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On the Hatching Mechanism of Quail Embryos: Participation of Ectodermal Secretions in the Escape of Embryos from the Vitelline Membrane

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ABSTRACT—The avian hatching process is considered to comprise a series of complicated phases including the digestion of vitelline membrane and egg white and the breakdown of shell membrane and the eggshell. The present study focuses on the first phase, *i.e.*, the digestion of vitelline membrane in Japanese quail *Coturnix japonica*. When embryos were subjected to immunocytochemical tests with an anti-*Xenopus* hatching enzyme antibody, staining was observed on day 0 of incubation in the outermost ectodermal cells of blastoderms, and later in the ectodermal cells of yolk sacs in the area vitellina. It was confirmed by electron microscopy that what had been specifically stained in these (two locations) were secretory granules in the cells. A 57 kDa protein was detected by immunoblot tests of the extracts of yolk sac material from the area vitellina. Ultrastructural disintegration of the vitelline membrane followed the advance of the yolk sac toward the vegetal pole. We propose that the step-by-step digestion of the vitelline membrane from the animal pole side toward the vegetal pole one is accomplished by an enzyme produced by the outermost ectodermal cells which show a temporary secretory activity.

INTRODUCTION

Hatching is a universal phenomenon in the animal kingdom; embryos escape from their protecting envelopes in order to get food for further growth or to implant in the uterus. Studies concerning the hatching mechanism have been conducted with sea urchins (Lepage and Gache, 1990; Nomura *et al.*, 1991, 1997; Ghiglion *et al.*, 1997), tunicate (D'Aniello *et al.*, 1997), fish (Schoots *et al.*, 1982; Yamagami *et al.*, 1992; Yasumasu *et al.*, 1994; Inohaya *et al.*, 1995; Yamagami, 1996), amphibians (Yoshizaki, 1991; Yoshizaki and Yamasaki, 1991; Fan and Katagiri, 1997; Katagiri *et al.*, 1997; Kitamura and Katagiri, 1998), and mammals (Perona and Wassarman 1986; Sawada *et al.*, 1990). The common mechanism found in them is a disintegration of protecting envelopes by proteolytic hatching enzymes secreted by hatching gland cells (fish and amphibians) or temporarily participating anonymous cells (sea urchins and mammals).

Though avian hatching is a subject of interest to chicken-breeders and people who are caring for near-extinct species, no analysis of its mechanism has been made. Most research interest has been directed to the development of an *in vitro*

culture system of embryos (Kamihira *et al.*, 1998) or to finding the mechanism of eggshell resorption that supports the rapid skeletal growth of embryos (Elaroussi and DeLuca, 1994).

The avian egg or embryo is surrounded by various envelopes. The inner layer of the vitelline membrane is produced by the oocyte itself or by granulosa cells (Kuroki and Mori, 1995; Waclawek *et al.*, 1998; Takeuchi *et al.*, 1999); all the other envelopes of the continuous membrane, the outer layer of the vitelline membrane, the egg white, the shell membrane, and the eggshell are produced by the oviduct (Bellairs *et al.*, 1963; Bain and Hall, 1969). Therefore, avian hatching is considered to involve either total or partial digestion of these envelopes.

The present study will deal with digestion of the vitelline membrane as a mean to fully understand the avian hatching mechanism. The main purpose of the present study is to identify the cells which are engaged in the production of the hatching enzyme in a quail embryo, using an anti-*Xenopus* hatching enzyme (αXHE) antibody (Katagiri *et al.*, 1997) as a probe. There is a possibility that the antibody may be able to recognize the quail hatching enzyme, since the gene whose translation is supposed to play a role in the degradation of eggshell matrix (Elaroussi and DeLuca, 1994), and whose predicted polypeptide has a high homology with hatching enzymes of both *Xenopus* (XHE; Katagiri *et al.*, 1997) and medaka fish (choriolytic enzymes; Yamagami *et al.*, 1996) is activated in

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the cells of chorioallantoic membrane of quail embryos. In the present study we will show an intimate relation between the behavior of the cells stained with the αXHE antibody and the mode of disintegration of the vitelline membrane.

MATERIALS AND METHODS

Procurement of embryos

Fertilized eggs of Japanese quail *Coturnix japonica* were collected around 10 o'clock in the morning and immediately put into an incubator at 39°C where they were automatically subjected to a see-saw motion. The embryonic development was measured in days of incubation from 0, the day of egg-laying, to 17, the day of "birth". Until day 2 of incubation, the embryo was separated from the albumin (egg white) by repeated decantation and immersed in the avian Ringer solution. After day 3 of incubation, the embryo with the albumin was directly immersed in the solution.

Immunocytochemistry

The IgG antibodies (anti-GST-UVS.2) used for immunocytochemical study were raised against a bacterially-expressed fusion protein comprising glutathione-S-transferase (GST) and UVS.2 (Katagiri *et al.*, 1997) by injection into rabbits. Since UVS.2 was shown to be a portion of the functional *Xenopus* hatching enzyme (Katagiri *et al.*, 1997), these antibodies will hereafter be referred to as the anti-XHE antibody.

