Developmental Biology **213,** 18–32 (1999)
Article ID dbio.1999.9365, available online at http://www.idealibrary.com on IDEAL®

Lipovitellins, Play Different Roles during Oocyte Maturation and Early Development of Barfin Flounder, *Verasper moseri*, a Marine Teleost that Spawns Pelagic Eggs

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Two forms of vitellogenin (Vg), Vg A and Vg B, were identified in serum from estrogen-treated barfin flounder (*Verasper moseri*). Structural changes of lipovitellins (Lvs) derived from the two Vgs were examined during vitellogenesis and oocyte maturation. Two Lvs, vLv A and vLv B, were identified electrophoretically and immunologically in postvitellogenic oocytes. Each appeared to be composed of distinct heavy chains (vLvH A, M_r 107,000, and vLvH B, M_r 94,000) and light chains (vLvL A, M_r 30,000, and vLvL B, M_r 28,000) when analyzed by SDS–PAGE. Results from N-terminal amino acid sequencing and Western blotting using antisera to vLvH A and vLvH B verified that there are two Vg polypeptides in serum from estrogen-treated fish, Vg A (M_r 168,000) and Vg B (M_r 175,000), which give rise to vLvH A–vLvL A and vLvH B–vLvL B, respectively. N-terminal sequencing revealed two sequences for both phosvitin and β'-component, supporting the concept of duality for all three classes of Vg-derived yolk proteins. During oocyte maturation, native dimeric vLv B was dissociated into a native M_r 170,000 monomer (oLv B). Meanwhile, vLv A was extensively cleaved including complete degradation of vLvH A into free amino acids. We propose that the quantitative ratio of vLv A to vLv B in postvitellogenic oocytes regulates the buoyancy of the spawned pelagic eggs by controlling availability of free amino acids which function as osmotic effectors during oocyte hydration. The vLv A/vLv B ratio likely also controls the proportional availability of different types of nutrients, free amino acids versus Lv, for use during embryonic development.

Key Words: **vitellogenin; yolk protein; lipovitellin; phosvitin; β'-component; vitellogenesis; yolk proteolysis; oocyte maturation; teleost.**

INTRODUCTION

Eggs of oviparous teleosts accumulate a large amount of yolk protein as a nutrient store required by developing embryos. During yolk protein accumulation, referred to as vitellogenesis, the yolk precursor protein (vitellogenin, Vg) is hepatically synthesized as regulated by estrogen, mainly estradiol-17β. Following incorporation into growing oocytes by receptor-mediated endocytosis, Vg is proteolytically cleaved into smaller yolk proteins (for review see Wiegand, 1982; Ng and Idler, 1983; Wallace, 1985; Mommsen and Walsh, 1988; Selman and Wallace, 1989; Specker and Sullivan, 1994). In amphibians and birds, Vgs give rise to at least two major classes of yolk proteins, an extensively lipidated lipovitellin (Lv) and a highly phosphorylated phosvitin (Pv) (Wallace, 1985). The native Vg of an amphibian, *Xenopus laevis*, has a molecular weight of 450,000–460,000 (Wallace, 1970; Redshaw and Follett, 1971) and yields a native Lv present as an approximate M_r 400,000 dimer

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0012-1606/99 $30.00

(Wallace, 1985) and two types of Pv of M_r 34,000 and 33,000 as well as two smaller phosphoproteins termed "phosvettes" (Wiley and Wallace, 1981). As in the higher oviparous vertebrates, teleost Vg is also constructed of Lv and Pv (see Wiegand, 1982; Ng and Idler, 1983). In addition, the third class of Vg-derived yolk protein, designated β'-component (β'-c) or E2-component, has been identified and characterized in some teleosts (Markert and Vanstone, 1971; Hara and Hirai, 1978; Campbell and Idler, 1980; Hara *et al.*, 1993; Matsubara and Sawano, 1995).

Aside from initial processing of Vg, the occurrence of additional proteolysis of the yolk proteins during final oocyte maturation has been discovered in *Fundulus heteroclitus* (Wallace and Begovac, 1985; Wallace and Selman, 1985). This second proteolysis of Vg-derived proteins occurs particularly in marine or brackish-water fishes, which exhibit remarkable hydration of their oocytes during final maturation (Greeley *et al.*, 1986; Carnevali *et al.*, 1992, 1993; Matsubara *et al.*, 1995; Thorsen *et al.*, 1996). This second proteolytic event is unique to teleosts (Byrne *et al.*, 1989). Regarding marine pelagic eggs, they contain a high quantity of free amino acids compared to demersal eggs. The increase in free amino acid content is thought to play a significant role in generating osmotic effectors needed for water influx during oocyte maturation and hydration (Craik and Harvey, 1987; Greeley *et al.*, 1991; Thorsen and Fyhn, 1996; Thorsen *et al.*, 1996). Based on results of studies of *in vitro* oocyte maturation using media with or without free amino acids, the source of free amino acids was suggested to be yolk proteins, which degrade during oocyte maturation (Thorsen and Fyhn, 1996). Our previous study demonstrated the occurrence of proteolysis in all three classes of Vg-derived yolk proteins during oocyte maturation of barfin flounder, *Verasper moseri* (Matsubara and Sawano, 1995). Reverse temporal correlation between the quantity of the yolk proteins versus free amino acids, coupled with a synchronous influx of water into the oocytes, strongly suggested that yolk protein proteolysis is the major cause of oocyte hydration (Matsubara and Koya, 1997). On the other hand, free amino acids are also believed to serve as a major substrate for generation of energy via aerobic metabolism and for protein synthesis during embryogenesis (Fyhn and Serigstad, 1987; Rønnestad and Fyhn, 1993; Rønnestad *et al.*, 1992, 1993; Finn *et al.*, 1995). Thus, there seems to be two significant functions of oocyte maturation-associated yolk proteolysis, acquisition of proper buoyancy and guaranteeing a usable nutrient supply for the embryo. In addition to β'-c and Pv, which both undergo extensive proteolysis during oocyte maturation of barfin flounder, Lv is degraded partially, remaining as a native M_r 170,000 monomer after oocyte maturation (Matsubara and Koya, 1997). Thus, the Lv molecule is likely processed to allow for quite different types of utilization before and after fertilization.

The strictly adjusted positive buoyancy of marine pelagic eggs with respect to their surrounding seawater (Thorsen *et al.*, 1996) raises the possibility that there is an allocation system by which Lv is degraded to varying degrees during oocyte maturation depending on the ultimate egg buoyancy required. Recent molecular studies of vertebrate Vg genes and mRNAs suggest that there are two or more Vg genes in *X. laevis* and chickens (see Byrne *et al.*, 1989) as well as some teleosts (Lee *et al.*, 1994; LaFleur *et al.*, 1995a). These studies raise the question of whether the plural Vg system is involved in regulation of selective utilization of the yolk proteins as osmotic effectors versus macromolecular nutrients in marine teleosts. In this study, we biochemically and immunologically analyzed Vg and its derived yolk proteins in barfin flounder, a marine teleost spawning pelagic eggs, to clarify the details of molecular alterations of the yolk proteins during their first and second proteolytic events discussed above. Furthermore, we discovered two forms of yolk proteins and their precursor Vgs and verified their precursor–product relationships. Considering the different roles of the two forms of Vg as regards yolk protein utilization, we propose a schematic flow chart for regulated proteolysis of yolk proteins involving the dual Vg system in barfin flounder.

MATERIALS AND METHODS

Samples

Adult female and immature male barfin flounders used in the present study were maintained in 40- and 50-kl aquaria at Akkeshi Station, Japan Sea-Farming Association, in Hokkaido. A total of eight adult females, 5 to 7 years of age (weight range 4.2–7.1 kg), were kept at a water temperature of 6°C during the spawning season in April. To collect postvitellogenic oocytes, about 1 g of ovarian follicles was obtained from the females by cannulation of their ovaries through the urogenital pore using a sterilized polyvinyl tube (4-mm i.d.) connected to a 10-ml syringe. The collected piece of ovary was washed with cold physiological saline for flounder, pH 7.4 (Hirano *et al.*, 1971), and then the fully grown postvitellogenic oocytes were collected in their intact follicles. Ovulated eggs were obtained in the same manner within 1 day after ovulation. The oocytes and eggs were each aliquated into 10 subsamples and then were stored at −80°C until use.

