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Original Article

Molecular cloning of *Xenopus* hatching enzyme and its specific expression in hatching gland cells

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ABSTRACT UVS.2 has been known as a cloned cDNA expressed selectively in the hatching gland cells of *Xenopus laevis*. To determine the molecular identity and function of UVS.2-encoded proteins, antibodies were raised against a bacterially-expressed fusion protein comprising glutathione-S-transferase (GST) and UVS.2. Anti-GST-UVS.2 antibodies inhibited the vitelline envelope digesting activity of the medium (hatching medium) in which dejellied prehatching embryos were cultured. On Western blotting, hatching medium contained 60 kDa and 40 kDa molecules reactive with these antibodies. Whole-mount immunostaining showed a specific localization of UVS.2 protein in the hatching gland cells which appeared first at stage 20, increased in number and intensity to stage 31 then decreased gradually thereafter. Immunoelectron microscopy revealed that UVS.2 protein is localized exclusively in the secretory granules in the hatching gland cells. A cDNA library from the dorsoanterior portion of stage 25 embryos was screened with UVS.2, and a 1.8 kb insert thus cloned contained additional 619bp and 204bp at the 5' and 3' ends of UVS.2, respectively. This clone, designated XHE, contained an open reading frame encoding 514 amino acids including both signal and propeptide sequences. The predicted mature enzyme comprising 425 amino acids consists of about 200 amino acid-long metalloprotease sequence of astacin family at the N-terminus, followed by two repeats of CUB domain each 110 amino acid-length. We conclude that UVS.2 represents an approximately 3/4 C-terminal portion of the hatching enzyme.

KEY WORDS: *hatching enzyme, gene expression, hatching gland cells, metalloprotease, Xenopus laevis*

Introduction

The hatching of *Xenopus* embryos is aided by an embryo-derived enzyme, the hatching enzyme, which partially digests the fertilization envelope and jelly (Carroll and Hedrick, 1974; Yoshizaki and Yamasaki, 1991). Although efforts have been made to isolate the hatching enzyme (Urch and Hedrick, 1981), its detailed characterization has been hampered by the extremely small amount of enzyme produced by embryos. That the hatching enzyme is produced and secreted by epidermal cells, the hatching gland cells, has been based on electron microscopic observations (Yoshizaki, 1973) and cytochemical localization of proteolytic activity in these cells (Yoshizaki, 1991). These cells were shown to be transient secretory cells that are located in a specific pattern in the anterodorsal region of prehatching embryos. Because of this strictly transient nature and location, hatching gland cells have recently attracted attention as a model for studying inductive and patterning mechanisms during early development. In these studies, the cDNA clone XA-1 (Sive *et al.*, 1989) and tyrosine hydroxylase (Drysdale and Elinson, 1991, 1993) have proved convenient molecular markers for detecting hatching gland cells, but the exact functional relevance of these markers is not known.

A cDNA clone, UVS.2, was identified as a gene selectively expressed in the dorsoanterior region of *Xenopus* embryos (Sato and Sargent, 1990). Its transcripts have been shown to be localized by *in situ* hybridization in the anterior neural fold of neurula embryos, and the UVS.2 protein was visualized by immunohistochemical means exclusively in the hatching gland cells. Sequence analyses of UVS.2 suggested the presence of a protease domain, but were not conclusive because this clone did not contain a full open reading frame.

Abbreviations used in this paper: GST, glutathione-S-transferase; HM, hatching medium; VE, vitelline envelope; XHE, *Xenopus* hatching enzyme, submitted to the DDBJ, EMBL and GenBank sequence data banks with accession number D89632.

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This study was undertaken to determine the functional significance of UVS.2 which has often been referred to as a developmentally regulated gene. We show in this study that (1) UVS.2 protein represents a portion of the *Xenopus* hatching enzyme, and (2) this enzyme is a metalloprotease belonging to the astacin family.

Results

Anti-GST-UVS.2 antiserum inhibits VE-digesting activity of hatching medium

When dejellied mature unfertilized eggs were placed in the hatching medium derived from the culture of 500 embryos/ml, their VEs were swollen during 30-45 min, and were completely digested in 60-80 min, resulting in the flattening of eggs. Dejellied eggs were incubated in hatching medium in the presence of various concentrations of immunoglobulins from anti-GST-UVS.2 sera or preimmune sera (control), and the rates of digestion of VEs were determined at various times after incubation. The VE-digesting activity of hatching medium was inhibited by 1:50-100 dilutions of antibodies (Table 1). Immunoglobulin from control preimmune serum was much less inhibitory, although at 1:50 dilution it caused a certain inhibition presumably due to some non-specific inhibition. These results indicate that our anti-GST-UVS.2 antiserum contains antibodies specifically reacting with the VE-digesting activity in the hatching medium.

The lysates of bacteria after induced expression of GST or GST-UVS.2 were electrophoresed on SDS-PAGE, and were Western-blotted using anti-GST-UVS.2 antiserum. The lysates containing GST did not react at all, whereas those containing GST-UVS.2 exhibited a band with apparent molecular weight at 60 kDa. Thus, our antiserum reacted specifically with UVS.2. Western-blots of hatching media from various preparations exhibited either one or both bands with apparent molecular weights at 60 kDa and 40 kDa (Fig. 1). The ratio of these components was variable according to preparations. Both bands were also present in the culture media of anterior embryonic halves possessing the VE-digesting activity (data not shown). Other weaker bands appeared inconsistently, and disappeared completely when transblots were reacted with the antiserum preabsorbed with the culture medium of posterior embryonic halves (Fig. 1, lane 2). These results indicate that both 60 kDa and 40 kDa components detected by our antiserum represent molecules exhibiting the VE-digesting activity.