Embryos were fixed in Bouin's solution and embedded in paraffin. Five μm thick sections of embryos were treated with the αXHE antibody (1:50 dilution) for 1 hr at 37°C, after which they were washed thoroughly with phosphate-buffered saline and treated with fluorescein isothiocyanate (FITC)-conjugated goat antiserum against rabbit IgG (E-Y Laboratories, San Mateo, USA) for 1 hr at 37°C. Control sections were treated with non-immune rabbit serum instead of αXHE antibody.

Electron microscopy

Embryos were immersed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer at pH 7.2 and the egg integuments or embryonic tissues that were isolated were further fixed in the same solution for 3 hr. For conventional electron microscopy, the specimens were rinsed in the buffer, post-fixed with 1% osmium tetroxide in the same buffer, dehydrated in acetone, and embedded in epoxy resin. Thin sections were stained with uranyl acetate and lead citrate.

For immunoelectron microscopy, the specimens were rinsed in the buffer, dehydrated in ethanol, and embedded in Lowcryl K4M resin (Yoshizaki and Yonezawa, 1994). Thin sections were treated with the αXHE antibody (1:200 dilution) for 1 hr at 37°C and then with a gold-conjugated goat antiserum against rabbit IgG (E-Y Lab.) for 1 hr as described previously (Yoshizaki and Yonezawa, 1994).

Western blot

The extracts of yolk sacs in Ringer solution were separated by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on the 5–20% gradient gel 'PAGEL' (Atto, Tokyo, Japan). Transblotted membranes were treated with the αXHE antibody (1:200 dilution) and then with horseradish peroxidase-conjugated goat antiserum (E-Y Lab.). The peroxidase was detected by a saturated concentration of 4-chloro-1-naphthol and 0.03% H_2O_2. The molecular weights of the proteins were estimated from a calibration curve obtained with the standard proteins in an LMW calibration kit (Pharmacia, Sweden), stained with Coomassie brilliant blue. Protein was determined by the method of Smith *et al.* (1975) with bovine serum albumin as a standard.

RESULTS

Ultrastructural change of vitelline membrane during embryonic development

The vitelline membrane of a new-laid egg (day 0 of incubation) was composed of three distinct layers: an inner layer facing the yolk (or "yolk cell"), an outer layer in contact with the egg white, and a continuous membrane lying between them (see Fig. 1c). The inner layer was 3–5 μm in width and consisted of a meshwork of cylindrical fibers of 300–600 nm in thickness. Between the fibers were substantial gaps containing granules or vesicles. The outer layer was 3–7 μm in width and consisted of a variable number of sublayers lying one above the other, each of which is composed of fine fibrils. The continuous membrane was 30–75 nm in thickness.

On day 0 of incubation, no difference was observed in the ultrastructure of the vitelline membrane in any location around the embryo or the yolk. On day 2 of incubation, the vitelline membrane at the animal pole side (Fig. 1a) showed a very distinctive feature from that of the vegetal pole side (Fig. 1c); the cylindrical fibers of the inner layer appeared to dissociate, the sublayers of the outer layer became indistinguishable, and the continuous membrane was hard to identify. Fig. 1b shows the vitelline membrane facing the yolk sac (peripheral extension of blastoderm) at the transition from its area vasculosa to area vitellina. The inner layer facing the area vasculosa shows the dissociation of the fibers whereas that facing the area vitellina shows unchanged cylindrical fibers.

Until day 2 of incubation, the embryo and yolk could be separated easily from the thick egg white by decantation. After day 2, the decantation procedure always disrupted the yolk, which meant the loss of integrity of the vitelline membrane and/or the thin egg white adhering it. Exactly, on day 3 of incubation, such egg white totally disappeared at the region above the embryo proper, and the vitelline membrane at the region also decreased its width to about half that of new-laid egg (Fig. 2a).

With a stereo-microscope, we could confirm the gradual disappearance of the vitelline membrane; it began at the animal pole side and advanced toward the vegetal pole side during the embryonic development. On day 4 of incubation, at the site where the vitelline membrane was completely digested, the egg white which once made a firm contact with underlying yolk sacs showed signs of being ingested. From day 5 of incubation on, the partially digested portion of the vitelline membrane was apart from the yolk and enfolded in the egg white (Fig. 2b). The latest stage when we recognized the vitelline membrane at the vegetal pole side was day 15 of incubation. This vitelline membrane, folded up like a ball, consisted of outer layer only (Fig. 2c). If unfolded, it covers approximately one-fourth area of the yolk surface.