For induction of vitellogenesis, three immature males (1.0–1.2 kg) were thrice injected intramuscularly with estradiol-17β dissolved in propylene glycol, once every 3 days. The dosage used was 5 mg estradiol per kilogram of body weight. Three days after the last injection, blood samples were collected from the dorsal vessels of each fish using a syringe containing a mixture of serine protease inhibitors, 10 mM phenylmethylsulfonyl fluoride (PMSF), and 10% aprotinin solution (Sigma) in 0.9% NaCl, at 10% of the volume of blood to be collected. The blood was allowed to clot at 4°C for 30 min followed by centrifugation at 3000*g* for 15 min to separate the serum which was either subjected immediately to biochemical analysis or stored at −80°C until use.

Gel Chromatography

Gel chromatography of serum and extracts of postvitellogenic oocytes and ovulated eggs were performed on a Pharmacia FPLC system using a prepacked column of Superose 6 HR 10/30 (Pharmacia, Uppsala, Sweden) equilibrated with 20 mM Tris–HCl, pH 8.0, containing 150 mM NaCl and 1 mM PMSF. Extracts of oocytes

and eggs were prepared by homogenizing them with the elution buffer at concentrations of 10 and 20% v/v, respectively, followed by centrifugation at 10,000*g* for 10 min. Serum was diluted 1:4 in the same elution buffer before chromatography. Approximately 200 μl of oocyte/egg extract or diluted serum was applied to the Superose 6 column. The flow rate through the column was 0.5 ml/min. The molecular weights of the relevant protein peaks were estimated by HMW and LMW Gel Filtration Calibration Kits (Pharmacia).

The elution positions of phosphoprotein phosphate in the chromatography fractions were measured as follows. An aliquot (0.5 ml) of each fraction was dried at 65°C, resuspended in 0.2 ml of 4 N NaOH, and then incubated at 100°C for 30 min. After neutralizing with a same volume of 4 N HCl, the sample was centrifuged at 10,000*g* for 10 min and 0.2 ml of the supernatant was collected. The phosphate content of the supernatant was measured according to Gamst and Try (1980).

Electrophoresis

Sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis (PAGE) was performed in the presence of 2-mercaptoethanol (2-ME) on 7.0 and 9.2% acrylamide gels and precast linear acrylamide gradient gels with gradients of total acrylamide concentrations from 5 to 20% and from 3 to 10% (Atto, Tokyo, Japan). The gels were stained with Coomassie Brilliant Blue R-250 (CBB) or by using a silver staining kit (Wako, Osaka, Japan). Estimation of molecular weight by SDS–PAGE was performed using molecular weight markers (SigmaMarker Wide Range; Sigma, St. Louis, MO; LMW Electrophoresis Calibration Kit; Pharmacia, U.S.A.) to calculate the relative molecular weight of protein bands.

Two-dimensional (2D) electrophoresis was carried out with a combination of isoelectric focusing as the first dimension and gradient SDS–PAGE as the second dimension. The isoelectric focusing in the first dimension was performed using an AE-6050A rectangular gel system (Atto) with carrier ampholytes, pH range 3–10 (Bio-Lyte 3/10; Bio-Rad), at a concentration of 3% v/v. Samples (4 μl) of 3 or 15% homogenates of postvitellogenic oocytes and ovulated eggs, respectively, were mixed with equal volumes of 60% glycerol solution without detergent, applied to the first-dimensional gel strips (2.5% total acrylamide, 7.5% glycerol, and 3% ampholytes, pH 3–10) and electrophoresed at 50 V for 40 min, 100 V for 40 min, 150 V for 150 min, and finally 250 V for 40 min. The gel strips were then electrophoresed against precast 5–20% linear gradient slab gels (Atto) as the second dimension. After electrophoresis, gels were either stained with CBB or subjected to immunoblotting analyses.

Preparation of Antisera

Polyvalent antisera were raised in rabbits against the purified Lv heavy chains A (vLvH A, M_r 107,000) and B (vLvH B, M_r 94,000) and Lv light chains A (vLvL A, M_r 30,000) and B (vLvL B, M_r 28,000). The antigens were isolated from excised gel pieces containing the desired bands after SDS–PAGE (Fig. 1) of partially purified native Lv fractions (pooled fractions 5–10 of Fig. 2A) from postvitellogenic oocytes. The samples (10 mg protein/ml) of pooled Lv fractions from postvitellogenic oocytes were mixed with equal volumes of SDS sample solution with 2-ME, then 200 μl of each was applied to the SDS–PAGE gels. To isolate Lv heavy chains and Lv light chains, four homogeneous gels of 7.5 and 9.2%, respectively, were used. After electrophoresis, gel strips from both sides of the gels were cut with the position marked by punch, stained with CBB, destained with 30% methanol and 10% acetic acid solution, and washed with distilled water, then the strips were put back to their original positions. The antigens were isolated from excised gel pieces containing the desired bands from unstained gels using an AE-6580 electroeluter (Atto). The antiserum to β'-component from vitellogenic oocytes was prepared as described by Matsubara and Sawano (1995). The antisera are named using the abbreviations a-vLvH A, a-vLvH B, a-vLvL A, a-vLvL B, and a-β'-c, respectively.

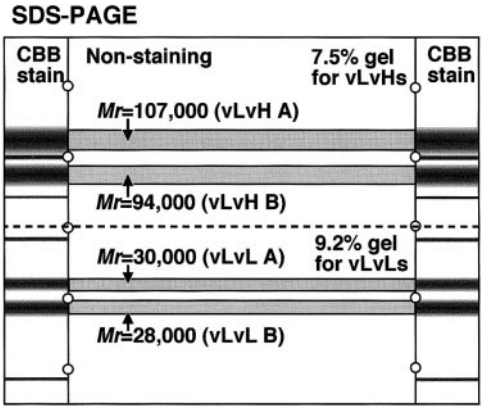

FIG. 1. Schematic drawing of the method to isolate Lv heavy chains A (vLvH A, M_r 107,000) and B (vLvH B, M_r 94,000) and Lv light chains A (vLvL A, M_r 30,000) and B (vLvL B, M_r 28,000) after SDS–PAGE. Partially purified native Lv fractions (pooled fractions 5–10 of Fig. 2A) from postvitellogenic oocytes were subjected to SDS–PAGE using 7.5% homogeneous gel for isolating the Lv heavy chains and 9.2% gel for Lv light chains. After electrophoresis, gel strips from both sides of the gels were cut with the position marked by punch and stained with CBB, then the strips were put back to their original positions. The antigens were isolated from excised gel pieces containing the desired bands (shaded area) from unstained gels using an electroeluter.

Immunoblotting

Semidry transblotting after SDS–PAGE was performed using a polyvinylidene fluoride (PVDF) membrane (Immobilon-P; Millipore) and a semidry transfer apparatus (Trans-Blot SD; Bio-Rad). Nonspecific binding of antibodies was blocked by a mixture of 1% bovine serum albumin and 5% powdered milk. Western blots were obtained using the specific antiserum as the primary antibody using 1:2000 to 1:5000 dilutions and a 1:3000 dilution of goat anti-rabbit IgG horseradish peroxidase conjugate (Bio-Rad) for the secondary antibody. Immunoreactive bands were made visible by HRP color development reagent (Bio-Rad) containing 4-chloro-1-naphthol. Negative control was obtained using 1:1000 dilution of normal rabbit serum instead of the primary antibodies.

