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[REVIEW]

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

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ABSTRACT—Enzymatic hatching of fish embryos is caused by a sequential occurrence of many elementary processes from the commitment of the hatching gland cells to the emergence of the embryos. Molecular biological approaches to the formation, properties and function of the hatching enzyme should be required for elucidation of the enzymatic hatching, since this enzyme is a key molecule to analyze these processes. Besides them, there are some other processes indirectly related to hatching, e.g., formation and hardening of the egg envelope. The present article describes the results of our studies on some of the above-mentioned problems in the fish, *Oryzias latipes*, which have been obtained mostly in the early 1990s.

INTRODUCTION

More than a half century has passed since the study on the mechanism of hatching of Medaka, *Oryzias latipes*, was initiated by Ishida in 1944 (Ishida, 1944a,b). The hatching is almost universally seen in metazoa during their development and is prerequisite to their independent life. However, it seems that this developmental phenomenon has scarcely aroused interests of many biologists, since the hatching may have been usually considered to be a temporary and simple phenomenon, which is not veiled by "the mystery of nature" and is not worth while to analyze. In fact, the actual emergence of e.g., a fish embryo from the egg envelope is obviously transient and seemingly simple as compared with some other intriguing developmental phenomena such as induction, pattern formation, morphogenesis, etc.

As schematized in Fig. 1, however, there are many sequentially occurring intra-embryonic processes (bold-lettered processes) that lead to the emergence of the embryo. They all together construct the whole phenomenon of enzymatic hatching, i.e., they are the fundamental or elementary processes of the phenomenon of enzymatic hatching (Yamagami, 1988). To comprehend the whole hatching phenomenon, we have to clarify the mechanism of every elementary process, which is constructed of many cellular and molecular events that are common to those of the other intriguing developmental phenomena mentioned above. Thus, when we go on analyzing the mechanism of hatching, we should solve the basic and common problems in developmental biology, exploiting some hatching-related key cells or key molecules as a probe(s).

In this context, the hatching enzyme has been considered by some investigators as a good probe for analysis of embryonic differentiation at cellular and molecular levels (Ishida, 1985; Yamagami, 1988), since this enzyme is highly specific for the hatching gland cells of an embryo and closely related to their differentiation. As shown in Fig. 1, the elementary processes of enzymatic hatching may be mostly explained in terms of the hatching enzyme. Moreover, if we could analyze all the processes from the commitment of a hatching gland cell to the hatching enzyme secretion, then we shall be able to explicate a life of a cell from its birth to death.

As regards choriolysis, i.e., breakdown of the egg envelope, the molecular architecture of the egg envelope (chorion) as well as the mechanism of the enzyme action is closely related to it. Although the molecular structure of the egg envelope is too complex to be clarified at present, we have now a clue to its analysis, since precursor proteins of the egg envelope subunits were isolated and their molecular structure has been more and more clarified. In addition, the egg envelope thus formed of its subunits should be hardened on fertilization for its playing a protective role. Thus, choriogenesis, i.e., egg envelope formation, and chorion

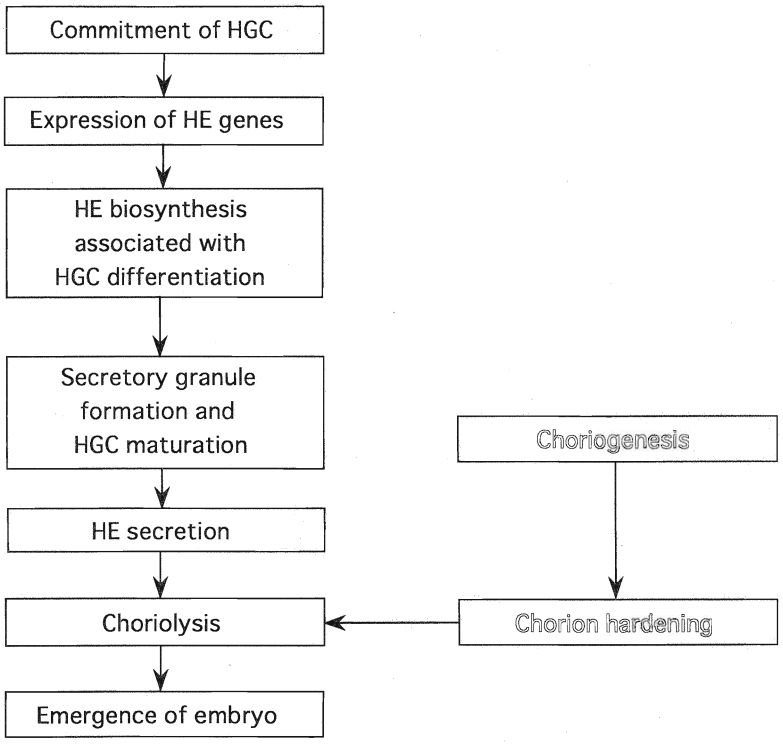

Fig. 1. Some elementary processes directly or indirectly related to the enzymatic hatching in fish. HE, Hatching enzyme. HGC, Hatching gland cell.

hardening (Fig. 1, outlined-lettered processes) are other elementary processes somewhat relevant to the hatching mechanism, which are also briefly dealt with in the present article.