Immunofluorescent observations of developing embryos

Sections of embryos on various days of incubation were stained immunofluorescently with an anti-*Xenopus* hatching enzyme (αXHE) antibody (Fig. 3). On day 0 of incubation, the
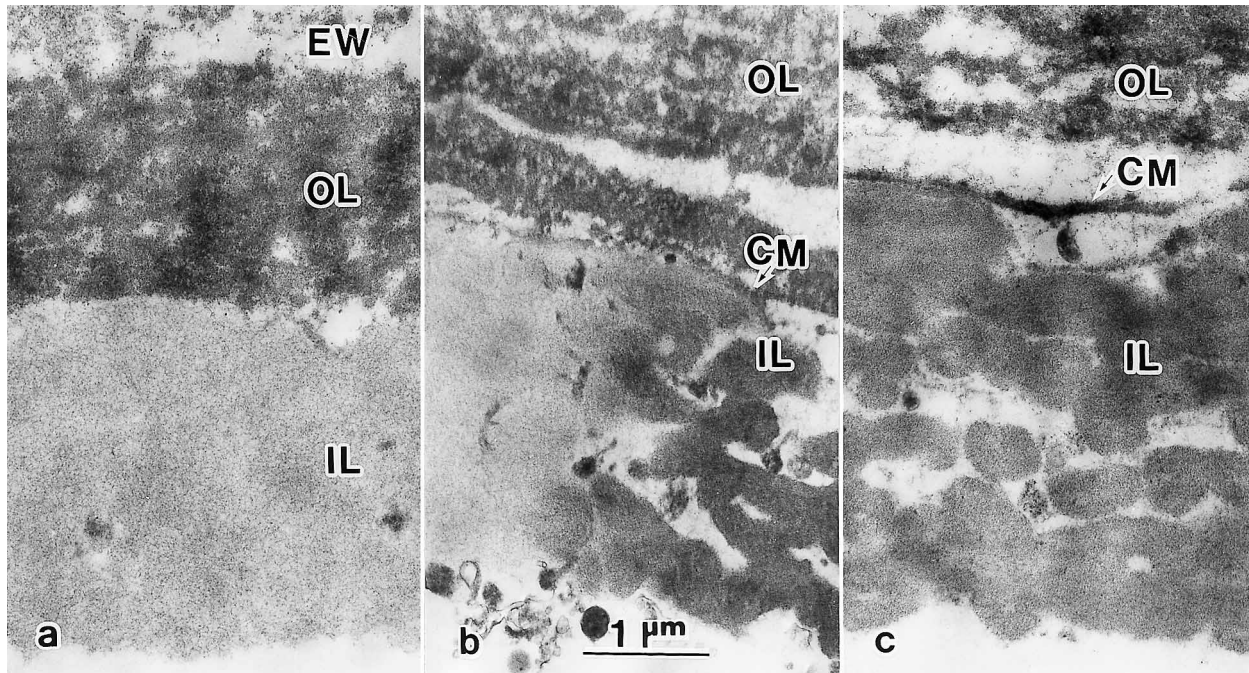
Fig. 1. Electron micrographs of the vitelline membrane around embryos on day 2 of incubation. (a) Animal pole side. Both the inner layer (IL) and the outer layer (OL) of the vitelline membrane show smooth appearance. Continuous membrane (CM) seen in Fig. b and c is indistinct. (b) Intermediate portion of vitelline membrane facing the boundary between area vitellina (right side) and area vasculosa (left side) of yolk sac. (c) Vegetal pole side. The IL consists of a network of cylindrical fibers, and the OL consists of a stack of sublayers, each of which is composed of fine fibrils. The CM is distinct. EW, egg white.
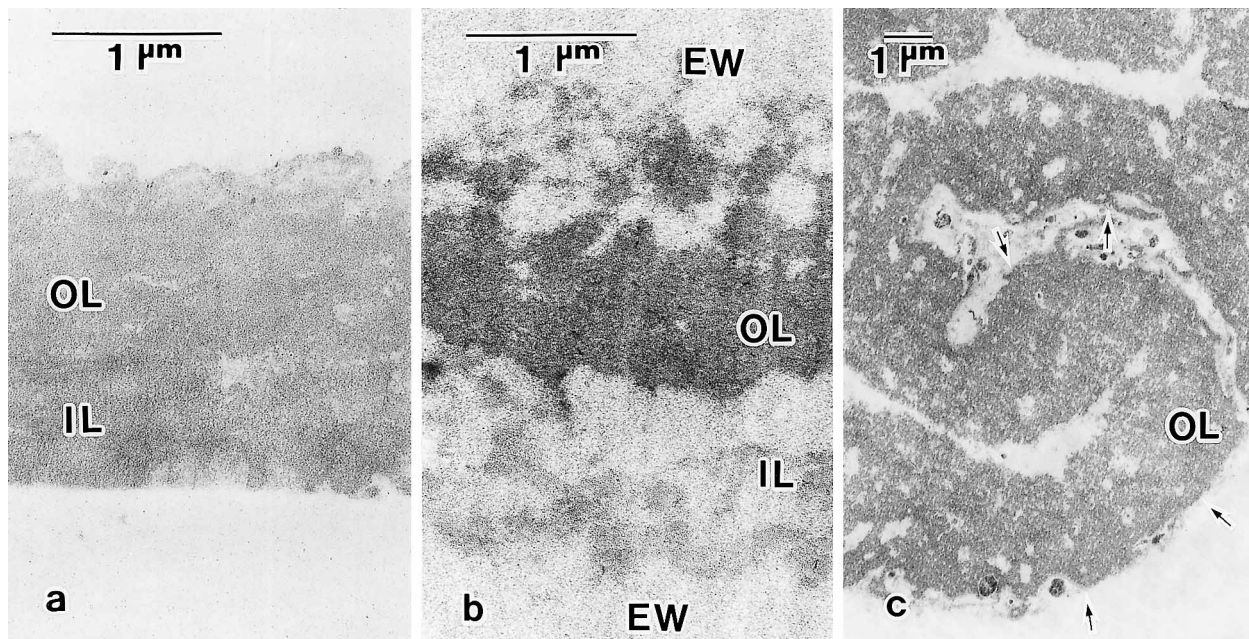

