva remained under this condition. In another experiment, both A4.0 and A2.9 were detected in RNAs from the tongue (data not shown), but adult tissues, including the brain, lung, spleen, cholecyst, urinary bladder, fat body did not show this positive signal. When swimming larvae were dissected along the anterior-posterior axis into the head, body and tail, there were no significant differences among the RNAs from these parts (data not shown).

Because completely different Northern profiles were obtained from adult and larval preparations, we studied the RNAs isolated from individuals around various metamorphic stages (Fig. 2). Before metamorphosis (St.40, St.50, St.58 in Fig. 2), L2.3 and L1.9 were invariably present. Upon metamorphosis, A4.0 and A2.9 appeared promptly and L2.3 and L1.9 decreased markedly (Fig 2, META). Because each RNA sample was prepared from one animal in this experiment, this observation indicated the coexistence of A4.0 and A2.9 plus L2.3 and L1.9 in that individual during metamorphosis. After metamorphosis, L2.3 and L1.9 disappeared (Fig. 2, YA).

DISCUSSION

Northern blots revealed at least five RNA products homologous to *ep*37 cDNA. Because A4.0 and A3.5 disappeared at higher stringency, while A2.9, L2.3 and L1.9 remained, at least two groups of genes might be present with respect to sequence similarity. This leads to the notion that *ep*37 is a member of a novel epidermis-specific gene family. The fact that PCR amplification of *Cynops* genomic DNA using the same set of primers for *ep*37 cDNA gave several products (data not shown) seems to support this speculation.

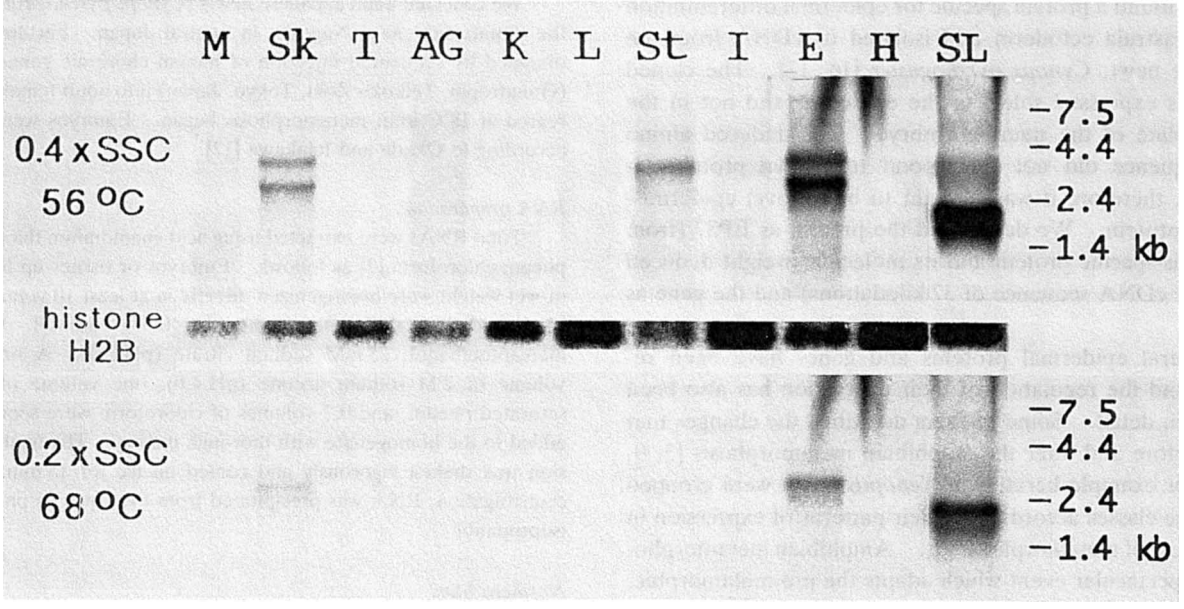

FIG. 1. Northern blot of *ep*37 expression in various adult tissues and in larva of *Cynops pyrrhogaster*. RNAs blotted onto nitrocellulose were hybridized with an *ep*37 cDNA probe, then washed in 0.4x SSC at 56°C (upper panel) and 0.2x SSC at 68°C (lower panel). The amounts of RNAs in each lane were verified using a chicken histone H2B probe (Oncor, P7010). M, muscle; Sk, skin; T, testis; AG, anal gland; K, kidney; L, liver; St, stomach; I, intestine; E, eye; H, heart; SL, swimming larva.