After the information about the hatching enzyme and the egg envelope of *Oryzias latipes* as of 1991 or 1992 was reviewed some years ago (Yamagami, 1992; Yamagami *et al.*, 1992), a considerable advance has been made in the study of these subjects at the cellular and molecular levels. In the present article, several pieces of information of the enzyme and its substrate, which have been collected these years are described.

MOLECULAR STRUCTURE OF HCE AND LCE, THE CONSTITUENT PROTEASES OF THE HATCHING ENZYME

The medaka hatching enzyme is an enzyme system consisting of high choriolytic enzyme (HCE, or choriolysin H; EC 3.4.24.67) and low choriolytic enzyme (LCE, or choriolysin L; EC 3.4.24.66), which are similar in protein-chemical characteristics but are different in the mode of choriolytic action (Yasumasu *et al.*, 1989a,b; Yamagami *et al.*, 1993). In other animal species, there has so far been no definitive evidence that the hatching enzyme comprises multiple enzymes. Whether the hatching enzyme is generally an enzyme system or not seems to be of enzymological and phylogenetic interest, but is now open to further examination of various animal species.

Cloning of the cDNAs for HCE and LCE has provided us with valuable information of their molecular characteristics and the evolutionary relationships between the two enzymes and between them and other related molecules (Yasumasu *et al.*, 1992b, 1994). Employing total RNA extracted from day 3 embryos (retinal pigmentation stage) where the highest level of expression of the hatching enzyme genes is occurring, a cDNA library was constructed with a λ gt 11 system. Two cDNA clones for HCE (HCE21 and HCE23) and a clone for LCE have been isolated from the library (Yasumasu *et al.*, 1992b). There is a high similarity in nucleotide sequence (92.8% identity) and predicted amino acid sequence (95.5% identity) between the two clones for HCE. HCE23 and HCE21 possess an open reading frame encoding 270 and 279 amino acids, respectively. Both signal peptides are 20 amino acids long, but the propeptide regions are 50 and 59 amino acids long for HCE23 and HCE21, respectively. Mature enzyme portions of them both consist of 200 amino acids. In a previous experiment, two isoforms of HCE, HCE1 and HCE2, were already isolated from the hatching liquid of the outbred medaka embryos (Yasumasu *et al.*, 1989a), and the isolated two cDNA clones are considered to correspond to these isoforms. As shown later, the genes corresponding to both the cDNA clones are found in the genome of embryos of an inbred strain. The cDNA clone for LCE includes an open reading frame of 271 amino acids, comprising a signal peptide of 20 amino acids, a propeptide of 51 amino acids and a mature enzyme of 200

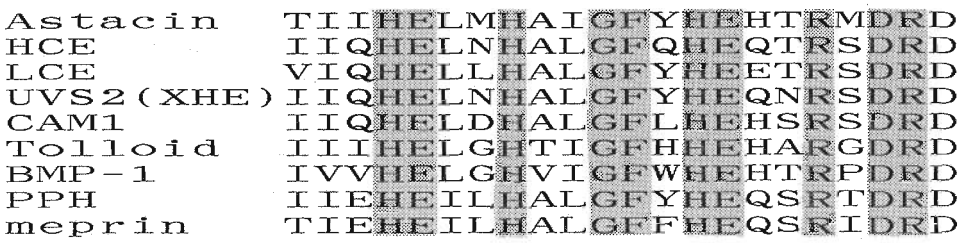

Fig. 2. Alignment of the amino acid sequences of the active sites of some astacin family proteases. Shaded columns represent the conserved amino acid residues. The sequence of UVS2 (putative *Xenopus laevis* hatching enzyme) is cited from the data of Katagiri, Ch. *et al.* (in preparation) by the courtesy of Dr. Ch. Katagiri.

amino acids (Yasumasu *et al.*, 1992b).

The deduced molecular weights of HCE21, HCE23 and LCE are 22900, 22700 and 23100, and these values are compatible with the apparent molecular weights of their mature enzymes as estimated on SDS-PAGE. There are putative N-glycosylation sites only in their propeptides, but not in their mature enzyme portions. N-glycanase digestion reduces the molecular weights of only their proenzymes but not of their mature enzymes. O-glycanase treatment does not alter the molecular weights of their proenzymes. Taken together, the mature HCE and LCE are considered to be non-glycosylated. Thus HCE and LCE are synthesized in the form of preproenzyme and stored as proenzyme or zymogen of glycoprotein nature in the secretory granules of the hatching gland (Yasumasu *et al.*, 1992a). The precursory forms of HCE and LCE were found in the prehatching embryo extracts that had been prepared in the presence of ethylenediaminetetraacetate (EDTA), while all the proenzymes were converted into mature forms on homogenization in the absence of EDTA (Yasumasu *et al.*, 1992b). This fact strongly suggests that the activation of the proenzyme, or the molecular conversion into an active form, is catalyzed by an EDTA-sensitive protease(s). Since the medaka hatching enzyme is found to be sensitive to EDTA (Yamagami, 1973; Yasumasu *et al.*, 1989a,b), there may be an autocatalytic activation mechanism in the medaka hatching enzyme system. It was found previously that the probable proenzymes of HCE and LCE were located in the same secretory granules with discrete arrangement; the proHCE being inside and the proLCE being outside (Yasumasu *et al.*, 1992a). The mechanism of packaging in such arrangement of the two proenzymes that may be simultaneously synthesized is interesting.