TABLE 1

INHIBITION OF VITELLINE ENVELOPE (VE)-DIGESTING ACTIVITY OF HATCHING MEDIUM (HM) BY ANTI-GST-UVS.2 ANTIBODIES

	No. of eggs with digested VE at 60 min	90 min
HM	10	10
HM + 1/50 antiserum[a]	0	0
HM + 1/100 antiserum	0	1
HM + 1/500 antiserum	3	8
HM + 1/50 normal serum[a]	4	8
HM + 1/100 normal serum	10	10
Steinberg's solution	0	0

Ten dejellied eggs were placed each in 0.5 ml HM containing various concentrations of antibodies, and the number of eggs with digested VE was scored. [a]Crude immunoglobulin fraction diluted to equivalent serum concentrations indicated.

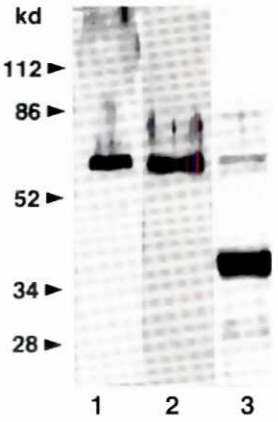

Fig. 1. Western blot analysis of hatching media using anti-GST-UVS.2 antiserum (lanes 1,3) or the same antiserum preabsorbed with culture medium of posterior embryonic halves (lane 2). *Lanes 1 and 2 were derived from the same hatching medium preparation. Numbers to the left refer to the molecular weight (kDa) of markers.*

Immunostaining of embryos

Whole-mount immunostaining was made on embryos at various stages. The embryos at stage 31 possessed strongly immunoreactive epidermal cells exclusively in the dorso-anterior portion of the head region. The positive signals comprising 5-6 cell-width were distributed along the dorsal midline anterior to the level of the ear vesicle, and extended into two arms of 2-3 cell-width in the form of an inverted Y at the forehead region (Fig. 2). This distribution of immunoreactive cells was exactly same as that described previously for hatching gland cells visualized by electron microscopy (Yoshizaki, 1973), or immunocytochemistry using antibodies against UVS.2 (Sato and Sargent, 1990) and tyrosine hydroxylase (Drysdale and Elinson, 1991). Embryos at stage 19 did not possess cells immunoreactive with anti- GST-UVS.2. The positive staining of hatching gland cells was first visible as weak and punctuating signals at stage 20, and during stage 20-25 both the immunoreactivity and the number of reactive cells increased (Fig. 2). Both the immunoreactivity and the number of positive cells were highest at stage 31, followed by a gradual decrease towards stage 35/36 and later (Fig. 3). A small number of scattered reactive cells were still present in the swimming larvae (stages 43-46).

In sections of stage 25-31 embryos, immunoreactivity with anti-GST- UVS.2 was confined to the cytoplasm apical to an accumulation of pigment granules in the hatching gland cells (not shown). This distribution of fluorescence was reminiscent of the previous observation using an antiserum different from ours (Sato and Sargent, 1990), and suggested an intracellular localization of immunoreactive molecules in the apical granules of hatching gland cells. Ultrathin sections of stage 31 albino embryos stained with the immunogold technique revealed a heavy accumulation of gold particles on the secretory granules in the apical cytoplasm of hatching gland cells (Fig. 4). No deposition of gold particles was seen in other regions in the hatching gland cells or in other types of cells in the embryo. All the results presented above indicate that our anti-GST-UVS.2 antibodies are directed against the hatching enzyme of *Xenopus laevis*.

Cloning of cDNA for Xenopus hatching enzyme

Eight positive clones were isolated through the screening of 8×10^4 recombinant phages. The inserts of these clones were identical in terms of the sequences of their 5' and 3' portions. Figure