Fig. 2. Electron micrographs of the vitelline membrane around embryos on various days of incubation. (a) Vitelline membrane of embryo on day 3 of incubation. The egg white is absent on the membrane at the region of animal pole side. (b) Vitelline membrane of embryo on day 6 of incubation. Partially digested portion of the membrane is enfolded in the egg white (EW). (c) Vitelline membrane of embryo on day 15 of incubation. Only the outer layer (OL) of the membrane remains. Arrows indicate the inner surface of the membrane. IL, inner layer of the vitelline membrane.

blastoderm consisted of several layers of cells, among which the outermost cells were stained with the antibody at their apical-most cytoplasm (Fig. 3a). On day 1 of incubation, the cells at the area pellucida of the blastoderm were not stained, whereas the outermost cells at the area opaca were stained (Fig. 3b). On day 2 of incubation, the embryonic body which
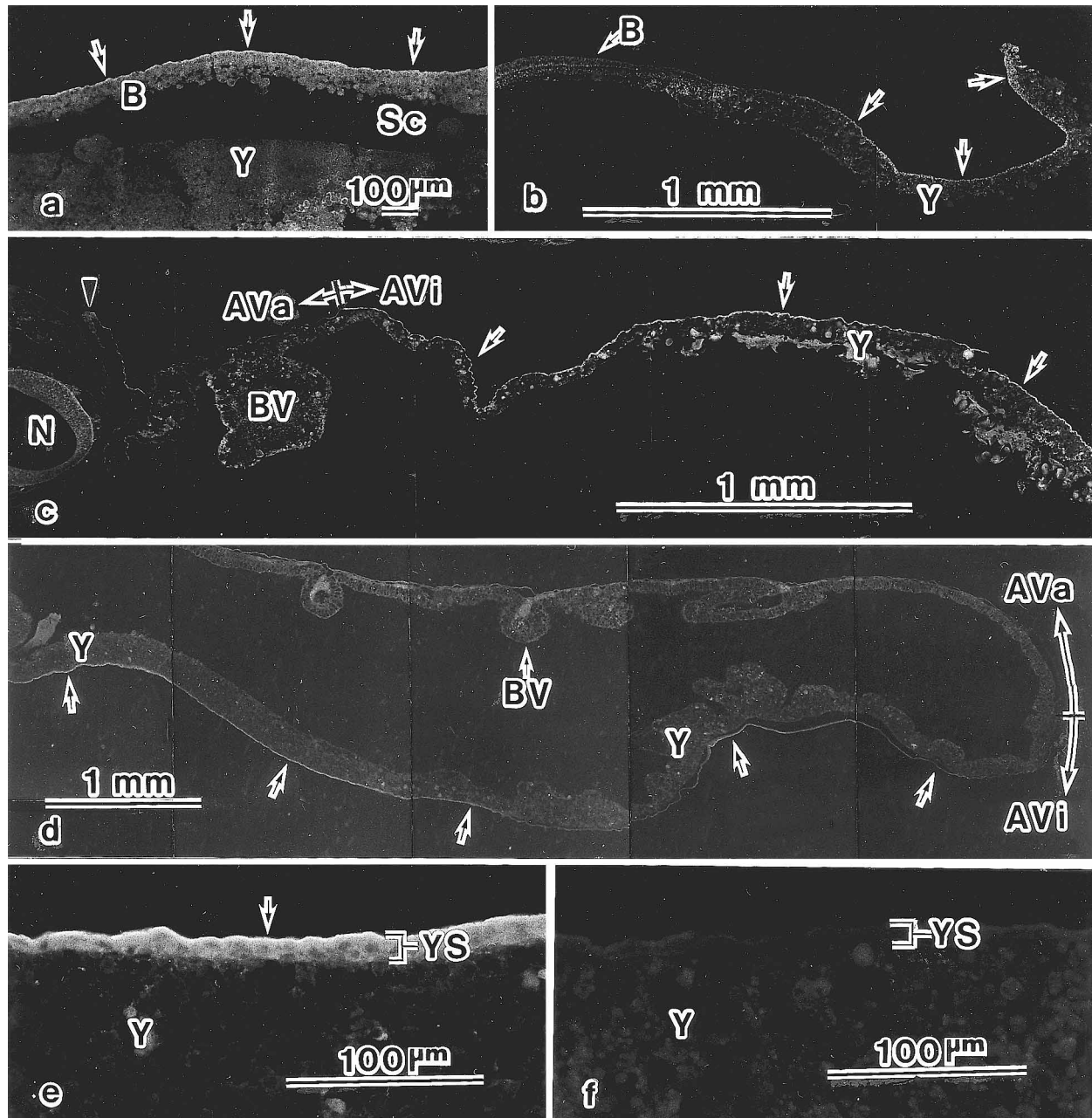
Fig. 3. Immunofluorescent micrographs of developing embryos, stained with anti-*Xenopus* hatching enzyme (αXHE) antibody and FITC-conjugated second antiserum. (a) Embryo on day 0 of incubation. Apical cytoplasm of outermost cells of blastoderm (B) is stained (arrows). (b) Embryo on day 1 of incubation. The cells at the area pellucida of the balstoderm are not stained, whereas the outermost cells at the area opaca are stained (arrows). (c) Embryo on day 2 of incubation. Yolk sac at area vitellina (AVi) is stained (arrows), whereas embryonic body and yolk sac at area vasculosa (AVa) are not stained. An arrowhead indicates a tip of extending chorioamniotic membrane. (d-f) Embryos on day 4 of incubation, showing extension of the stained area at AVi (arrows in d) with a strong fluorescence in the apical cytoplasm (arrow in e) of ectodermal cells in yolk sac (YS). Most yolk mass was removed; thus the yolk sac is deformed. Control shows no stain (f). Blood cells in blood vessels (BV in c and d) show non-specific fluorescence. N, neural tube; Sc, subgerminal cavity; Y, yolk (or yolk cell).