A high sensitivity of the medaka hatching enzyme to EDTA (Yamagami, 1973) allowed us to suppose that the constituent enzymes were metalloproteases. Biochemical analysis of the highly purified HCE and LCE has revealed that both of them are Zn-proteases (Yasumasu *et al.*, 1989a,b). One of the most intriguing characteristics of the primary structures of HCE and LCE is the presence of a consensus amino acid sequence for the active site of the astacin family protease, HExxHxxGFxHExxRxDR (Dumermuth *et al.*, 1991; Bond and Beynon, 1995), in both of them (Fig. 2). Astacin (EC 3.4.24.21) is a well-characterized Zn-protease found in the alimentary tract of the crayfish, *Astacus fluviatilis* (=*astacus*) (Titani *et al.*, 1987; Stöcker *et al.*, 1988). There are high similarities in molecular characteristics between astacin and each of HCE and LCE. These facts indicate that HCE and LCE belong to this protease family (Yasumasu *et al.*, 1992b, 1994). Although HCE and LCE, as well as astacin, consist of only a protease domain, there are many other members of this family, whose molecules are composed of some other domains in addition to the astacin protease domain. Thus HCE, LCE and astacin are the smallest members of this family (Bond and Beynon, 1995). Among the other members are Tolloid (a differentiation factor in *Drosophila*; Shimell *et al.*, 1991), BMP-1 (a human bone morphogenetic factor; Wozney *et al.*, 1988), PPH (a human intestinal protease; Dumermuth *et al.*, 1991, 1993), CAM1 (a factor for degradation of egg shell matrix proteins in quail embryo; Elaroussi and DeLuca, 1994) and meprin (a membrane protease of mouse kidney; Jiang and Bond, 1992; Jiang *et al.*, 1992). At present, however, the physiological role of the astacin protease domain in these molecules is not yet well elucidated. It seems that the astacin family is related to some other zinc protease families including the matrix metalloprotease (matrixin) family (Jiang and Bond, 1992), since the proteases of these families possess a consensus amino acid sequence, HExxHxxGxxH, in their active site (Bode *et al.*, 1993). The matrix metalloprotease family includes some endopeptidases such as collagenase, gelatinase and stromelysin, and participates in degradation and/or remodelling of extracellular matrices (Matrisian and Hogan, 1990). The sea urchin hatching enzyme belongs to this family but not to the astacin family (LePage and Gache, 1990). These facts would remind us of a fact that the egg envelope is also a structure similar to extracellular matrices in the morphology, origin and in biochemical nature. Recently, we have obtained the results that the hatching enzymes of some other fish than medaka, such as zebrafish and amago salmon, are also astacin family proteases (Inohaya *et al.*, unpublished data). Moreover, putative amphibian hatching enzyme, UVS-2, is conjectured to be a member of astacin family (Sato and Sargent, 1990; Katagiri Ch, personal communication). The above-mentioned CAM 1 is proposed to be related to the quail hatching (Elaroussi and DeLuca, 1994). Thus, the difference in the type of protease between the sea urchin hatching enzyme and the hatching-related enzymes of vertebrates such as bird, amphibian and fish seems to be a problem to be clarified.

BIOCHEMICAL PROPERTIES AND ENZYMATIC SOLUBILIZATION OF THE INNER LAYER OF THE EGG ENVELOPE

Fish egg envelope (chorion) generally consists of two or three structurally different layers. In medaka eggs, the envelope is usually divided into two layers; a thin outer layer and a thick inner layer (Yamamoto and Yamagami, 1975). The hatching enzyme digests only the inner layer by eventually converting most of its scleroprotein into a mass of soluble glycoproteins (Iuchi and Yamagami, 1976), without concomitant production of significant amounts of free amino acids (Yamagami, 1970). It is also known that the process of the digestion consists of two steps, i.e., choriolytic swelling caused by HCE and subsequent solubilization of the swollen structure by LCE (Yasumasu *et al.*, 1989a,b). It is conjectured that such a unique cooperative action of HCE and LCE in digestion of the inner layer should be closely related with the structural characteristics of the inner layer.