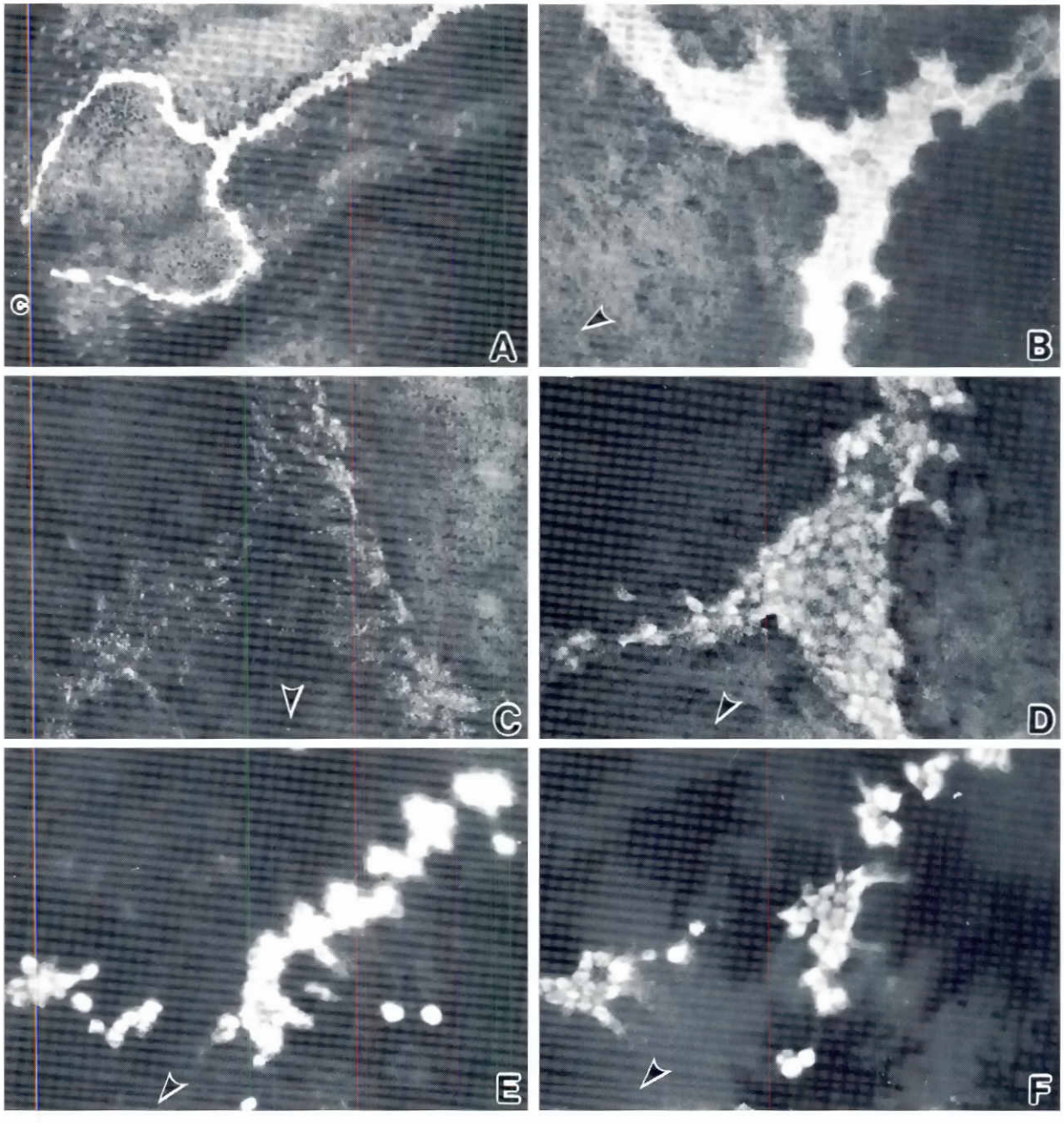

Fig. 2. Whole-mount immunostaining of embryos with anti-GST-UVS.2 antiserum and rhodamine B-conjugated anti-rabbit IgG, showing distribution of reactive cells at st. 31 (A,B), st. 20 (C), st. 25 (D), st. 35/36 (E), and st. 43 (F). (B) *Higher magnification of* **(A)**. *Arrowheads indicate the frontal side of embryos. c, cement gland. A, x12; B-F, x320.*

5 shows the complete nucleotide sequence of the longer insert, comprising 1,802bp, together with the deduced amino acid sequence. As seen in the figure, the sequence from the 620th to 1,598th bp was identical to that of UVS.2. Thus, in comparison with our cDNA, UVS.2 lacks 619bp and 204bp at both 5' and 3' regions, respectively. We hereafter refer to this clone as "*Xenopus* Hatching Enzyme (XHE)" instead of UVS.2. Northern blot analyses using various portions of dissected embryos and whole-mount *in situ* hybridization studies indicated that, as reported for UVS.2 (Sato and Sargent, 1990), the XHE transcripts appear first at stage 17 and are distributed in the pattern reminiscent of hatching gland cells (data not shown). XHE contains a long open reading frame encoding 514 amino acids, of which 19 amino acids starting from the first methionine are regarded as a signal peptide region because of its high content of hydrophobic residues and a signal peptide cleavage pattern. Based on the (-3,-1) rule (Von Heijne, 1984), the cleavage site by signal peptidase is predicted to be in between Ser19 and Ser20.

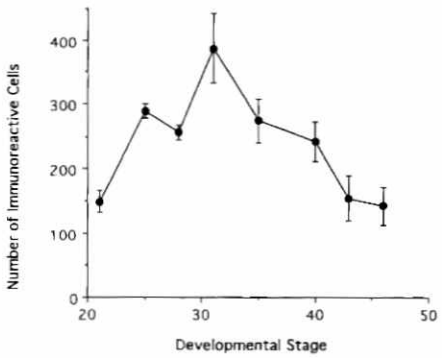

Fig. 3. Number of anti-GST-UVS.2 reactive cells per embryo during pre- and posthatching stages.

It was previously suggested that UVS.2 belongs to the astacin family of proteases, because 100 amino acids in the N-terminus of UVS.2 have a 40% identity to the C-terminal half of astacin (Dumermuth *et al.*, 1991). The proteases or protease domains of the astacin family are composed of about 200 amino acids. Comparison of the deduced amino acid sequences between XHE and the established proteases of the astacin family suggests that the XHE cDNA encodes the entire protease sequence of the astacin family, spanning 197 amino acid residues from Ser90 to Asn287. This protease domain in XHE contains the active-site consensus sequence of astacin family proteases, HExxHxxGFxHE, which is located immediately next to the most N-terminal region of UVS.2 (Dumermuth *et al.*, 1991). The similarity search using Bork and Beckmann (1993) as reference revealed that the remaining 226 amino acid residues following the protease domain encode two repeats of the CUB (Complement subcomponents C1r/C1s, Uegf, Bmp1) domain, each of which spans 110 amino acid residues. Taken together, we propose that XHE is synthesized as a preproenzyme with a signal sequence of 19 amino acids, a propeptide sequence of 90 amino acids, and a mature enzyme of 425 amino acids. In terms of domain structure, the mature enzyme of XHE is composed of the *Astacus* protease domain and two repeats of CUB domain (Fig. 6). The calculated molecular weight of the predicted mature enzyme is 47,182 Da.