consists of neural tube, notochord, somites, blood vessels, and covering ectoderm was not stained, whereas the peripheral-most yolk sac, which is called the area vitellina, was stained (Fig. 3c). In the course of the incubation, the yolk sac extended and enclosed the yolk. On day 4 of incubation, the half diameter of the yolk sac area was about 15 mm. The cells at the area vitellina, corresponding to the peripheral 7 mm zone of yolk sac, were stained with the αXHE antibody (Fig. 3d). The strongest stain was observed at the apical-most cytoplasm of such cells (Fig. 3e). No stain was observed with a non-immune rabbit serum (Fig. 3f). At the area vasculosa, corresponding to the centripetal 8 mm zone of yolk sac, the blood cells were stained with the antibody (Fig. 3d), but they were also stained with the non-immune serum (data not

shown).

On day 8 of incubation, the yolk sac had completely enclosed the yolk. The cells stained with the antibody were observed at the area vitellina, the width of which zone was then about 3 mm, but on day 10 of incubation they could not be found in the yolk sac (data not shown).

Ultrastructural observations of yolk sac

The area vitellina of the yolk sac was composed of two layers of cells: the outer ectodermal cells and inner endodermal cells, both of flat shape (Fig. 4a). The outer ectodermal cells might be a participant in the production of enzyme which affects the overlying vitelline membrane. These cells possessed many long microvilli, well-developed endoplasmic reticula, and some secretory granules (Fig. 4b). Secretory granules tended to line up beneath the apical plasma membrane. Multi-vesicular bodies, of obscure significance, were occasionally observed in these cells .

The area vasculosa of the yolk sac was composed of outer ectodermal cells, inner endodermal cells, and various mesodermal cells of mesenchyme, blood cells, and blood vessels located between them (Fig. 4c). The outer ectodermal cells in this area lacked the secretory granules and their microvilli were short and few in number (Fig. 4d).

When the area vitellina of the yolk sac was processed for immuno-electron microscopy using the αXHE antibody, the secretory granules were specifically stained (Fig. 5a,b). Since the secretory granules formed a line beneath the apical plasma membrane, a number of them varied from cell to cell depending on the cutting plane. No stain was observed on the secretory granules when reacted with the non-immune serum (Fig. 5c).