Compared with other biological structures, the fish egg envelope seems somewhat simple at the molecular level, since it is composed of a relatively small number of component protein species. However, clarification of the molecular architecture is only just starting. Fish egg envelope (especially its innermost layer) is regarded as a structure equivalent to the vitelline envelope and has been conventionally considered to originate from the growing oocyte. Recently, there have been reports describing that the precursors of inner layer proteins of the egg envelope of some fish species are synthesized in the liver of the mother fish under the influence of estrogen, like vitellogenin (Hamazaki *et al.*, 1984, 1987a, 1989; Oppen-Berntsen *et al.*, 1992a,b; Yamagami *et al.*, 1994; Murata *et al.*, 1994, 1995). Among the precursors, those found in medaka, *Oryzias latipes*, have been isolated and characterized (Hamazaki *et al.*, 1987b; Murata *et al.*, 1993), and examined of their predicted primary structures with the cloned cDNAs (Murata *et al.*, 1995a,b). Also in the winter flounder, *Pseudopleuronectes americanus*, the cDNA and the gene for a putative precursor of egg envelope protein have been cloned and analyzed (Lyons *et al.*, 1993).

For the medaka egg envelope, the inner layer is known to be composed of two groups of glycoprotein subunits. One group is tentatively named the ZI-1,2 group, since it was considered, at the beginning of our studies (Hamazaki *et al.*, 1987b), to be composed of two distinct subunit proteins, ZI-1 and ZI-2. On native- and SDS-PAGE, this group represents a mass of proteins with molecular weights ranging from 74K to 76K. Recently it has become clear that this group consists of not two but three protein bands (Murata *et al.*, 1993; Sugiyama *et al.*, unpublished data). The other group, ZI-3, comprises only one protein with molecular weight of 49K (Hamazaki *et al.*, 1987b; Murata *et al.*, 1993; Sugiyama *et al.*, 1996). The precursors of these two protein groups have been named SF (spawning female-specific) substances, i.e., H-SF (high-molecular-weight SF) substances for proteins of the former(ZI-1,2) group, and L-SF (low-molecular-weight SF) substance for the latter (Murata *et al.*, 1991; Yamagami *et al.*, 1994). As mentioned above, the cDNA for L-SF has been cloned by the use of a cDNA library constructed from poly(A)$^+$RNA of the liver of spawning female fish or estrogen-treated male fish (Murata *et al.*, 1995). The clone, L-SF41, contains an open reading frame encoding a signal peptide of 19 amino acids and L-SF protein of 420 amino acids (Fig. 3). The protein is considered to be N-glycosylated and possesses a domain similar to ZP-domains in ZP3 (zona pellucida 3) of some mammalian species. Cloning of the cDNAs for H-SF has also been done and the molecular structures of the components of H-SF are clarified in detail. Therefore, compared to vitellogenin, we propose the general name of choriogenin for such liver-derived precursors of the fish egg envelope (chorion), i.e., medaka choriogenin H and L for H-SF and L-SF, respectively. It is hardly likely that a considerable biochemical modification of the precursor proteins occurs prior to their assembly to construct the inner layer of the egg envelope, since there is only a little difference in biochemical characteristics such as amino acid composition and molecular weight between the subunits and their respective precursors.

The inner layer thus formed is that of the oocyte (or unfertilized egg) envelope, which is not yet hardened. It seems likely that the assemblage of the subunits in such an unhardened envelope is done through non-covalent bonding such as hydrogen bonding, electrostatic (or hydrophobic) interaction, etc. between them. On fertilization (or activation), the egg envelope becomes hardened probably by a hardening enzyme (Zotin, 1958; Oppen-Berntsen *et al.*, 1990; Masuda *et al.*, 1991, 1992; Iwamatsu *et al.*, 1995) and the hardening is considered to occur through formation of some crosslinks between the subunits, which gives rise to insoluble polymers of the subunits. In fish, one of the most responsible crosslinks is reportedly γ-Glu-ε-Lys isopeptide (Hagenmaier *et al.*, 1976; Oppen-Berntsen *et al.*, 1990; Iuchi *et al.*, 1991, 1996; Lee *et al.*, 1994). Formation of this type of crosslink is known to be catalyzed by transglutaminase in some animal tissues (Folk and Chung, 1973). It seems highly probable that this enzyme plays a crucial role also in the hardening of fish egg envelopes (Hagenmaier *et al.*, 1976; Oppen-Berntsen *et al.*, 1990; Iuchi *et al.*, 1991, 1996). Analysis of the hardening mechanisms including the mode of action of transglutaminase should be one of the interesting problems from fertilization physiology, protein biochemistry and enzymology. It is the hardened inner layer that serves as the natural substrate for the hatching enzyme.

As mentioned above, the first step of the egg envelope digestion is performed by HCE; HCE causes a remarkable swelling of the inner layer with concomitant release of some polypeptides from it. The inner layer becomes very susceptible to digestion by LCE only after it was swollen. Thus the choriolytic swelling of the inner layer by HCE is a prerequisite process to solubilization of the egg envelope by the hatching enzyme. Recently, it was found that some of the peptides released by HCE at the choriolytic swelling contained a high concentration of Pro in the form of Pro-x-y repeat sequence,