Discussion

Previous demonstration of the UVS.2 protein as a specific marker of *Xenopus* hatching gland cells (Sato and Sargent, 1990) was based on its immunohistological localization identical to the morphological description of hatching gland cells (Yoshizaki, 1973). Using our anti-UVS.2 antibodies, we show in this study that the immunoreactive molecules are present exclusively in the secretory granules of hatching gland cells and in the culture medium ("hatching medium") of prehatching embryos. Our stage by stage observations of whole-mount immunostaining match well with the chronological fate of secretory granules in the hatching gland cells during the pre- to post-hatching stages of embryos (see Yoshizaki, 1973). These observations, combined with the antibody-mediated inhibition of VE-digesting activity in hatching medium, indicate that the UVS.2 proteins with apparent molecular weights at 60 kDa and 40 kDa represent the hatching enzyme *per se* of *Xenopus laevis*. We have also found (unpublished observation) that our antibodies specifically stain the hatching gland cells and react with a 55 kDa protein in the hatching medium of distant anuran species *Rana pirica* (previously referred to as *R. chensinensis*).

We have not identified the minor components which occasionally appear in Western blots. They also occur in culture media of both anterior and posterior embryonic halves (unpublished observation), and exhibit no specific cellular localization discernible in immunofluorescence light microscopy. Possibly these cross-reactive molecules represent proteases other than hatching enzyme: e.g. *Xenopus* bone morphogenetic protein-1 which possesses 38.3% homology in amino acid sequence with UVS.2 (Maeno *et al.*, 1993).

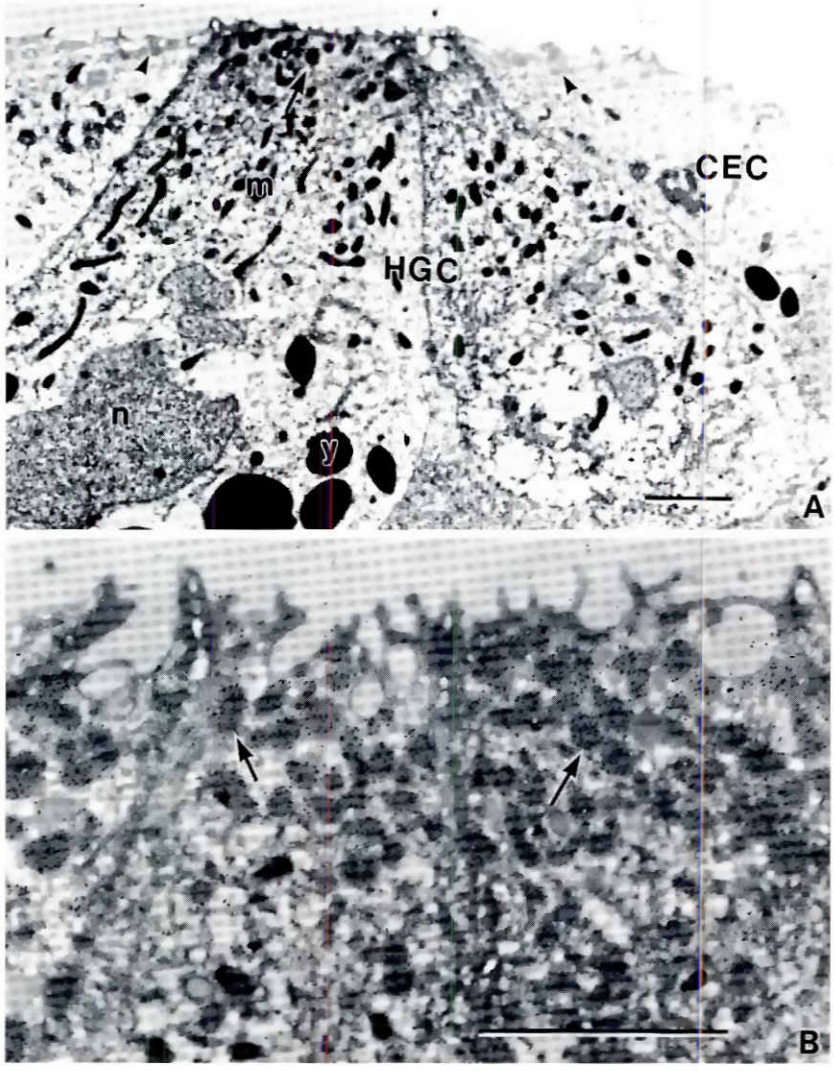

Fig. 4. Immunoelectron microscopic localization of UVS.2 in ultrathin section of stage 31 embryo, *showing hatching gland cells (HGC) and common epidermal cell (CEC), and colloidal gold particles distributed exclusively in secretory granules (arrows) in apical cytoplasm of HGC but not in those in CEC (arrowheads). m, mitochondrion; n, nucleus; y, yolk platelet. Bar, 3 μm.*

Among other markers of hatching gland cells so far described are tyrosine hydroxylase which Drysdale and Elinson (1990, 1993) utilized for studying inductive events in the differentiation and patterning of hatching gland cells. The relevance of tyrosine hydroxylase to the function of hatching gland cells is not known; nor is known the subcellular localization of this enzyme. Although in the embryonic epidermis tyrosine hydroxylase is expressed specifically in the hatching gland cells, this protein is reportedly expressed also in the brain and the spinal cord in later developmental stages (stage 34 and later). In comparison with the chronology of UVS.2 expression, tyrosine hydroxylase is first seen in hatching gland cells at stage 18/19: a stage slightly earlier than the first appearance of the UVS.2 protein (hatching enzyme) (stage 20) and later than that of UVS.2 or XHE transcripts (stage 17, by *in situ* hybridization: Sato and Sargent, 1990). Another hatching gland cell marker XA-1 (Sive *et al.*, 1989) is expressed earlier in the late gastrula stage (stage 11.5). This cDNA, also expressed in cement gland cells, may be involved in the anterior patterning of epidermal cells. In view of the experiments with explanted epidermal tissues showing that hatching gland cells are determined at stage 12-12.5 (Drysdale and Elinson, 1991), it is of interest to determine whether the expression of XA-1 is a prerequisite for the expression of XHE.