Amino Acid Sequence Analysis

N-terminal amino acid sequence analysis was carried out in the Takara Biomedical Center (Shiga, Japan). The sequences of the Vg and yolk polypeptides were determined on an automated peptide

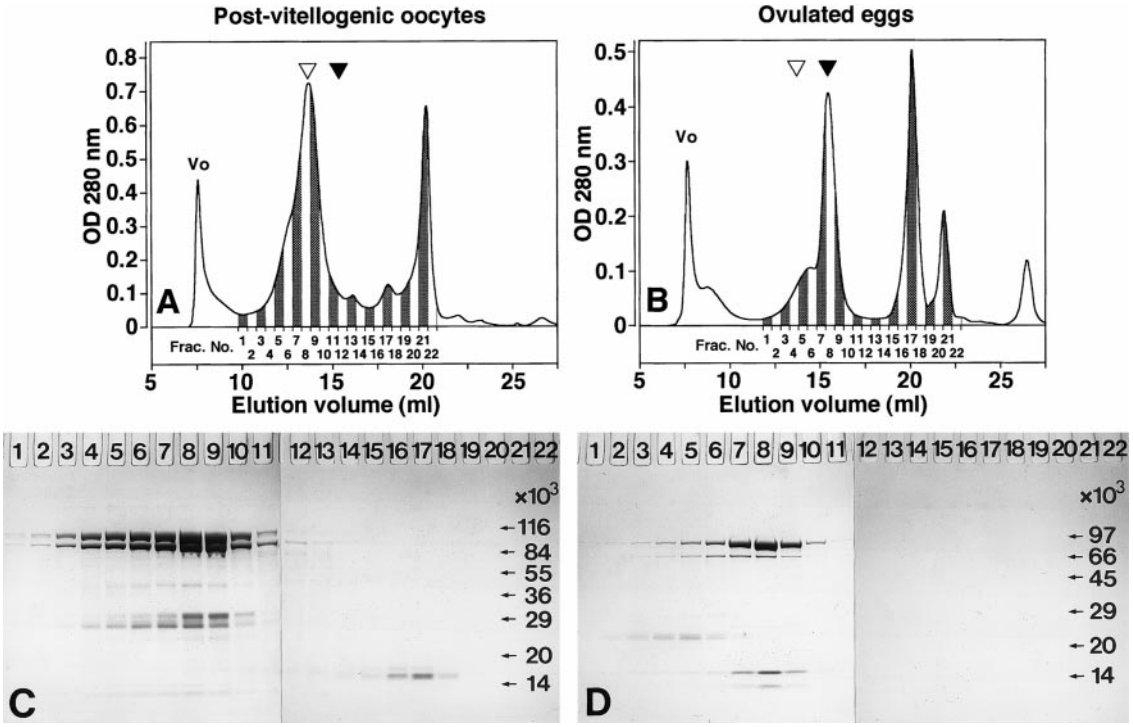

FIG. 2. Elution profiles of the homogenates from postvitellogenic oocytes (A) and ovulated eggs (B) after Superose 6 gel chromatography and 5–20% gradient SDS–PAGE of the chromatographic fractions from postvitellogenic oocytes (C) and those from ovulated eggs (D). The same homogenates from postvitellogenic oocytes and ovulated eggs are depicted after being resolved by Superose 6 gel chromatography and SDS–PAGE. Numbered fractions (0.5 ml each) are indicated by alternating bands. Open and closed arrowheads in the Superose 6 gel chromatography identify peak positions of M_r 410,000 lipovitellin in postvitellogenic oocytes and M_r 170,000 lipovitellin in ovulated eggs, respectively. Lane numbers in the electrophoreograms correspond to fraction numbers in the corresponding chromatograms. Molecular weight values on the right in C and D indicate migration positions of marker proteins.

sequencer (HP G1005A; Hewlett Packard) using excised blotting membrane chips (Immobilon-P_{SQ}; Millipore) containing the desired polypeptide bands. All the polypeptides were analyzed more than twice.

Nomenclature of Yolk Protein Subunits

In the present study, we utilized abbreviations of yolk protein subunits according to the following scheme: (1) The first lowercase letter indicates maturational stage of the oocyte (v, vitellogenic oocyte; o, ovulated egg). (2) The next pair of letters indicates the yolk protein class (Lv, lipovitellin; Pv, phosvitin). (3) The next uppercase letter indicates the kind of subunit chain (H, heavy chain; L, light chain in the case of Lv). (4) The last uppercase letters (A or B) indicate the identity of the parental Vg molecule (Vg A or Vg B).

RESULTS

Identification of Two Forms of Lipovitellin

The elution profiles of homogenates from oocytes versus ovulated eggs after Superose 6 gel chromatography were remarkably different, indicating significant biochemical changes in the yolk proteins during oocyte maturation (Figs. 2A and 2B). The main UV-absorbing peak of native Lv from vitellogenic oocytes corresponding to about M_r 410,000 (Fig. 2A) disappeared after ovulation, and another smaller peak (M_r 170,000) was observed instead (Fig. 2B). The chromatography fractions containing the major UV-absorbing peaks from oocytes and eggs were further subjected to gradient SDS–PAGE (Figs. 2C and 2D). For Lv-peak fractions from postvitellogenic oocytes, two major bands comigrated in a molecular weight range 90,000 to 110,000. The most densely staining bands were observed for fractions corresponding to the Lv peak. These Lv fractions also contained two minor bands of lower apparent molecular weight (M_r 30,000 and 28,000) and another faint band (M_r 42,000). The elution positions of the two minor bands after Superose 6 chromatography were quite different in the Lv fractions and the M_r 28,000 band eluted ahead of the M_r 30,000 and 42,000 bands. A small UV-absorbing peak designated β'-c from vitellogenic oocytes (Matsubara and Sawano, 1995) eluted at a position of M_r 19,000 after Superose 6 gel chromatography (fraction 17 in Fig. 2A) and

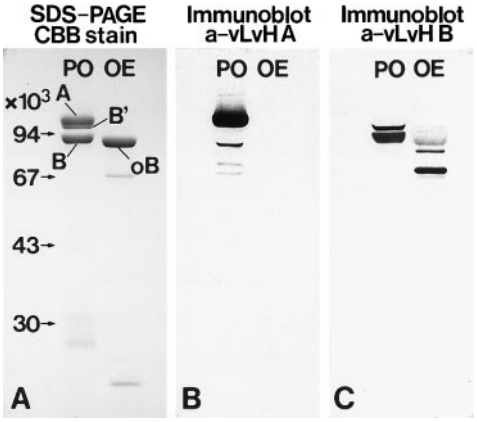

FIG. 3. SDS–PAGE (A) and immunoblotting (B, C) of postvitellogenic oocyte homogenate (lane PO) and ovulated egg homogenate (lane OE). (A) SDS–PAGE on a 9.2% gel stained with Coomassie brilliant blue; (B) immunoblotting using antiserum to the M_r 107,000 polypeptide (a-vLvH A); and (C) immunoblotting using antiserum to the M_r 94,000 polypeptide (a-vLvH B). Labels A, B′, B, and oB correspond to the M_r 107,000, 102,000, 94,000, and 92,000 bands, respectively.

appeared after SDS–PAGE as a distinct band of M_r 17,000 when electrophoresed in the presence of 2-ME. Ovulated egg homogenates gave rise to a main UV-absorbing Lv peak of M_r 170,000 with a shoulder of approximately M_r 330,000 as seen in Fig. 2B. When these chromatography fractions were subjected to gradient SDS–PAGE, the Lv-peak fractions migrated as a major band of M_r 92,000 with minor bands of M_r 67,000 and 15,000 as well as a faint band of M_r <10,000. The fraction containing the M_r 330,000 shoulder contained the M_r 92,000 and 67,000 bands as well as a smaller M_r 22,000 band when run on SDS–PAGE.

To verify the relationship between the major yolk polypeptide bands, immunoblotting analyses were carried out (Fig. 3). Results of 9.2% acrylamide gel SDS–PAGE revealed that homogenate of postvitellogenic oocytes formed two major bands having apparent molecular weights corresponding to 107,000 and 94,000 and a faint band of M_r 102,000, while homogenates of ovulated eggs produced a single main band of M_r 92,000 (Fig. 3A). Subsequent Western blots performed using specific antisera raised against purified polypeptides from the M_r 107,000 (a-vLvH A) and 94,000 (a-vLvH B) bands are shown in Figs. 3B and 3C, respectively. Although the former antiserum strongly reacted with the M_r 107,000 band, no immunoreaction was observed against the M_r 94,000 and 102,000 bands or other bands with the exception of a sharply staining thin band just beneath the M_r 94,000 band from homogenates of postvitellogenic oocytes (Fig. 3B). Using this antiserum, no immunostaining bands from ovulated egg homogenates were visible in Western blots. On the other hand, the antiserum raised against the M_r 94,000 polypeptide (a-vLvH B) reacted strongly to both a M_r 102,000 and a M_r 94,000 band from homogenates of postvitellogenic oocytes and less strongly to the M_r 92,000 band from ovulated egg homogenates (Fig. 3C). This antiserum also cross-reacted to a thin M_r 80,000 band along with a M_r 67,000 band from ovulated egg homogenates, which were not obvious after CBB staining. It thus appeared that, during oocyte maturation, the M_r 94,000 and 102,000 polypeptides were degraded to a M_r 92,000 polypeptide, whereas the M_r 107,000 polypeptide was degraded to the extent that no epitopes recognized by the antiserum remained intact.