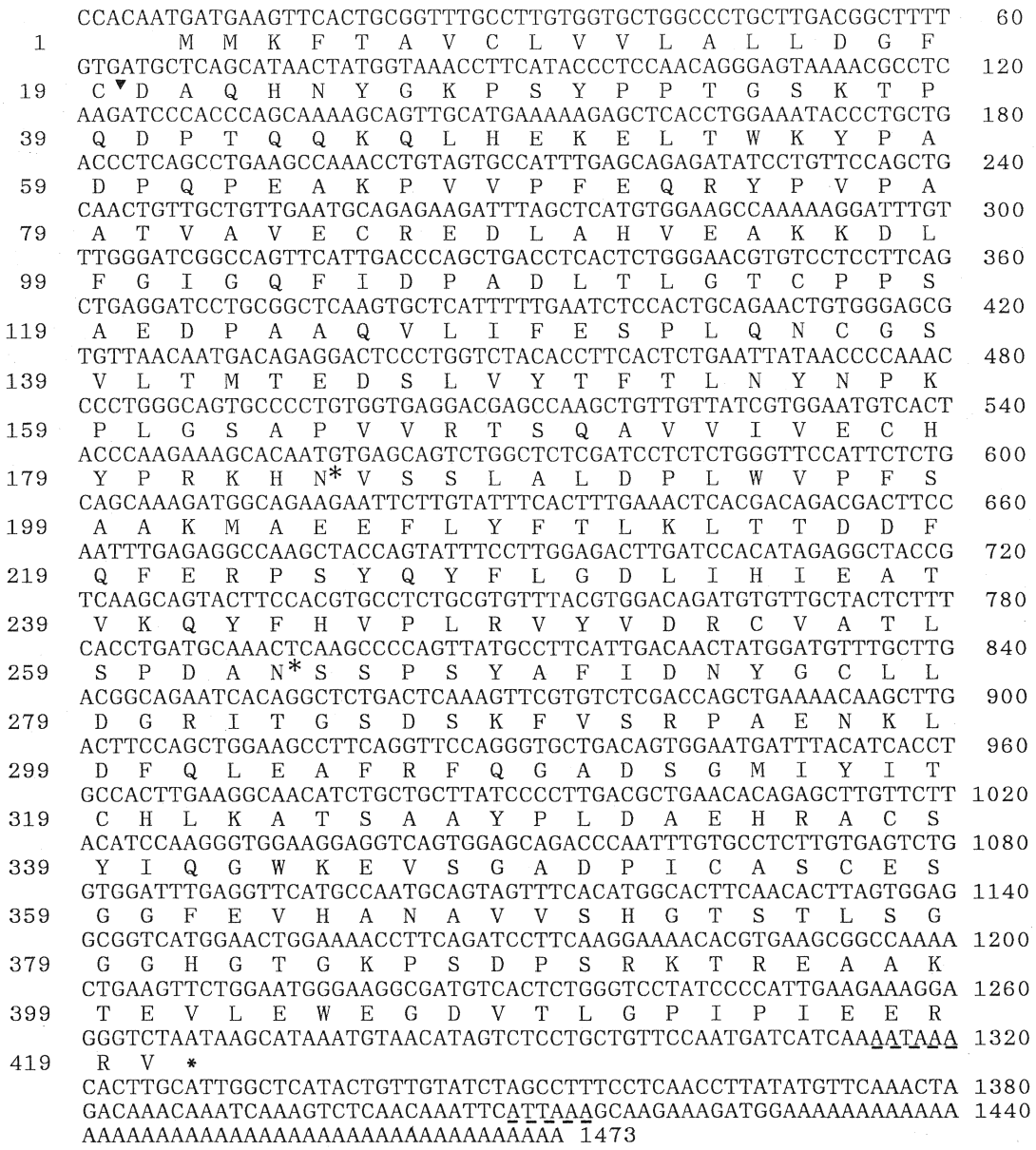

Fig. 3. Nucleotide and predicted amino acid sequences of a cDNA clone for L-SF (choriogenin L) (L-SF41). A black triangle refers to the cleavage site of the signal peptide. Large asterisks refer to possible N-glycosylation sites and a small asterisk indicates the stop codon. Dashed underlines are putative poly(A)-addition signals.

where x was mostly Glx (Lee *et al.*, 1994). Moreover, the peptides contain many γ-Glu-ε-Lys isopeptides. This Pro-rich sequence is found in a part of the subunits of the Zl-1,2 group (Sugiyama, H., personal communication), and also in the predicted amino acid sequence of a cDNA clone for its precursor, H-SF (choriogenin H) (Murata *et al.*, 1995b). A similar repeat sequence has been found in a cDNA clone for an egg envelope protein that was isolated from a female liver of the winter flounder (Lyons *et al.*, 1993). Although the detailed mechanism of enzymatic solubilization of the medaka egg envelope is still obscure, clipping the peptide(s) containing the Pro-rich region from the subunits of the Zl-1,2 group by HCE seems to be one of the causes that bring about the swelling and subsequent solubilization of the inner layer.