Western blot tests on yolk sac

Immunoblot tests were made to reveal the nature of molecules which reacted with the αXHE antibody (Fig. 6). The
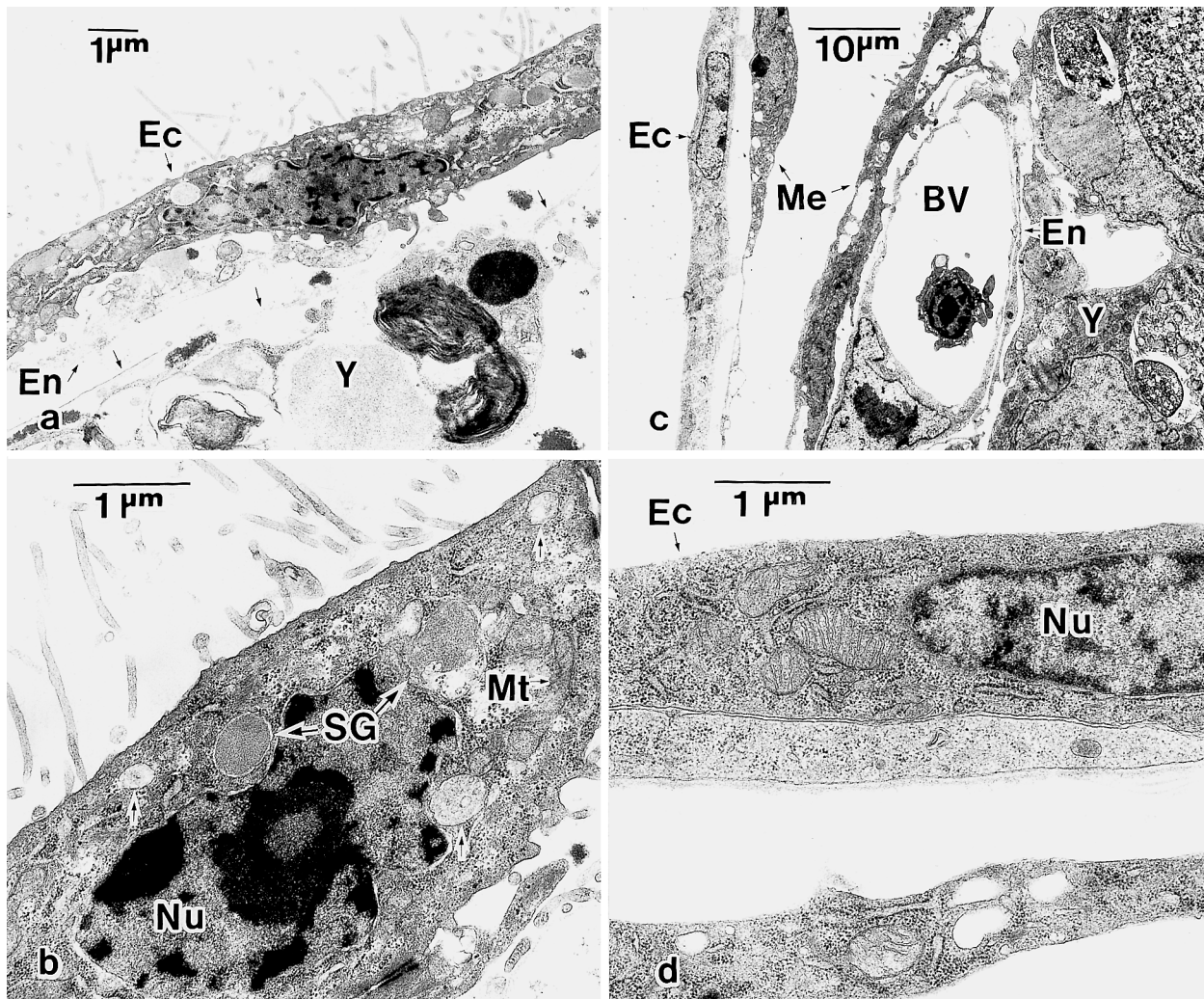

Fig. 4. Electron micrographs of yolk sacs of embryos on day 4 of incubation. (a,b) Yolk sacs at area vitellina. Ectodermal cells (Ec) possess secretory granules (SG) mostly in apical cytoplasm. Many long microvilli are prominent. Arrows in Fig. a indicate the plasma membrane bounding the yolk (Y). Arrows in Fig. b indicate multivesicular bodies. (c,d) Yolk sacs at area vasculosa. Secretory granule is absent and microvilli are poor in Ec. Mesodermal cells (Me) including blood vessel (BV) are invading between Ec and endodermal cells (En). Mt, mitochondrion; Nu, nucleus.
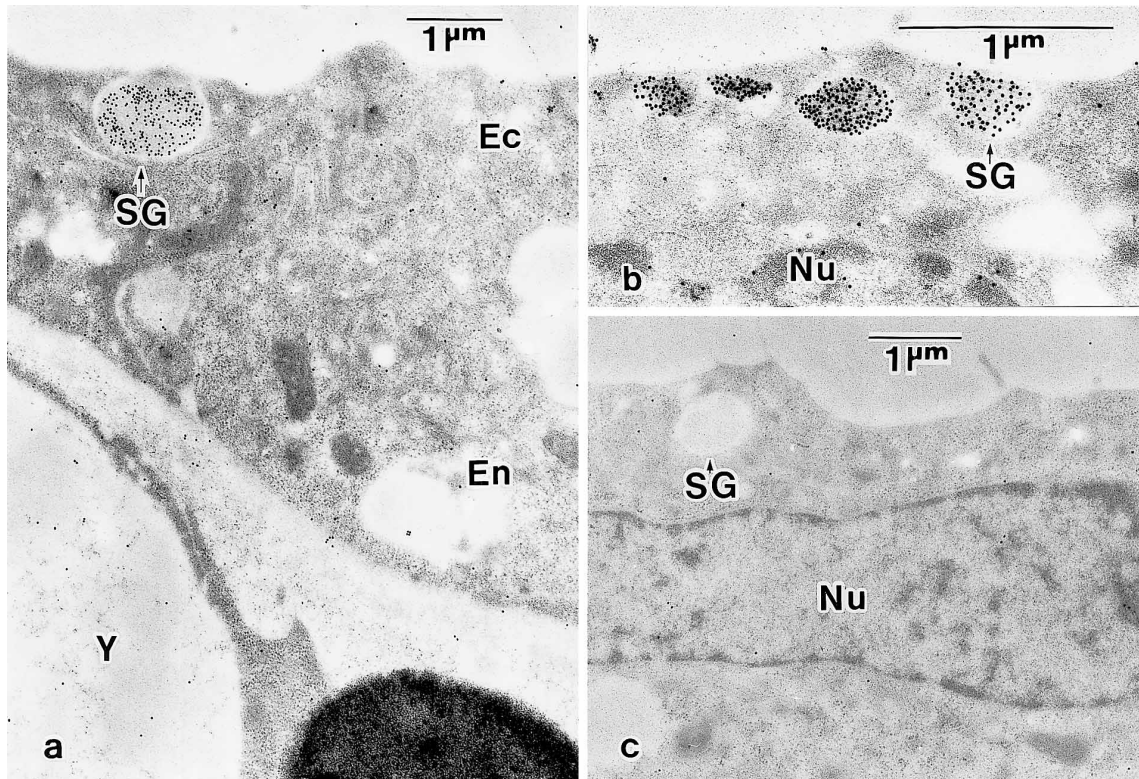
Fig. 5. Immunoelectron micrographs of yolk sacs at area vitellina of embryos on day 4 of incubation, stained with αXHE antibody. (a,b) Secretory granules (SG) are specifically stained. The number of SG in ectodermal cells (Ec) varies depending on the cutting plane. (c) Control, showing no stain. En, endodermal cell; Nu, nucleus; Y, yolk cell.
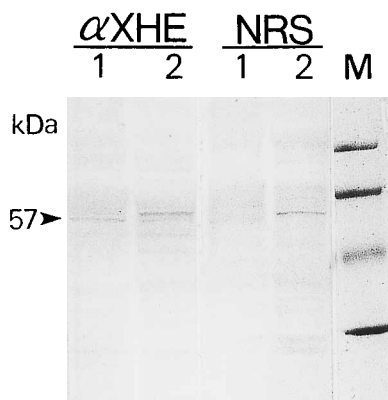