N-terminal amino acid sequences obtained for the M_r 107,000, 102,000, and 94,000 polypeptides from postvitellogenic oocytes and the M_r 92,000 polypeptide from ovulated eggs are provided in Fig. 4. The N-terminus of the M_r 107,000 polypeptide was similar to that of the M_r 94,000 polypeptide, with 44% identity over 25 amino acid residues. On the other hand, the N-terminus of the M_r 94,000 polypeptide was completely identical to the corresponding sequences for the M_r 102,000 polypeptide. The N-termini of these polypeptides had significant similarity to those of various teleost Vgs (Folmar *et al.*, 1995) corresponding to the Lv heavy-chain domain of the Vg molecule (Byrne *et al.*, 1989). Thus, the M_r 107,000, 94,000, and 102,000 polypeptides are suggested to be Lv heavy chains of barfin flounder Vgs and were designated vLvH A, vLvH B, and vLvH B′, respectively. The N-terminal amino acid sequence of the M_r 92,000 polypeptide from ovulated eggs was identical with those of vLvH B and vLvH B′ after the fifth amino acid residue. Agreement of the various N-terminal sequences

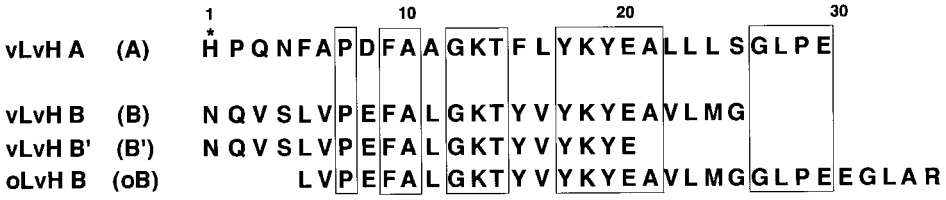

FIG. 4. N-terminal amino acid sequences of barfin flounder lipovitellin heavy chains. Labels vLvH and oLvH B are from postvitellogenic oocytes and ovulated eggs, respectively. Abbreviations in parentheses correspond to the labels of protein bands in SDS–PAGE shown in Fig. 3. Ambiguity is indicated with an asterisk.

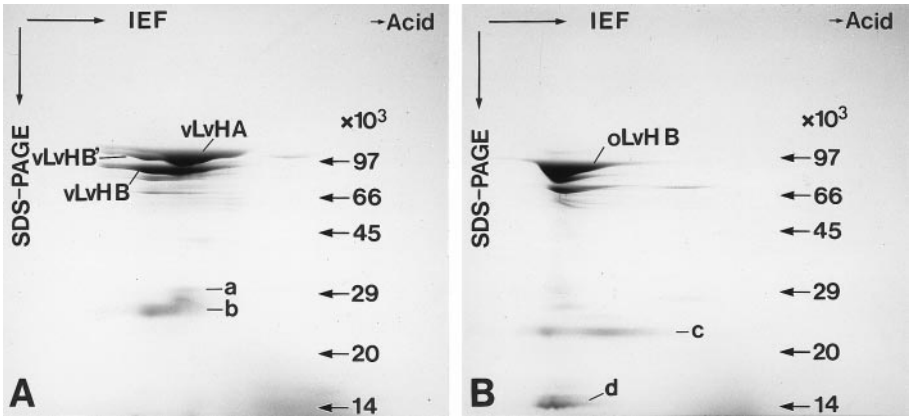

FIG. 5. Two-dimensional gel electrophoresis of postvitellogenic oocyte homogenate (A) and ovulated egg homogenate (B). Lipovitellin heavy chains are indicated in the same abbreviations used in the text. Labels a, b, c and d correspond to the M_r 30,000, 28,000, 22,000, and 15,000 spots, respectively. Molecular weight values on the right indicate migration positions of marker proteins.

supports the results of the immunoblotting analysis and verifies that the M_r 92,000 polypeptide is the proteolytically degraded product previously identified as oLvH B, produced from either vLvH B or vLvH B′ or most probably from both of them.

In order to further verify the subunit composition of the native Lv molecule(s), homogenates of postvitellogenic oocytes and ovulated eggs were subjected to two-dimensional gel electrophoresis (Fig. 5). The results, coupled with those from Western blotting using a-vLvH A and a-vLvH B (data not shown), demonstrated differences in isoelectric points among native vLvH A, vLvH B, and vLvH B′. Moreover, vLvH A and a minor spot of M_r 30,000 (spot a in Fig. 5A) comigrated closely in the first-dimensional isoelectric focusing, whereas vLvH B comigrated in this dimension with a minor spot of M_r 28,000 (spot b in Fig. 5A). This evidence suggests that the M_r 30,000 and 28,000 polypeptides are components of native vLv A and vLv B, respectively. In ovulated egg homogenates, the oLvH B comigrated during isoelectric focusing with a minor spot of M_r 15,000 (spot d in Fig. 5B). Thus, the M_r 15,000 polypeptide seemed likely to be a component of oLv B. Another minor spot of M_r 22,000 migrated to rather acidic position compared to the M_r 15,000 polypeptide and had no obvious counterpart in the first dimension of 2D electrophoresis.

Figure 6 shows the patterns produced by postvitellogenic oocyte and ovulated egg homogenates after gradient SDS–PAGE ± Western blotting using antisera raised against the gel-purified M_r 30,000 polypeptide (a-vLvL A) and 28,000 polypeptide (a-vLvL B), respectively. In addition to the vLvHs present in postvitellogenic oocyte homogenates, three distinct bands having molecular weights corresponding to M_r 30,000, 28,000, and 17,000 and one faint band of M_r 42,000 were visible after CBB staining (Fig. 6A, lane PO). On the other hand, two distinct bands of M_r 22,000 and 15,000 and a minor band of M_r <10,000 were observed for ovulated egg homogenates (Fig. 6A, lane OE). Western blots using antiserum against the M_r 30,000 polypeptide (a-vLvL A) visualized two distinct bands of M_r 30,000 and 42,000 from postvitellogenic oocyte homogenates and also cross-reacted strongly to the two bands of M_r 22,000 and 20,000 from ovulated egg homogenates (Fig. 6B). In contrast, the antiserum raised against the M_r 28,000 polypeptide (a-vLvL B) reacted strongly to the M_r 28,000 band and less strongly

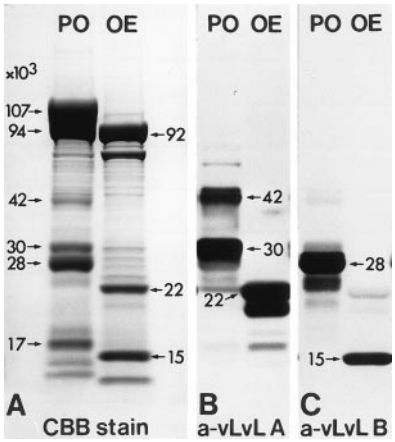

FIG. 6. SDS–PAGE (A) and antigenic profiles in immunoblotting (B, C) of postvitellogenic oocyte homogenate (lane PO) and ovulated egg homogenate (lane OE). (A) SDS–PAGE of a 5–20% gradient gel stained with Coomassie brilliant blue; (B) immunoblotting using antiserum to the M_r 30,000 polypeptide originating from postvitellogenic oocytes (a-vLvL A); (C) immunoblot using antiserum to the M_r 28,000 polypeptide originating from postvitellogenic oocytes (a-vLvL B). The major bands corresponding to M_r 107,000, 94,000, 42,000, 30,000, 28,000, 17,000, 92,000, 22,000, and 15,000 were labeled using their molecular sizes.