Our base sequence analyses of cloned XHE cDNA indicate that UVS.2 constitutes an approximately 3/4 C-terminal portion of functional *Xenopus* hatching enzyme. Particularly noticeable with XHE is the presence of a consensus amino acid sequence, HExxHxxGFxHE, immediately next to the N-terminal end of UVS.2. This sequence is known as the active site of the astacin family zinc-metalloproteases with a variety of biological functions, e.g., the digestive enzyme of the crayfish *Astacus*, differentiation factors such as human bone morphogenetic protein-1 (BMP-1) and Tolloid in *Drosophila*, a membrane protease of mouse kidney (meprin), hatching enzymes of the fish medaka (HCE and LCE), and a factor involved in the degradation of quail egg-shell matrix (QuCAM-1) (see Yamagami, 1996). The N-terminal domain of mature XHE has a 33-52% amino acid sequence homology with these known members of this family. Among these, the highest homology with XHE is found for the medaka hatching enzyme constituents HCE (51.3%) and LCE (47.1%) (Yasumasu *et al.*, 1994), and the quail CAM-1 (50.5%) (Elaroussi and De Luca, 1994). These highly homologous proteins of the astacin family are functionally similar in that they participate in the breakdown or degradation of embryo coats in a strictly regulated fashion during development.

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   1 CAGAGAGAAGGAGAAATATACCGATACATCTTTAAGATGGATGTAAAAATATCAGCCATT
                                         M  D  V  K  I  S  A  I     8
  61 CTGCTGGCCTGCATTATACAATATGCTGTGAGCTCACCTATACAGGTTTTCTACTCAGGT
     L  L  A  C  I  I  Q  Y  A  V  S ↓ S  P  I  Q  V  F  Y  S  G    28
 121 GCTAAAATACTTGCTGAAGAAGATGCAATGGCCAAGGAGGACATTCTCAAAGCAATAGAA
     A  K  I  L  A  E  E  D  A  M  A  K  E  D  I  L  K  A  I  E    48
 181 AAAGCAGATCCAGGAAGCGTGAAGACACAGGACTCCATGGACATACTCAGTCAGATATTA
     K  A  D  P  G  S  V  K  T  Q  D  S  M  D  I  L  S  Q  I  L    68
 241 GAGGCAAACAAAGGTATTAAGCTGCAAACCCAAGAAGGAGACATTATTCAAAAACAAGGT
     E  A  N  K  G  I  K  L  Q  T  Q  E  G  D  I  I  Q  K  Q  G    88
 301 CGTAGCGCCATAAATGATGCAAGATTCCTTTGGCCTAAGTCTGCCGATGGGATTGTCCCT
     R ▼ S  A  I  N  D  A  R  F  L  W  P  K  S  A  D  G  I  V  P   108
 361 GTGCCTTACAACCTCTCCTACAGCTATAATGCGGATCAGTTAGCTCTCTTTAAGAAGGCC
     V  P  Y  N  L  S  Y  S  Y  N  A  D  Q  L  A  L  F  K  K  A   128
 421 ATTCAGGAGTTTGAAGCCTTAACCTGTGTGAGATTTGTACCTTGGACAACAGAAGTCAAT
     I  Q  E  F  E  A  L  T  C  V  R  F  V  P  W  T  T  E  V  N   148
 481 TTTCTCAACATCATGTCTAATGGCGGTTGTGGATCTTTAATTGGGAAAAATGGAGGAGCT
     F  L  N  I  M  S  N  G  G  C  G  S  L  I  G  K  N  G  G  A   168
 541 CAGAGATTGGAGTTGGATGCAAATGGCTGCATGAATATGGGGATCATCCAGCATGAACTG
     Q  R  L  E  L  D  A  N  G  C  M  N  M  G  I  I  Q  H  E  L   188
 601 AACCATGCCCTGGGCTTCTATCATGAACAGAACAGAAGCGACCGGGATGACTATGTCATT
     N  H  A  L  G  F  Y  H  E  Q  N  R  S  D  R  D  D  Y  V  I   208
 661 ATACATACTGAGAATATCATACCAGACTTTCTCAAAATGTTCGAAAAATACAACACCAAT
     I  H' T  E  N  I  I  P  D  F  L  K  M  F  E  K  Y  N  T  N   228
 721 AACCTTGGGATTGAATATGACTACGCTTCAGTGATGCATTACTCACGTTATCACTATAGT
     N  L  G  I  E  Y  D  Y  A  S  V  M  H  Y  S  R  Y  H  Y  S   248
 781 ATCAACGGAGACATCACCATTGAGCCCAAGCCTGATCCAAATGTTCCCATTGGACAAAGG
     I  N  G  D  I  T  I  E  P  K  P  D  P  N  V  P  I  G  Q  R   268
 841 GATGGACTTAGTATTCTGGACATTTCTAAGATTAACAAACTATATGAGTGCAATGTGTGC
     D  G  L  S  I  L  D  I  S  K  I  N  K  L  Y  E  C  N  V  C   288
 901 TCCAATTTGCTCCCTTACTCCAATGGGATGATGATTTCAGCCAACTACCCCTCTGCCTAC
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 961 CCTAATAATGCCAACTGTGTTTGGTTGATTAGAACTCCATCTGGCCAGGTGACCCTGCAA
     P  N  N  A  N  C  V  W  L  I  R  T  P  S  G  Q  V  T  L  Q   328
1021 TTCCAAGCCTTTGATATCCAGTCATCTTCGGGCTGCGTCTCTGACTACATTAAGATCTAT
     F  Q  A  F  D  I  Q  S  S  S  G  C  V  S  D  Y  I  K  I  Y   348
1081 GATGGTCCTACTAAGGCATTCCCTGTGTTAGTAAACAGGGCATGTGGTACAGGACTGATC
     D  G  P  T  K  A  F  P  V  L  V  N  R  A  C  G  T  G  L  I   368
1141 CCTCTACAGATTGCCTCCACTAACCAGATGCTGGTTGAGTTTGTCAGTGATAGAGCAGTT
     P  L  Q  I  A  S  T  N  Q  M  L  V  E  F  V  S  D  R  A  V   388
1201 ACTGGGACCGGCTTCAAAGCAACATATGGCTCAATTCAGTGTGGTGGAGCTTTCTATTCC
     T  G  T  G  F  K  A  T  Y  G  S  I  Q  C  G  G  A  F  Y  S   408
1261 TCACCCAAGACTTTCACATCTCCGAATTACCCTGGAAACTATACAACTAACACAAACTGC
     S  P  K  T  F  T  S  P  N  Y  P  G  N  Y  T  T  N  T  N  C   428
1321 ACCTGGACAATCACAGCTCCCGCTGGATTCAAGGTATCACTGCGCATCACTGACTTTGAA
     T  W  T  I  T  A  P  A  G  F  K  V  S  L  R  I  T  D  F  E   448
1382 CTTGAGATCGGAGCTTCGTGCAGATATGACTATCTAAACATTTACAATTCAACTCTTGGT
     L  E  I  G  A  S  C  R  Y  D  Y  L  N  I  Y  N  S  T  L  G   468
1441 GCTGTCATGGGCCCATACTGTGGCCCCATAGATTTCCATTCTGCTATTGTTTCCAAATCA
     A  V  M  G  P  Y  C  G  P  I  D  F  H  S  A  I  V  S  K  S   488
1501 AATTCCATGATGATCACGATGAACAGCGACTTCTCGAAGCAATATAAAGGCTTCAGCGCT
     N  S  M  M  I  T  M  N  S  D  F  S  K  Q  Y  K  G  F  S  A   508
1561 ACCTATACTTTTGTGAGGTAAAGAGAATGGAAACGAAGGAATTCATTGTTACTGAACAAG
     T  Y  T  F  V  R                                             514
1621 GAGAGATGGGCGTCTGCAGTATCTTGTCCACATTAAATGAAATAAATGTGAAGGATCTAC
1681 AAGAAATTATACAGTTAATCATTTTTGGGAAGGGTTGTTSCTGTGTGATATAATTAT
1741 GTTTGAGGAATTTTAACAAGACTAAATAAACCATGATTTTGTAAATATAAAAAAAAAAAA
1801 AA
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Fig. 5. Nucleotide sequence and predicted amino acid sequence of XHE. *The numbers to the left and right refer to those of nucleotides and amino acids, respectively. Arrow and arrowhead indicate the putative signal sequence cleavage site and the putative N-terminus of mature enzyme, respectively. An active site consensus sequence of HExxHxxGFxHE motif of astacin family is indicated by shaded boxes. Underline indicates the poly(A) signal sequence.*