Fig. 6. Immunoblot analysis of yolk sac extracts of embryos on day 4 of incubation, stained with αXHE antibody or non-immune rabbit serum (NRS). Extracts (70 μg each) from area vitellina (lanes 1) or area vasculosa (lanes 2) of yolk sacs were analyzed by SDS-PAGE. Western-blotted membranes were treated with the antiserum and then with peroxidase-conjugated second antiserum. The 57 kDa protein is specifically immunostained in area vitellina extracts. Its faint stain is also present in area vasculosa extracts. The stain at 59 kDa in the area vasculosa extracts is not specific. M, molecular markers of 94, 67, 43, and 30 kDa from top to bottom.

Fig. 7. Schematic illustration of digesting process of quail vitelline membrane. On day 0 of incubation (a), the outermost ectodermal cells of blastoderm (dotted) possess secretory granules, presumably containing hatching enzyme. The vitelline membrane (VM) and egg white (EW; hatched) are not affected yet. On day 2 of incubation (b), the ectodermal cells of yolk sac at area vitellina (AVi) possess secretory granules, whereas those of blastoderm do not. However, the vitelline membrane over the blastoderm is disintegrated. On day 4 of incubation (c), the yolk sac advances toward the vegetal pole, and the ectodermal cells at area vitellina possess secretory granules, whereas those at area vasculosa do not. The vitelline membrane as well as the egg white over the area vasculosa and embryonic body are disintegrated or completely digested. Y, yolk (or yolk cell). This illustration shows that predicted hatching enzyme secreted by ectodermal cells of yolk sac at area vitellina digests the vitelline membrane, and such digestion gradually extends from the animal pole side to the vegetal pole side with the advance of the yolk sac.

extract of yolk sac at the area vitellina gave a specific band at 57 kDa when treated with the antibody. The same band appeared in the extract of the yolk sac at area vasculosa, but it was extremely faint. The heavy stain at 59 kDa in the latter
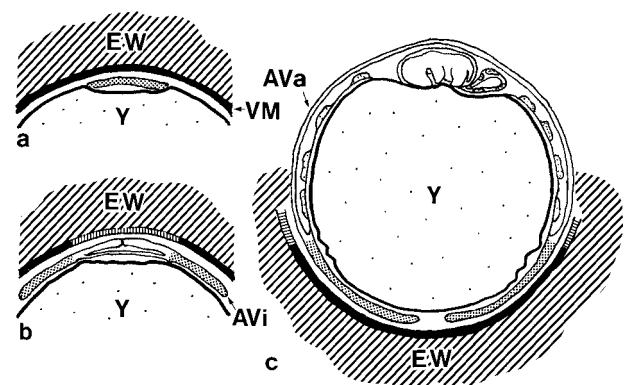
extract was also observed by means of a treatment with the non-immune serum, and therefore it was non-specific.

DISCUSSION

A summary of the present study is illustrated in Fig. 7. The anti-Xenopus hatching enzyme (αXHE) antibody stained the outermost ectoderm of the blastoderm at the beginning of development, and also stained the yolk sac ectoderm at the area vitellina at later developmental stages. The staining occurred in the secretory granules located in the apical-most cytoplasm of the cells. Such stained cells were always located at the leading zone of the extending yolk sac. Disintegration of the vitelline membrane and then the disappearance of the egg white occurred just behind the leading zone. Therefore, the membrane-digesting enzyme (presumably, a hatching enzyme) of quail might be produced by the cells of yolk sac ectoderm at the area vitellina.