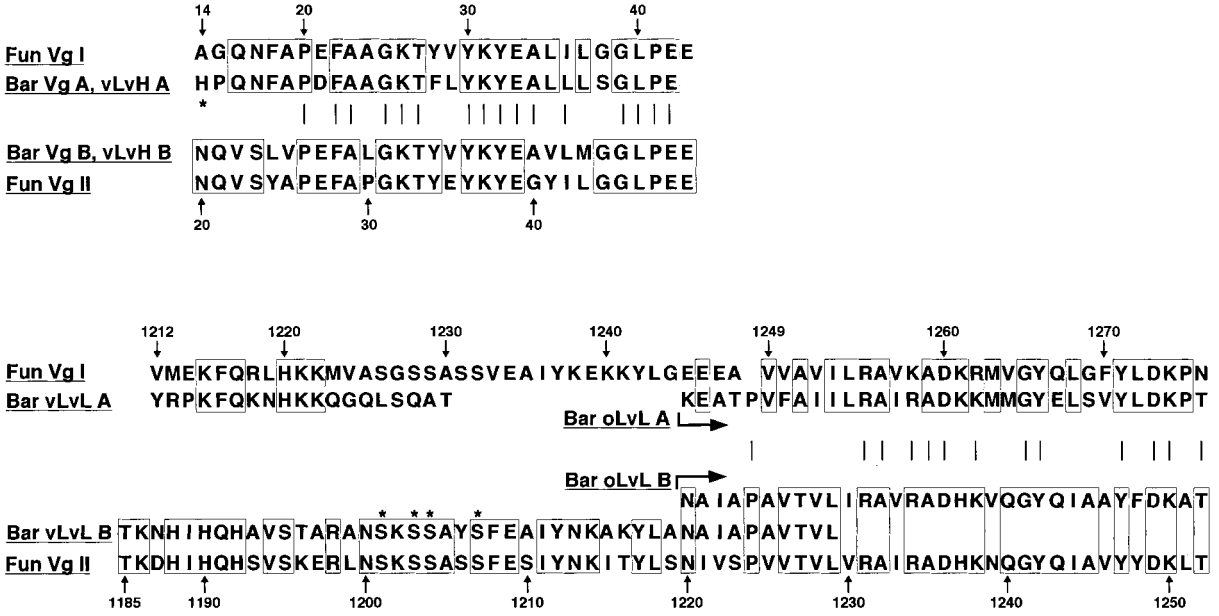

FIG. 7. N-terminal amino acid sequences of barfin flounder vitellogenins and lipovitellin heavy chains (top) and lipovitellin light chains (bottom) of postvitellogenic oocytes and ovulated eggs, aligned to corresponding amino acid sequences of *F. heteroclitus* vitellogenin I and II deduced from cDNA (LaFleur *et al.*, 1995a,b). Lipovitellin heavy and light chains are indicated using the same abbreviations used in the text. Ambiguities are indicated with asterisks. Bar, barfin flounder; Fun, *F. heteroclitus.*

to some smaller bands from postvitellogenic oocyte homogenates and also cross-reacted strongly to the M_r 15,000 bands from ovulated egg homogenate (Fig. 6C). These results clearly demonstrate that the M_r 22,000 and 15,000 polypeptides from ovulated eggs are proteolytically derived from the M_r 30,000 and 28,000 polypeptides, respectively, in postvitellogenic oocytes.

A further round of N-terminal amino acid sequence analysis was performed to identify whether the smaller yolk polypeptides represent Lv light chains. The amino acid sequences obtained are presented in Fig. 7 (bottom) along with a comparison of the corresponding amino acid sequences deduced from cDNA encoding the *F. heteroclitus* Vg I (LaFleur *et al.*, 1995b; GenBank Accession No. U07055) and Vg II (LaFleur *et al.*, 1995a; GenBank Accession No. U70826). Among 19 amino acid residues, there were no sequences identical between the N-termini of the M_r 30,000 and 28,000 polypeptides. However, 39% identity was observed between the N-termini of the M_r 22,000 and 15,000 polypeptides (13 positions of 33 residues). Considering the N-terminal alignment of the immunologically related polypeptide pairs (M_r 30,000–22,000 and M_r 28,000–15,000), their sequences were compared with and mapped to the deduced amino acid sequences of *Fundulus* Vg I and II. These comparisons showed that the M_r 30,000 and 22,000 polypeptides had significant similarities to *Fundulus* Vg I, aligning with it from amino acid positions 1212 and 1245, respectively. The M_r 28,000 and 15,000 polypeptides aligned best with *Fundulus* Vg II from amino acid positions 1185 and 1220, respectively. Identity between the available sequences shared by the M_r 30,000–22,000 polypeptides to *Fundulus* Vg I and II were 58% (47 of 81 residues) and 41% (33 of 81 residues), respectively. Corresponding values for the M_r 28,000–15,000 polypeptides were 45% (42 of 94 residues) and 70% (66 of 94 residues). These polypeptides aligned to regions of the *Fundulus* Vgs thought to give rise to Lv light chain (Lv_2) domains (LaFleur *et al.*, 1995b). Coupled with the results from 2D electrophoresis and Western blotting, the peptide sequence analysis suggested that the M_r 30,000, 28,000, 22,000, and 15,000 polypeptides represent Lv light chains of barfin flounder. These were designated vLvL A, vLvL B, oLvL A, and oLvL B, respectively.

The N-terminal amino acid sequence, KKILAPGLKD, of the M_r 42,000 polypeptide, which had strong immunoreactivity to a-vLvL A, was completely different from the N-terminal sequence of vLvL A or oLvL A (Fig. 7). The N-terminus of the M_r 42,000 polypeptide shared some identity (7 of 10 residues) with the stretch from position 1080 to 1089 of the amino acid sequence of rainbow trout Vg deduced from its cDNA (Mouchel *et al.*, 1996). This corresponds to the region just before the serine-rich (Pv) domain of trout Vg. Moreover, the purified M_r 42,000 polypeptide contained a sixfold higher level of phosphate (mol phosphate/mol protein) than vLvL A (data not shown), suggesting that it is a Pv–vLvL A complex.

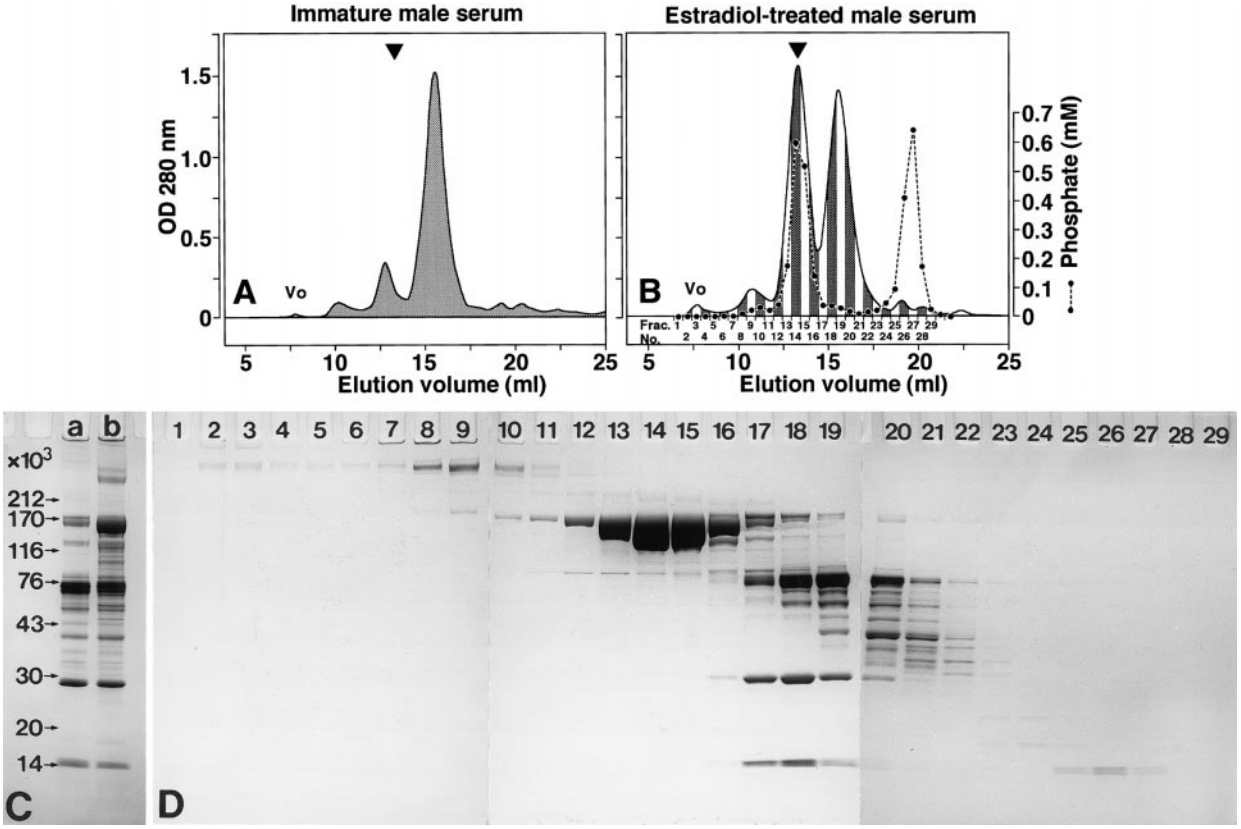

FIG. 8. Elution profiles of serum samples from immature male fish (A) and estradiol-17β-treated male fish (B) after Superose 6 gel chromatography; 5–20% gradient SDS–PAGE of the same samples from immature male fish, lane a, and estradiol-17β-treated male fish, lane b (C); and 5–20% gradient SDS–PAGE of chromatographic fractions generated using estradiol-17β-treated fish serum (D). Numbered fractions (0.5 ml each) are indicated by alternating bands. Closed circles with a dotted line in B indicate the phosphate concentration in each fraction. Closed arrowheads identify the position (M_r 520,000) of the vitellogenin peak. Lane numbers in the electrophoreogram shown in D correspond to fraction numbers in the chromatogram shown in B. Molecular weight values on the left in C indicate the migration positions of marker proteins.