ORGANIZATION OF THE GENES FOR HCE AND LCE

Among the elementary processes of the enzymatic hatching mentioned above, the mechanism of genetic control of the hatching enzyme synthesis in relation to the hatching gland cell differentiation is one of the most intriguing problems to be solved. Prior to analysis of the mechanism, we have to know the structure and organization of the genes for the hatching enzyme. On the other hand, as will be described in the next section of this article, temporal and spatial patterns of the expression of the genes should be also documented in

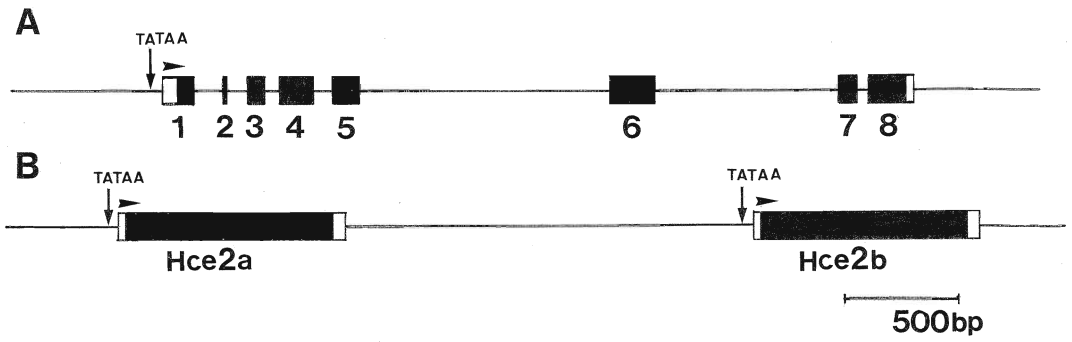

Fig. 4. Structure and organization of the genes for LCE and HCE. A, The LCE gene comprising 8 exons and 7 introns. B, Two HCE genes (*Hce2*) among seven functional genes are shown, each containing no introns. The dark boxes are coding regions and the open boxes show untranslated regions. Arrowheads show the direction of transcription.

relation to the process of the hatching gland cell differentiation.

By the use of the *Eco*RI fragments of the cDNAs for LCE and HCE(HCE23) as probes, the genes for LCE and HCE have been isolated from the EMBL-3 genomic libraries constructed from DNA of the inbred drR strain fish (Yasumasu *et al.*, 1994, 1996) (Fig. 4). The LCE gene is composed of eight exons and seven introns, and the total length is 3.6 kbp. A single copy of this gene is found in a genome as examined by Southern blot analysis. A TATA box consensus sequence is located 28 bp upstream of the major transcription start site as determined by primer extention analysis. Compared with the LCE gene, the HCE gene is markedly different in structure and organization. The HCE gene is a multigene, i.e., there are eight copies of it in a genome. Except one of them, which seems to be an unfunctional pseudogene, four copies are the genes for HCE23 and three are for HCE21. Six copies of them (three HCE23 and three HCE21) are situated in a cluster (Fig. 4). Every HCE gene has no introns, being about 1 kbp in total length. Like the LCE gene, the upstream region of every HCE gene is found to contain a TATA box. In the range of 200-400 bp of the 5'-flanking regions of all the HCE genes, there are highly conserved nucleotide sequences, and this may be related to a concerted expression of the HCE genes. However, there is no overall similarity in the range of 1.5 kb of 5'-flanking regions between the LCE gene and the HCE genes, while the expression of the LCE and HCE genes is well regulated to occur simultaneously. It would be hoped to find out a *cis*-regulatory element(s) by examining their 5'-flanking regions and 3'-flanking regions as well in future. Thus the analyses of the structure and organization of the LCE and HCE genes have revealed two interesting differences between them, i.e., (1) the difference in the copy number and (2) that in the exon/intron organization (Yasumasu *et al.*, 1994, 1996).

Concerning the first difference (in the copy number), it should be noted that the amount of HCE recovered from the hatching liquid is several times as much as that of LCE (Yasumasu *et al.*, 1992a), and that a larger amount of HCE than LCE is needed to perform choriolytic swelling on account of a tight binding of HCE to the egg envelope (Yasumasu *et al.*, 1989c). Since most of the HCE genes seem to be transcribed, the multiple copies of HCE gene would stand for the larger amount of HCE over LCE. The second difference (in the exon/intron organization) seems to be intriguing as well as puzzling, considering that both HCE and LCE have been evolved probably from a common ancestral gene. This problem may have to be considered by comparatively examining the genes for other members of the astacin family. An explanation may be possible that the intron-less HCE genes are derived from a retroposon that was brought about via an mRNA transcribed from a common ancestral gene of LCE and HCE, followed by its multiplication and divergence (Yasumasu *et al.*, 1994, 1996). In such a case, a regulatory element(s) common to the LCE gene and the retroposed HCE genes may be working in a genome, since the expression of both the LCE gene and most HCE genes appears to be under a strict "spatial and temporal regulation" (see later). At present, the hatching enzyme system consisting of plural constituent proteases is found only for medaka. Whether it is the case also for other animal species or not remains open to studies in future as an interesting problem in comparative zoology. In any case, the elucidation of the structure and organization of the upstream region of the genes for the medaka hatching enzyme should be needed as a basis for analysis of a genetic mechanism regulating a coordinative synthesis of an enzyme system.