Another characteristic of XHE is the occurrence of two repeats of the CUB motif at the C-terminal side of the metalloprotease domain. QuCAM-1 possesses one repeat and the medaka HCE and LCE lack it. There are a number of diverse proteins containing both zinc-metalloprotease and CUBs, such as Tolloid, BMP-1, Uegf, and A5 protein, which are involved in the regulation of developmental processes (Bork and Beckmann, 1993). Another protein in this group may be the spermadhesins which are located

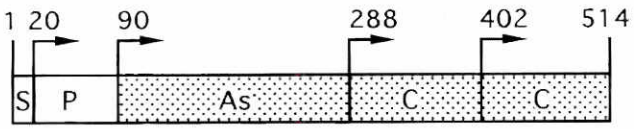

Fig. 6. Domain structure of XHE. *Structure of mature enzyme including Astacus protease domain (AS) and CUB domain (C) is represented by shaded boxes. S, signal sequence; P, prosequence. Numbers at the top refer to the starting amino acid residues of the domain structures.*

in the sperm head and act as a zona pellucida- and carbohydrate-binding protein (Sanz *et al.*, 1992). In analogy with the function elucidated for spermadhesins, the CUB motifs in XHE could function as an embryo coat-binding domain to aid in successful degradation of the substrate envelope molecules.

Given a cDNA-based structural framework of XHE and the appropriate antibodies reactive with its translational products, it is now possible to study the developmental regulation of XHE expression as well as the enzymatic characterization of the *Xenopus* hatching enzyme. Definition of XHE as coding for a metalloprotease on the basis of cDNA sequence is compatible with the previous report that the hatching enzyme of *Xenopus* (Urch and Hedrick, 1981) is sensitive to EDTA and $ZnCl_2$. The apparent molecular mass of 60 kDa (Fig. 1) is also approximately similar to what was estimated from sucrose-density gradient sedimentation (Urch and Hedrick, 1981), but is larger than the value of 48 kDa predicted from our amino acid sequence data. It remains to be determined whether this discrepancy is due to a mobility artefact in the SDS-PAGE system or to glycosylation of functional enzyme molecules. There is evidence that 40 kDa molecules are derived from 60 kDa molecules during manipulation of enzyme preparations (Fan and Katagiri, 1997), suggesting the possibility of autodigestion during the purification procedure. Efforts are currently being made to characterize the hatching enzyme and to study the regulation of its production during development.