Identification of Two Forms of Vg

Experiments were conducted to identify the specific precursor Vg molecules which give rise to the two forms of Lv in barfin flounder. The elution profiles in Superose 6 column chromatography of serum from immature and estradiol-17β (E_2)-treated male flounder are shown in Fig. 8. E_2-treated male serum produced a new UV-absorbing peak at an elution position corresponding to M_r 520,000 (Fig. 8B) and slightly retarded from the second major peak, excepting the peak of void volume, produced by immature male serum (Fig. 8A). At the same position as this E_2-induced UV-absorbing peak, a phosphorus-rich peak was observed when the fractions were subjected to a measurement of their phosphorus content (Fig. 8B). Thus this peak contained an E_2-induced, UV-absorbing phosphoprotein which, based on these characteristics, was assumed to be a barfin flounder Vg(s). E_2 injection induced the appearance in male flounder of serum protein(s) with approximate molecular weight 160,000–180,000 as estimated by gradient SDS–PAGE in the presence of 2-ME (Fig. 8C). When chromatographic fractions of E_2-treated male serum were individually subjected to SDS–PAGE, these bands were derived from the putative Vg-peak fractions (Fig. 8D).

To identify the precursor Vg molecules of vLvH A and B, immunoblotting analysis of the chromatographic fractions constituting the Vg peak (fractions 12–17 in Fig. 8) was performed (Fig. 9). Western blots using a-vLvH A and a-vLvH B demonstrated that both antisera strongly visualized the putative Vg band (Figs. 9B and 9C). However, the fractions most intensely immunostained by the two antisera differed, being about one fraction apart (fraction 14 versus 15), and the a-vLvH B-reactable polypeptide eluted earlier than the a-vLvH A-reactable one. Meanwhile, results of immunostaining with a-β'-c were the same as for CBB staining (Fig. 9D), indicating that the M_r 160,000–180,000 band consisted of two Vg polypeptides. Transverse arrange-

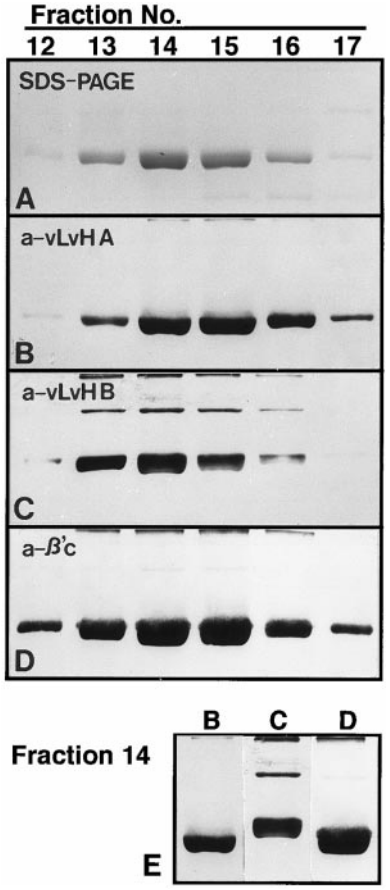

FIG. 9. SDS–PAGE (A) and immunoblots (B, C, D) of chromatographic fractions (12–17) shown in Fig. 8C collected from estradiol-17β-treated fish serum. Four identical sample sets were submitted to SDS–PAGE and Western blotting. (A) SDS–PAGE on a 7% gel stained with Coomassie brilliant blue; (B, C, and D) immunoblotting using antisera against the M_r 107,000 polypeptide (a-vLvH A), M_r 94,000 polypeptide (a-vLvH B), and β'-component (a-β'-c), respectively; (E) transverse arrangement of the lanes of fraction 14 from three separate gels shown in B, C, and D, to emphasize the contrast in relative band migrations.

ment of results (lanes) from the different types of staining done on fraction 14 made it apparent that the a-vLvH A-reactable band was a little more anodic compared to the a-vLvH B-reactable one (Fig. 9E). Figure 10 shows the results of Western blots of fractions 13 and 16 done using a-vLvL A and a-vLvL B to verify the identity of the parent Vg molecules based on differences in qualitative ratios of immunoreactivity between fractions. The immunostaining results clearly indicate that the major constituents of fraction 13 and 16 were a-vLvL B- versus a-vLvL A-reactable polypeptides, respectively, in accordance with those shown for the a-vLvHs in Fig. 9.

Highly diluted samples of the pooled Vg chromatography fractions electrophoresed in the presence of 2-ME on 3–10%

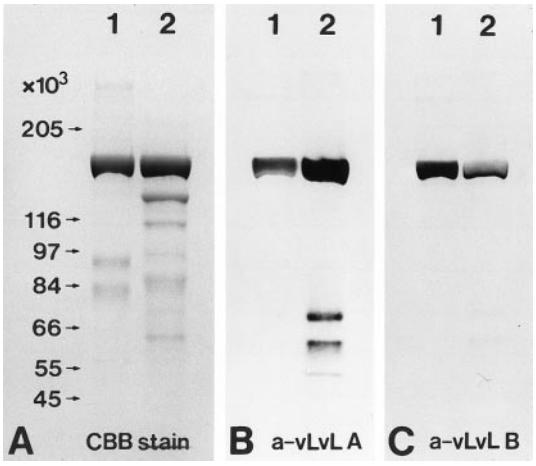

FIG. 10. SDS–PAGE (A) and immunoblots (B, C) of chromatographic fractions (No. 13, lane 1, and No. 16, lane 2) shown in Fig. 8C generated using estradiol-17β-treated fish serum. Three identical sample sets were submitted to SDS–PAGE and Western blotting. (A) SDS–PAGE on a 3–10% gradient gel stained with Coomassie brilliant blue; (B and C) immunoblotting using antisera against vLvL A (a-vLvL A) and vLvL B (a-vLvL B), respectively.

gradient SDS–PAGE gels and then subjected to silver staining yielded two Vg polypeptides having apparent molecular weights corresponding to 168,000 and 175,000 (Fig. 11, lane 1). The qualitative ratios of M_r 168,000 and 175,000 Vg polypeptides between fraction 13 and 16 were different (Fig. 11, lane 2 and 3), being in agreement with the results from immunoblottings (Figs. 9 and 10). Transblots of these M_r

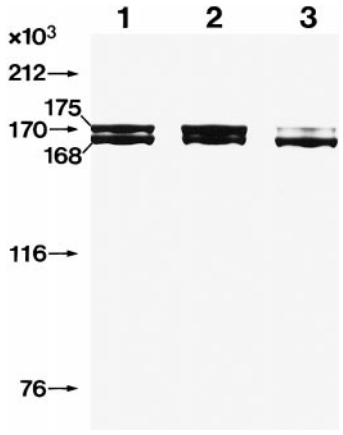

FIG. 11. SDS–PAGE of a 3–10% gradient gel of partially purified vitellogenin fractions at high dilution. Lane 1, pooled fractions 13–16; lane 2, fraction 13; lane 3, fraction 16 in Fig. 8C. The gel was stained with silver as described in the text. Molecular weight values on the left with arrows indicate the migration positions of marker proteins.