EXPRESSION OF THE HATCHING ENZYME GENES AND DEVELOPMENT OF THE HATCHING GLAND CELLS

Although the genes for HCE and LCE represent quite different structures and organizations as described above, their expression in the developing embryos is considered to be strictly regulated temporally and spatially, as both the enzymes should be fully synthesized and stored in the hatching gland cells by the time of secretion at hatching. In fact, Northern blot analysis reveals that the gene transcripts for HCE and LCE are expressed at approximately the same stage in day after fertilization (Yasumasu *et al.*, 1992b). This result has been confirmed in a more recent experiment (Inohaya *et al.*, 1995a),

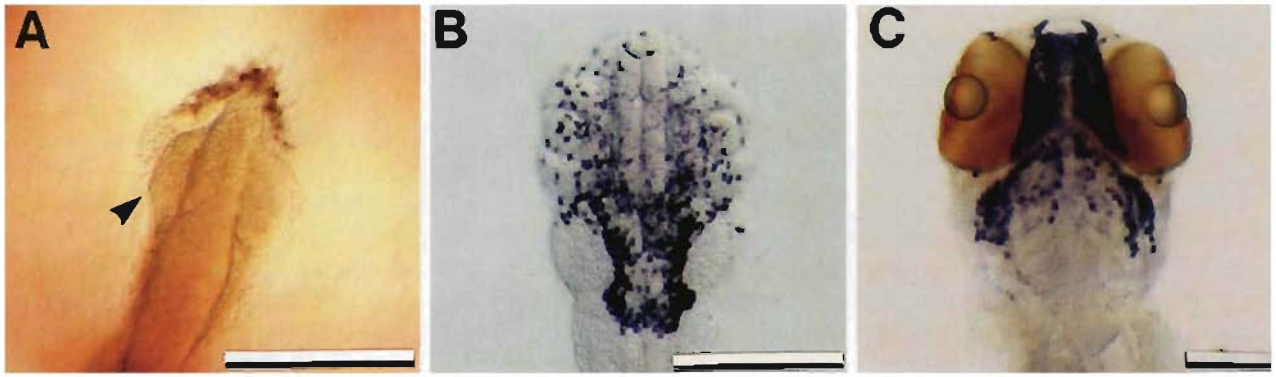

Fig. 5. Expression of the HCE gene in developing embryos of *Oryzias latipes*, as visualized by whole mount *in situ* hybridization employing the HCE23 probe. A, An embryo with 3-4 somites. The arrowhead indicates an optic vesicle. B, An embryo just before heart-beating. C, An embryo just before hatching. Scale bars, 250 μm. Other explanations, see text.

in which the expression signals for both genes can be first detected in the embryos at the stage of brain differentiation and lens formation.

It is quite difficult, however, to identify the exact time when the gene expression starts in an embryo. When we visualize the signal of gene expression by whole mount *in situ* hybridization of an embryo with an antisense RNA probe raised from HCE23, followed by its sectioning, the time of the first gene expression can be traced back to the stage as early as late gastrula (ca 90% epiboly). The first signal is observed in a restricted group of cells at the front of the hypoblast. The cell group, after once projected anteriorly like a beak-like structure, retracts to the anterior end of the embryonic axis and the HCE-positive cells are aligned along the front margin of the head rudiment and both optic vesicles. Section of the *in situ* hybridized embryo demonstrates that these HCE-positive cells are located in the Polster, which is regarded to be corresponding to the prechordal plate (cf: Ballard, 1973, 1982). Also in zebrafish, the hatching gland cells originate from the Polster (Inohaya *et al.*, 1995b). These results are also compatible with the finding that the progenitors of the hatching gland cells originate from the organizer region in fish (Kimmel *et al.*, 1990). At present, whether *all* or *a part* of the Polster cells are HCE-positive remains obscure.

Afterward, the Polster unites with the covering ectoderm, and the HCE-positive cells markedly increase in number, disperse in the endoderm beneath the brain and optic vesicles, and migrate posteriorward to the pharyngeal pouch and branchial pouches. The location of a large number of HCE-positive cells is again changed from the pharyngeal pouch to their final location, the mouth cavity, by moving anteriorward in parallel with elongation of the lower jaw of the embryo (Fig. 5).

The study on the sequential change of the pattern of gene expression for HCE has revealed the followings: (1) the progenitor (or immature) hatching gland cells are transiently located in the Polster in the process of development. (2) the progenitor (or immature) hatching gland cells start the expression of the hatching enzyme gene(s) just prior to their localization to the Polster at early gastrula. (3) the progenitor (or immature) hatching gland cells seem to concurrently perform gene expression for the hatching enzyme, mitosis, and migration (Inohaya *et al.*, 1995a).

It was generally considered that the hatching gland cells of fish and amphibian were ectodermal, although the hatching glands of medaka and sturgeon were reported to be endodermal (see Yamagami, 1988). The Polster is regarded as an equivalent to prechordal plate in amphibian, and the HCE-positive cells were located at the very front of hypoblast before they are localized to the Polster. These results strongly suggest that the medaka hatching glands are mesoendodermal. The mesoendodermal hatching gland cells migrate through an ectodermal ventral wall of the head rudiment to the endodermal pharyngeal wall. Although, as mentioned above, it is difficult to identify the exact time of the first gene expression for the hatching enzyme, it seems that the gene expression does not start before the progenitor gland cells reach the front region of hypoblast. We do not know, at present, when the progenitor hatching gland cells are committed and whether there is a determinant or an inducer.