Materials and Methods

Embryos

Mature females of *Xenopus laevis* were induced to ovulate by injection of 500 IU of human chorionic gonadotropin (Gonatropin: Teikoku Zoki Co., Tokyo, Japan). Embryos were obtained by artificial insemination, and were reared in chlorine-free tap water. Staging of embryos was made according to Nieuwkoop and Faber (1967).

Preparation of hatching medium

Stage 20 embryos were dejellied with 2.5% sodium thioglycolate (pH 8.3) in an agar-based Petri dish, and were cultured in 10% Steinberg's solution. At stage 22, fertilization envelopes were manually removed and resulting denuded embryos were transferred to Steinberg's solution containing antibiotics (100 IU/ml of Penicillin G potassium and 100 mg/ml of Streptomycin sulfate) at the density of 150 embryos in 2 ml solution, and were cultured for 16-20 h at 24°C until embryos attained stage 35/36. The culture medium was collected by decantation of embryos, centrifuged at 15,000g for 30 min, and was concentrated by centricon-10 (Amicon) to a 500 embryo/ml equivalent concentration, or lyophilized. The resulting solution will be referred to as "hatching medium". In some experiments, stage 20 embryos were cut into anterior and posterior halves at the posterior end of the gill region, and these embryonic halves were cultured in the same way as for collecting hatching medium.

Assay of vitelline envelope (VE) digesting activity

Mature unfertilized eggs were dejellied with sodium thioglycolate, washed and stored in De Boer's solution. Dejellied eggs were incubated with a gentle shaking in hatching medium in an agar-based 24-well dish at room temperature, and the digestion of VE was assessed by periodic observation by binocular microscope, taking the flattening of eggs as indication of VE digestion.

Anti-GST-UVS.2 antibodies and immunostaining

The fusion protein GST-UVS.2 was produced and purified according to Guan and Dixon (1991). Briefly, the cDNA for UVS.2 (Sato and Sargent, 1990) was ligated to the *Eco*RI site of expression vector pGEX-KG (Pharmacia), and the gene for glutathione-S-transferase (GST)-UVS.2 was expressed in *E. coli* strain TB-1. The cells were harvested, lysed, and sonicated, and the fusion protein was affinity-purified through glutathione-Sepharose 4B. A rabbit was immunized by two injections via subscapular route of total 1.5 mg of GST-UVS.2 in Freund's complete adjuvant. Crude immunoglobulin preparations were obtained by precipitation with 33% saturation of ammonium sulfate.

For immunostaining on whole-mount preparations, stage 19-46 embryos fixed with Dent' s fixative (Dent *et al.*, 1989) were pretreated with 5% non-fat dry milk, incubated with anti-GST-UVS.2 antiserum (1:2,000 dilution), followed by incubation with rhodamine-B-conjugated goat anti-rabbit IgG (1:100 dilution) (TAGO Inc., Camarillo, CA, USA). Controls consisted of treatment with preimmune rabbit serum instead of anti-GST-UVS.2 antiserum. For sectioned preparations, embryos fixed with Carnoy's fixative were embedded in paraffin, and 5 μm-thick sections were immunostained as for whole-mount preparations, except that the primary antiserum was preabsorbed with extracts of posterior halves of stage 25 embryos.

For immuno-electron microscopy, embryos were fixed in a mixture of 4% paraformaldehyde, 0.2% glutaraldehyde and 0.1 M sucrose in 0.05 M PIPES buffer (pH 7.2), embedded in LR-Gold (London Resin, Surrey, UK) at -5°C for polymerization with UV light in closed gelatine capsules, and ultrathin sections were placed on Formval-coated nickel grids. After pretreatment with 10% FCS, sections were treated successively with anti-GST-UVS.2 (1:1,000 dilution) and goat anti-rabbit IgG conjugated to colloidal gold particles (15nm, E-Y Lab., San Mateo, CA. USA) (1:100 dilution). The rabbit and goat antibodies were both diluted with phosphate-buffered saline containing 10% FCS. Control sections were incubated with non-immune rabbit sera instead of antisera. Sections were finally stained with uranyl acetate.

Western blotting

Samples were electrophoresed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) according to Laemmli (1970), and blotted onto nitrocellulose sheets (0.2 μm pore size) by the method of Towbin *et al.* (1979). The transblotted sheets were incubated with a 1:1,000 dilution of rabbit anti-GST-UVS.2 antiserum and a 1:500 dilution of the alkaline phosphatase-conjugated goat anti-rabbit IgG (Zymed, South San Francisco, CA, USA), and processed for examination of phosphatase activity.

Isolation of cDNA

Fertilization envelopes were manually removed from dejellied stage 25 embryos which were dissected so that the portions of embryos containing the dorsal part anterior to the gill region (dorso-anterior portion) were collected. The isolated embryonic portions were put into isogen medium (Nippon Gene Co., Toyama, Japan), homogenized, and total RNA was extracted according to the instruction manual. Poly(A)$^+$ RNA was purified by oligo(dT)-cellulose column. The cDNA was synthesized with a TimeSaver cDNA Synthesis kit using 5 μg of poly(A)$^+$RNA and an oligo(dT)primer, and the resulting cDNA was ligated into λExCell arms (Pharmacia). 8×10^4 plaques were screened with a 979-bp *Eco*RI-fragment of UVS.2 (Sato and Sargent, 1990) which was labeled with [α-^{32}P]dCTP, using a random-primed DNA labeling kit (Amersham Int. Plc.). After screening under high stringency conditions, the positive clones were subcloned by *in vivo*

phagemid release, and overlapping subclones were generated using a stepwise deletion method (Heinkoff, 1984). Both strands were sequenced by using Taq Dye primer Cycle sequencing kit with a 373A DNA sequencer (Applied Biosystems, Foster City, CA).