168,000 and 175,000 polypeptides on PVDF membranes after SDS–PAGE of fraction 13 and 16, respectively, were further subjected to N-terminal amino acid sequence analysis. The sequences obtained are listed in Fig. 7 (top) along with the corresponding deduced amino acid sequences of *Fundulus* Vg I (LaFleur *et al.,* 1995b; GenBank Accession No. U07055) and II (LaFleur *et al.,* 1995a; GenBank Accession No. U70826). The N-termini of the M_r 168,000 and 175,000 Vg polypeptides were identical to those of vLvH A and vLvH B, respectively, and so the two Vgs were designated Vg A and Vg B. Identity between Vg A and Vg B of their N-terminal amino acid sequences was 55% (16 of 29 residues). Higher identities were recognized in comparisons between Vg A and *Fundulus* Vg I (76%, 22 of 29 residues) and Vg B and *Fundulus* Vg II (73%, 22 of 30 residues).

DISCUSSION

The results of the present study clearly demonstrate the presence of two types of Vg molecules in barfin flounder which yield two different forms of Lv having distinct roles with respect to their manner of utilization during oocyte maturation and embryonic development. We previously demonstrated that Vg is proteolytically cleaved into the three classes of yolk proteins, Lv, Pv, and β'-c, during vitellogenesis in barfin flounder and that all three classes of yolk proteins undergo selective degradation during maturation-associated yolk proteolysis, a feature of final oocyte maturation in this species (Matsubara and Sawano, 1995). This process of yolk proteolysis involves disappearance of most Pv and β'-c and molecular alteration of native Lv molecules from M_r 410,000 to 170,000 with a partial loss in total mass. Thus, ovulated eggs mostly contain a single class of yolk protein, Lv, and a considerable amount of free amino acids derived from Lv and the other yolk proteins during oocyte maturation (Matsubara and Koya, 1997).

Using combinations of gel chromatography and SDS–PAGE in the present study, Lv in postvitellogenic oocytes was verified to be composed of plural components in general. In SDS–PAGE, a nonsymmetrical UV-absorbing chromatography peak of Lv yielded two closely parallel major bands including a mixed M_r 107,000 and 102,000 band and a distinct M_r 94,000 band, as well as two definite minor bands of M_r 30,000 and 28,000. The results from N-terminal amino acid sequencing coupled with a comparison of the sequences to the amino acid sequences deduced from *Fundulus* Vg I (LaFleur *et al.,* 1995b; GenBank Accession No. U07055) and II (LaFleur *et al.,* 1995a; GenBank Accession No. U70826) cDNA indicated that the three major polypeptides aligned to the N-terminal Lv heavy-chain domain of Vg, whereas the two minor polypeptides were positioned in the Lv light-chain domain of the Vg molecule. The observations that both major and minor polypeptides had similar but distinct N-termini, coupled with the finding that there was no immunological cross-reactivity between either the two major bands or the two minor bands, suggested the presence of two forms of Lv molecules in postvitellogenic oocytes. The M_r 107,000, 94,000 and 102,000 polypeptides in postvitellogenic oocytes of barfin flounder were thus identified as Lv heavy chains, designated vLvH A, vLvH B, and vLvH B′, respectively, whereas the M_r 30,000 and 28,000 polypeptides were identified as Lv light chains, namely vLvL A and vLvL B, respectively. The possible combinations of heavy and light chains within Vg molecules were considered to be vLvH A–vLvL A and vLvH B (or B′)–vLvL B. Considering its native molecular weight of about 410,000 with a lipid content of approximately 20.7% by weight (Matsubara and Sawano, 1995), the native vLv of barfin flounder likely has dimeric structure, consisting of two sets of heavy- and light-chain complexes. In the present study, it was not clarified whether the native Lv molecules involve randomly combined forms of the two Lv monomers. Further work is needed to resolve the connection between the Lv molecules and the structure of their native parent Vg molecules.

All of the distinct polypeptides comprising vLvHs and vLvLs that were observed in postvitellogenic oocytes disappeared during oocyte maturation. The native M_r 170,000 Lv seen in ovulated eggs yielded two other polypeptides (M_r 92,000 and 15,000) in SDS–PAGE. Immunoblotting analysis using both a-vLvHs (A and B) demonstrated that, whereas the M_r 92,000 polypeptide possesses common antigenicity to vLvH B, there is no polypeptide present in ovulated eggs which immunostains with a-vLvH A. This result suggests that the M_r 92,000 polypeptide in ovulated eggs is oLvH B, originating from the M_r 94,000 vLvH B polypeptide and/or the M_r 102,000 vLvH B′ polypeptide. N-terminal amino acid sequencing defined a proteolytic removal of 4 amino acid residues from the N-terminus during this change from vLvH B to oLvH B. The M_r 15,000 polypeptide was a constituent of oLv B derived from vLvL B as shown by the results of 2D electrophoresis and immunoblotting. As in the case of *X. laevis* Lv light chains (Wallace *et al.,* 1990), N-terminal amino acid sequencing effectively distinguished the M_r 22,000 and 15,000 polypeptides from one another and aligned them with corresponding Lv light-chain domains deduced from cDNAs of *Fundulus* Vg I and II (LaFleur *et al.,* 1995a,b). Accordingly, the M_r 22,000 and 15,000 polypeptides most likely represent barfin flounder Lv light chains and were designated oLvL A and oLvL B, respectively. The complete identity of 10 amino acid sequences of vLvL B over 36–45 residues from the N-terminus of oLvL B clearly demonstrated the N-terminal proteolytic truncation of vLvL B to oLvL B. The combined molecular weight of oLvH B and oLvL B shows good agreement with that of the peptide part of native Lv (M_r 170,000) after subtraction of 25.7% of its mass to account for the lipid fraction (Matsubara and Sawano, 1995). Native Lv in ovulated eggs is thus suggested to consist of oLvH B plus oLvL B.

A native protein which contained oLvL A eluted at a position of M_r 330,000 in gel filtration chromatography as a shoulder just before the main Lv peak (Fig. 2B). The par-

tially purified M_r 330,000 protein fraction contained more than 54% lipids by weight with a lipid composition similar to that of Lvs (Matsubara and Ohkubo, unpublished data). Therefore, the M_r 330,000 protein seems to be a novel, highly lipidated yolk protein which is reconstructed from oLvL A and vLv-bound lipids. A similar protein fraction was discovered in winter flounder, *Pleuronectis americanus*, and designated heat-labile Lv based on its instability during heat treatment (Hartling *et al.*, 1997).

Based on the existence of two forms of Lv molecules, we predicted involvement of two forms of parent Vg molecules in vitellogenesis and oocyte growth of barfin flounder. Gel chromatography of serum from E_2-treated fish revealed a symmetrical Vg peak, which appeared at a position of M_r 520,000, showing no evidence of the existence of two forms of Vg molecules. However, immunoblotting analysis of the Vg fractions using a-vLvH A, a-vLvH B, a-vLvL A, and a-vLvL B gave rise to discrepancies in stainability of the various fractions to each antiserum and suggested the presence of two forms Vg. The estimated molecular weights of the two forms of Vg polypeptides, Vg A and Vg B, were 168,000 and 175,000, respectively. Information generated by immunoblotting analyses of gel chromatography fractions allowed tentative estimation of the native molecular weights of Vg A and Vg B, which were approximated as 500,000–520,000 and 530,000–550,000, respectively. These values show good agreement with the estimated molecular weights of native Vg in other flatfish, 550,000 in *Platichtys flesus* (Emmersen and Petersen, 1976) and 530,000 in *Scophthalmus maximus* (Silversand and Haux, 1989). Both native Vg molecules from barfin flounder seem likely to be dimers considering their lipid contents of 18.7% by weight (Matsubara and Sawano, 1995).