Here, we have to consider about the relationship between the expression of the hatching enzyme genes and the differentiation of the hatching gland cells. Although the expression of the hatching enzyme genes cannot be regarded as a sole sign of the hatching gland cell differentiation, it must be one of the representative features of the terminal differentiation of the gland cells. In this context, it should be hoped to clarify a hierarchy of expression of the genes in the gland cell leading to the ultimate expression of the hatching enzyme genes. There have recently been reported several genes expressing their transcripts in the organizer region of some animals. Among them, *goosecoid* (*gsc*), *Lim1*, *Isl1*, *forkhead* and *hgg1* are found also in fish embryos such as zebrafish and/or medaka. The expression of these genes occurs more early than does that of the hatching enzyme genes. For example, the expression of *gsc* is seen in the organizer region and is probably overlapping with that of the hatching enzyme genes in zebrafish and in medaka (Thisse *et al.*, 1994; Inohaya *et al.*, 1995c). At present, however, it remains uncertain whether *gsc* is directly related to the

expression of the hatching enzyme genes. Among the genes mentioned above, *Lim1* seems to be one of the most interesting genes, considering that this gene is responsible for the development of an anterior half of embryonic head in vertebrates (Taira *et al.*, 1994a,b; Shawlot and Behringer, 1995).

Another interesting problem is how the route of migration of the differentiating (or maturing) hatching gland cells is determined. The final location of the fish hatching gland cells varies from species to species (Yanai, 1966; Ishida, 1985). In zebrafish, the gland cells are finally distributed semi-circumferentially on the anterior surface of yolk sac, while they *were* also located in the Polster in the course of development (Inohaya *et al.*, 1995b). Thus the immature gland cells in the Polster migrate antero-ventrally in zebrafish, but they migrate posteriorly in medaka. It is considered that the hatching gland cell differentiation is also accompanied with the expression of some genes relevant to mechanism of specific cell migration.

CONCLUDING REMARKS

In the present article, some results of the recent studies on the hatching enzyme and its substrate, the egg envelope, of medaka were described as a sequel to the previous information as of 1991/1992.

Concerning HCE and LCE, at least two major lines of study would be possible in future: One is to analyze the mechanism of the spatio-temporal regulation of their gene expression. The genic basis of this study is to identify some *cis*-regulatory element(s) in the genes for both enzymes. The cellular basis of this study may be closely related to the differentiation of the gland cells. It seems highly probable that there are many genes whose expression occurs in the cells which are related to the progenitor hatching gland cells. Finding-out of a hierarchy of these genes leading to the expression of the hatching enzyme genes would be one of the typical approaches. Whether there is present any inducing factor(s) for the gland cell differentiation or not, this line of study would be promising when it is performed in conjunction with a search for a *trans*-regulatory factor(s).

The other is to approach to the hatching enzyme from the phylogenetic aspects. The molecular characteristics of HCE and LCE as the members of the astacin family and their molecular relevance to other metalloendopeptidases should be more deeply analyzed. This line of study is necessarily related to the analyses of their gene structures as well as their protein conformations. There have been found many physiologically active molecules which contain an astacin protease domain in addition to other domains. Some of these molecules are serving as hatching enzymes, as digestive enzymes or as morphogenetic principles. But the physiological role of the remaining is still obscure. On the other hand, there are non-astacin hatching enzymes in some animals such as sea urchins. At present, it remains dubious whether the hatching proteases are different between vertebrates and invertebrates. Thus the evolutionary aspects of the hatching enzyme implicate (1) the molecular evolution of the astacin proteases and (2) the different adaptation of metalloproteases of different families to act as the hatching enzyme in various animals.

Regarding the egg envelope, there are also two major problems to be clarified. One is concerned with its molecular architecture. Comparison of the molecular structure of every subunit of the egg envelope with that of the corresponding precursor would intrigue us to analyze the mechanism of molecular assembly of the precursors, which probably occurs around a growing oocyte possibly under the *guidance* of some surface molecules of the oocyte and/or the granulosa cells. Subsequent establishment of molecular architecture of the egg envelope would be highly concerned with the mechanism of the egg envelope hardening. It would become clear in the near future that the egg envelope itself is a dynamic system for establishing its functional structure, while it has ever been regarded as an inert structure.

The other is the mechanism of hepatic synthesis of the precursors. Endocrinological and molecular biological analyses should be needed of an coordinative expression of the genes for every subunit precursor (choriogenin) and for vitellogenin(s) as well. Also in the case of egg envelope synthesis, we have to encounter the problem of adaptation not only at the molecular level but also at the tissue and/or organ level, since there seems to be a diversity of the pattern of egg envelope synthesis in various animals.

In conclusion, the study of the hatching enzyme and the egg envelope should be proceeded both comparatively and comprehensively.

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