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References

BORK, P. and BECKMANN, G. (1993). The CUB domain, a widespread molecule in developmentally regulated proteins. *J. Mol. Biol. 231:* 539-545.

CARROLL Jr., E.J. and HEDRICK, J.L. (1974). Hatching in the toad *Xenopus laevis*: Morphological events and evidence for a hatching enzyme. *Dev. Biol. 38:* 1-13.

DENT, J.A., POLOSON, A.G. and KLYMKOWSKI, G.M. (1989). A whole mount immunocytochemical analysis of the expression of the intermediate filament protein vimentin in *Xenopus laevis*. *Development 105:* 61-74.

DRYSDALE, T.A. and ELINSON, R.P. (1991). Development of the *Xenopus* hatching gland and its relationship to surface ectoderm patterning. *Development 111:* 469-478.

DRYSDALE, T.A. and ELINSON, R.P. (1993). Inductive events in the patterning of the *Xenopus laevis* hatching and cement glands, two cell types which delimit head boundaries. *Dev. Biol. 158:* 245-253.

DUMERMUTH, E., STERCHI, E.E., JIANG, W., WOLZ, R.L., BOND, J.S., FLANNERYI, A.V. and BEYNON, R.J. (1991). The astacin family of metalloprotease. *J. Biol. Chem. 1266:* 21381-21385.

ELAROUSSI, M.A. and DE LUCA, H.F. (1994). A new member to astacin family of metalloproteases: a novel 1, 25-dihydroxyvitamin D-3-stimulated mRNA from chorioallantoic membrane of quail. *Biochim. Biophys. Acta 1217:* 1-8.

FAN, T. and KATAGIRI, CH. (1997) The mode of action on the vitelline envelope of *Xenopus* hatching enzyme as studied by its two molecular forms. *Zool. Sci. 14.* (In press).

GUAN, K. and DIXON, J.E. (1991). Eukaryotic proteins expressed in *Escherichia coli:* an improved thrombin cleavage and purification of fusion proteins with glutathione S-transferase. *Anal. Biochem. 192:* 262-276.

HEINKOF, S. (1984). Unidirectional digestion with exonuclease III creates targeted break points for DNA sequencing. *Gene 28:* 351-359.

LAEMMLI, U.K. (1970). Cleavage of structural proteins during assembly of the head of bacteriophage T4. *Nature 227:* 680-685.

MAENO, M., XUE, Y., WOOD, T.I., ONG, R.C. and KUNG, H.F. (1993). Cloning and expression of cDNA encoding *Xenopus laevis* bone morphogenetic protein-1 during early embryonic development. *Gene 134:* 257-261.

NIEUWKOOP, P.D. and FABER, J. (1967). *Normal Table of Xenopus laevis (Daudin).* North-Holland, Amsterdam.

SANZ, L., CALVETE, J.J., MANN, K., SCHAEFER, W., SCHMID, E.R., AMSELSGRUBER, W., SINOWATZ, F., EHRHARD, M. and TOEPFER-PETERSEN, E. (1992). The complete primary structure of spermadhesin Awn, a zone pellucida- binding protein isolated from boar spermatozoa. *FEBS Lett. 300:* 213- 218.

SATO, M. and SARGENT, T.D. (1990). Molecular approach to dorsoanterior development in *Xenopus laevis*. *Dev. Biol. 137:* 135-141.

SIVE, H.L., HATTORI, K. and WEINTRAUB, H. (1989). Progressive determination during formation of the anteroposterior axis in *Xenopus laevis*. *Cell 58:* 171-180.

TOWBIN, H., STAEHELIN, T. and GORDON, J. (1979). Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. *Proc. Natl. Acad. Sci. USA 76:* 4350-4354.

URCH, U.A. and HEDRICK, J.L. (1981). Isolation and characterization of the hatching enzyme from the amphibian, *Xenopus laevis*. *Arch. Biochem. Biophys. 206:* 424-431.

VON HEIJNE, G. (1984). How signal sequences maintain cleavage specificity? *J. Mol. Biol. 173:* 243-251.

YAMAGAMI, K. (1996). Studies on the hatching enzyme (choriolysin) and its substrate, egg envelope, constructed of the precursors (choriogenins) in *Oryzias latipes:* a sequel to the information in 1991/1992. *Zool. Sci. 13:* 331-340.

YASUMASU, S., IUCHI, I. and YAMAGAMI, K. (1994). cDNA and the genes of HCE and LCE, two constituents of the medaka hatching enzyme. *Dev. Growth Differ. 36:* 241-250.

YOSHIZAKI, N. (1973). Ultrastructure of the hatching gland cells in the South African clawed toad, *Xenopus laevis*. *J. Fac. Sci. Hokkaido Univ. Ser. VI. Zool. 18:* 469-478.

YOSHIZAKI, N. (1991). Changes in surface ultrastructure and proteolytic activity of hatching gland cells during development of *Xenopus laevis*. *Zool. Sci. 8:* 295-302.

YOSHIZAKI, N. and YAMASAKI, H. (1991). Morphological and biochemical changes in the fertilization coat of *Xenopus laevis* during the hatching process. *Zool. Sci. 8:* 303-308.

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