As far as we know, the hatching gland cells specified for the production and secretion of hatching enzyme have been identified only in fish (Yamamoto *et al.*, 1979; Yamagami *et al.*, 1992) and amphibians (Yamasaki *et al.*, 1990; Yoshizaki, 1991). In sea urchins (Ghiglion *et al.*, 1997) and mammals (Perona and Wassarman, 1986), the supposed cells produce the hatching enzyme but finally differentiate into cells bearing another function. In quail, the cells stained with the αXHE antibody existed at different locations of the embryo at different developmental stages. However, the cells of the blastoderm in chickens move around only in the realm of the blastoderm, never radially toward the yolk sac (Spratt and Haas, 1965). Most probably, newborn ectodermal cells in aves produce the hatching enzyme temporarily and such potentiated cells are continuously recruited at the leading zone of the yolk sac. They may differentiate into nerve cells, epidermis, or ectodermal covering of the yolk sac at the area vasculosa, or to the chorion at the end of secretory activity.

An interesting phenomenon that was observed was the occurrence of firm contact of the egg white with the yolk sac at the location where the vitelline membrane had disappeared. We have ultrastructural evidence that shows an endocytotic uptake of the white by yolk sac ectoderm (unpublished). Although it is not yet known how the hatching enzyme participates in this process, such contact may confine secreted enzyme to a perivitelline space.

In *Xenopus* (Urch and Hedrick, 1981; Fan and Katagiri, 1997; Katagiri *et al.*, 1997) and medaka fish (Yasumasu *et al.*, 1989a, b, 1992; Yamagami, 1996), the hatching enzymes were well characterized and their cDNAs were cloned. The XHE has 51.3% and 47.1% homology with the medaka hatching enzyme constituents HCE and LCE, respectively (Katagiri *et al.*, 1997). They share the consensus sequence of the astacin family (Yamagami, 1996) which was found on astacin, a digestive protease of the crayfish, *Astacus fluviatilis* (Titani *et al.*, 1987; Dumermuth *et al.*, 1991). Although no direct evidence of the presence of the hatching enzyme in quail has yet been presented, a cDNA, which was cloned from chorio-

allantoic membrane (Elaroussi and DeLuca, 1994), deserves attention. The predicted polypeptide has a molecular mass of 40,890 Da, a 50.5% homology with XHE (Katagiri *et al.*, 1997), and an astacin consensus sequence. It is supposed to be a metalloendopeptidase which plays a role in the degradation of eggshell matrix at 11–12 days of incubation. It is not known at present whether the 57 kDa protein detected in the present study is the translation product of the cDNA. But it could be because (1) the cells of the chorion, or their precursor cells, were the constituents of yolk sac ectodermal cells, if development is traced back a few days; thus the same gene may be reactivated. And (2) the cells of the chorion were also stained with the αXHE antibody (unpublished). The value of the 57 kDa which exceeds that of 40,890 Da may be explainable by glycosylation of functional enzyme molecules, as reported for XHE whose molecular mass is 48 kDa for the predicted form and 60 kDa for the native one (Katagiri *et al.*, 1997). The occurrence of glycosylation of the hatching enzyme was experimentally confirmed in *Rana pirica* (Kitamura and Katagiri, 1998).

It was suggested by Waclawek *et al.* (1998) and Takeuchi *et al.* (1999) that the glycoprotein constituting the vitelline membrane of avian embryo is a ZP (zona pellucida)-domain-containing protein homologous to that of *Xenopus* (Kubo *et al.*, 1997; Yang and Hedrick, 1997) and medaka (Murata *et al.*, 1997) embryos. Then it is probable that the vitelline membrane degradation in avian embryo occurs on the same enzyme-substrate system as that in *Xenopus* and medaka embryos, that is, a system of astacin-like metalloproteinase and ZP-domain-containing glycoproteins.

As shown in several animals (sea urchins, fish, amphibians and mammals), hatching is the escape of embryos from their protecting envelopes in order to get food (or to absorb nutrients in the uterus) for further growth. Historically, avian hatching is considered to be an escape from all envelopes–vitelline membrane, egg white, shell membrane and eggshell. So even in *in vitro* culture, successful normal development is called "hatching" (Kamihira *et al.*, 1998). However, the components of egg white (Sugimoto *et al.*, 1999) and eggshell (Tuan, 1987) are apparently ingested as nutrients through chorioallantoic membranes by embryos. It seems to be similar to the nutrient absorption occurring at the placenta of mammals. The breakdown of shell membrane and the eggshell by pipping also seems to be an analogous phenomenon to mammalian birth in which fetuses undergo a transition from water to land living. So we propose to apply the term *hatching* restrictively to digestion of the vitelline membrane in aves.

The present study focused on the disintegration of the vitelline membrane by the embryo. However, the isolation and characterization of avian hatching enzyme are not done yet. Future study will be carried out to elucidate such an aspect of the avian hatching.

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