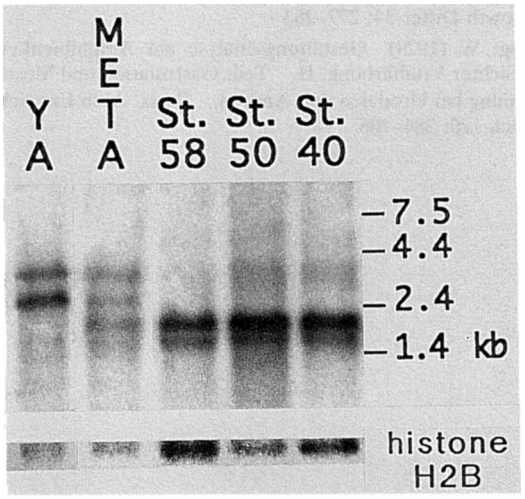

FIG. 2. Northern blot of *ep*37 expression at different developmental stages of *Cynops pyrrhogaster*. RNAs blotted onto a membrane were hybridized by *ep*37 cDNA probe and washed in 0.4x SSC at 56°C. The amounts of RNAs blotted were verified using a histone H2B probe. St.40, embryo just before hatching; St.50, swimming larva; St.58, larva just before metamorphosis; META, metamorphosing larva; YA, post-metamorphic young adults.

Whether A2.9, L2.3 and L1.9 were produced by alternative splicing or by transcription of independent genes remains unclear. Another possibility is that a longer product such as L2.3 is the unprocessed RNA of a mature product such as L1.9. Although L1.9 and L2.3 were detected as two discrete bands, we cannot exclude the possibility that L1.9 was the degraded form of L2.3. To clarify these issues, all the cDNAs corresponding to the five RNA products should be cloned.

As the EP37 was identified as an epidermis-specific protein, we predicted that adult skin would express the *ep*37 gene(s). However, we cannot explain why it is expressed in the eye, tongue and stomach of the adult newt. Since we prepared RNA from the whole eyeball, we could not identify which part of eye expressed the transcripts. Because some parts of eye (lens, for example) are derived from embryonic epidermal tissues [7, 8, 15], the expression in eye should be examined in more detail. In the context of epidermal specificity, the expression of *ep*37 genes in the tongue and stomach was unexpected, because the tongue consists of endodermal and mesodermal derivatives, and the stomach is solely derived from endoderm. Concerning that all of these tissues face the external environment, these results may reflect a yet unknown function of EP37 protein(s).

The switching of mRNA species relating to the *ep*37 gene during metamorphosis might reflect a change in the structure and function of the epidermis from larval to adult type [5, 11]. We did not discriminate whether the adult and larval type products were synthesized simultaneously in the metamorphosing animal. Because the larval products were abundant just before metamorphosis and decreased drastically thereafter, one possibility is that the detected larval product is a remnant mRNA of the pre-metamorphic stage and that larval products are not synthesized during metamorphosis. Another issue to be resolved is whether the same subset of epidermal cells sequentially expresses both the larval and the adult messages. As the major subsets of cells in skin undergo drastic changes during metamorphosis [11], it is likeky that cells synthesizing adult type products propagate in the tissue, while those synthesizing larval products are eliminated. The detailed localization of EP37 subtypes in conjunction with the reorganization of metamorphosing skin remains to be clarified.

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