Heretofore, multiple forms of Vg proteins have been discovered in the chicken, Vg I, II, and III (Wang and Williams, 1980; Wang *et al.*, 1983). In *X. laevis*, three Vg polypeptides (M_r 182,000, 188,000 and 197,000) and four Vg genes (A1, A2, B1, and B2) have been discovered (Wiley and Wallace, 1978; Wahli *et al.*, 1979; Germond *et al.*, 1984). In teleosts, the presence of two forms of Vg has been verified in two tilapia species, *Oreochromis auratus* (Ding *et al.*, 1989) and *O. mosambicus* (Kishida and Specker, 1993), and in the mummichog, *F. heteroclitus* (LaFleur *et al.*, 1995a). The tilapia Vg polypeptides have distinctly different molecular weights of 180,000 and 130,000 in *O. auratus* (Ding *et al.*, 1989) and 200,000 and 130,000 in *O. mosambicus* (Kishida and Specker, 1993). A unique feature of the smaller tilapia Vg is thought to be a deletion of the Pv domain in its gene, based on its very low content of phosphorus (Kishida and Specker, 1993). On the other hand, the two Vg polypeptides appear to have very similar molecular weights in barfin flounder, 175,000 and 168,000. Both Vg A and Vg B of barfin flounder likely give rise to distinct forms of Lv, Pv, and β'-c. This expectation is strongly supported by our finding of two different N-termini for all three classes of yolk proteins in postvitellogenic oocytes. The amino acid sequences of Pv were KKILAPGLKDGSLSSSSS and KKILVPGLKDNTSSSSS, and those of β'-c were AKAGAAECFARDTLTTFNNRKYRTEF and TKAHAAECTMTKDTVVTFNNRKYKNEM, respectively (T. Matsubara, unpublished data). The N-termini of the β'-c class of yolk proteins aligned with residues starting from amino acid position 1437 of *Fundulus* Vg I and 1416 of *Fundulus* Vg II. These positions appear to sequentially follow the LvL (Lv_2) domain of the Vg gene and are near the C-terminal end of Vg cDNAs. In *X. laevis*, it has been pointed out that the C-terminal end of Vg, the cysteine-rich region, is not accounted for by any known yolk protein (Gerber-Huber *et al.*, 1987; Wallace *et al.*, 1990). Recently, Yamamura *et al.* (1995) isolated a novel yolk glycoprotein of M_r 40,000 (YGP40) from the β-livetin fraction of chicken yolk plasma and identified it as a C-terminal, cysteine-rich fragment of Vg II. The discovery of YGP40 in the chicken and β'-c in some teleost fish leads us to expect cysteine-rich yolk polypeptides to be present in other oviparous vertebrates as well. Although it has not been verified from which parent Vg polypeptides the Pvs and β'-cs of barfin flounder are derived, the present data support the concept that two forms in all three classes of yolk proteins are present in vitellogenic oocytes of this species. Based on these results coupled with those from our previous reports (Matsubara and Sawano, 1995; Matsubara and Koya, 1997), we have constructed a flow chart which describes the detailed molecular alterations of the two forms of Vg and three classes of yolk proteins during vitellogenesis and oocyte maturation for barfin flounder (Fig. 12).

The free amino acid content of barfin flounder oocytes drastically increases during the time of oocyte maturation, showing a temporal correlation with degradation of the yolk polypeptides (Matsubara and Koya, 1997). Yolk protein proteolysis to free amino acids is thought to create a major part of the osmotic potential needed for oocyte hydration in plaice, *Pleuronectes platessa*, and lemon sole, *Microstomus kitt* (Thorsen and Fyhn, 1996). Although involvements of other possible osmotic mechanisms for water influx into the maturating oocytes, such as Na^+,K^+-ATPase in Atlantic croaker *Micropogonias undulatus* and spotted seatrout *Cynoscion nebulosus* (LaFleur and Thomas, 1991), translocation of inorganic cations from follicle cells to the oocytes (Wallace *et al.*, 1992) via heterologous gap junctions (Cerdá *et al.*, 1993) in *F. heteroclitus*, and phosphorus metabolism (Craik and Harvey, 1986), are still unclear in barfin flounder, a significant portion of the water influx is explainable by the osmotic pressure of increased free amino acids (Matsubara and Koya, 1997). Moreover, it has been suggested that the free amino acids are consumed as major substrates for aerobic energy production and material for protein synthesis in developing embryos and larvae of marine pelagic egg spawners (Fyhn and Serigstad, 1987; Rønnestad and Fyhn, 1993; Rønnestad *et al.*, 1992, 1993; Finn *et al.*, 1995). Contents of free amino acids and Lv in ovulated eggs were almost constant among barfin flounder females (Matsubara and Koya, 1997). Constancy in free amino acid content was also observed in ovulated eggs of

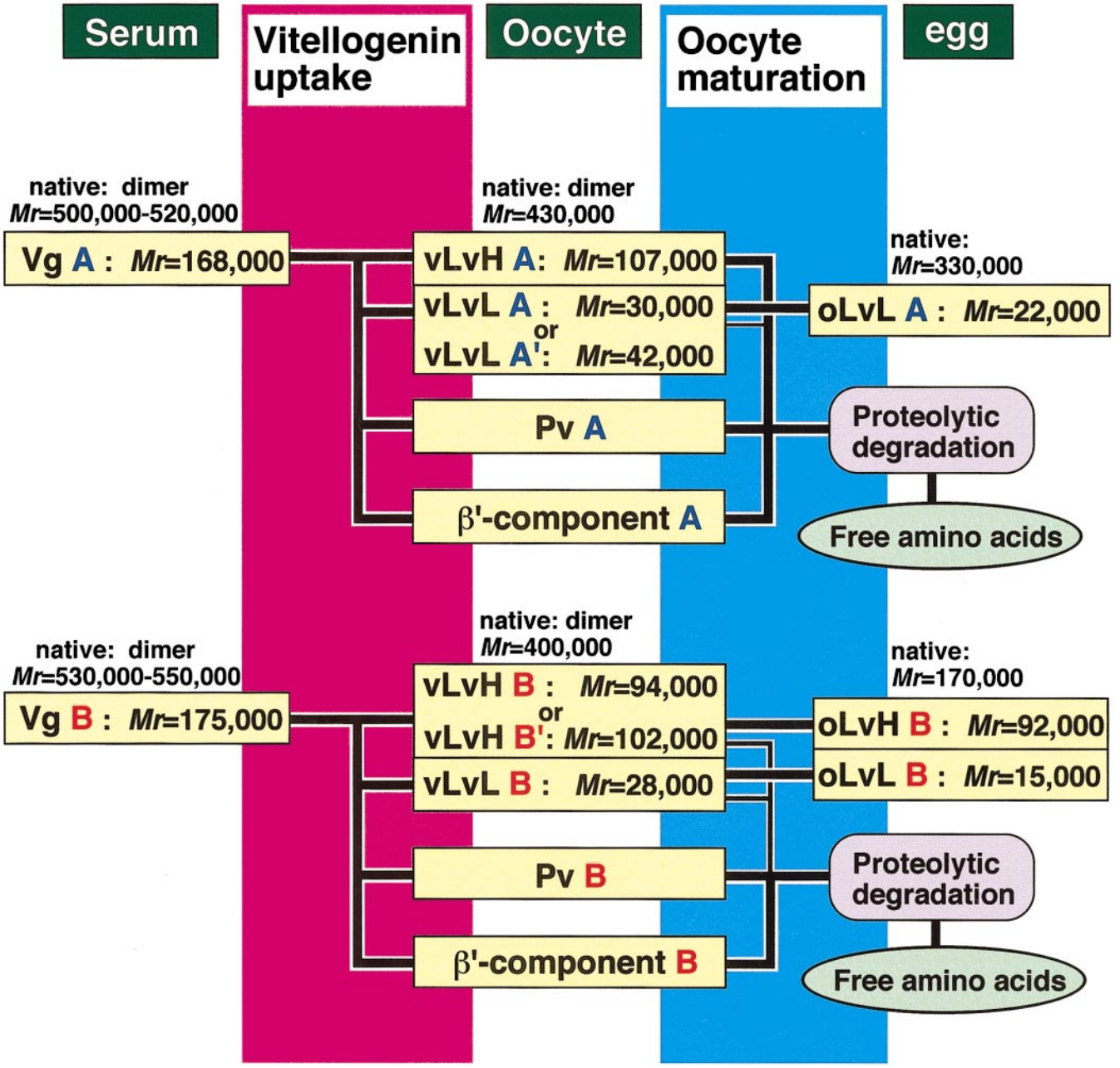

FIG. 12. Schematic drawing of the flow chart describing molecular alterations of the two forms of vitellogenin and vitellogenin-derived yolk polypeptides during vitellogenesis and oocyte maturation in barfin flounder.

other marine teleosts which lay pelagic eggs (Thorsen and Fyhn, 1996; Thorsen *et al.*, 1996). This phenomenon seems to be a very important factor for keeping the amount of major osmotic effectors at a specific level, so as to adjust the specific gravity of spawned eggs at a constant level with respect to the environmental seawater. After oocyte maturation in barfin flounder, vLvH A seems to be completely degraded into free amino acids; however, vLvH B remains as a major yolk protein reserve of M_r 170,000. Estimated proportional ratios of the completely degraded part of the polypeptides were 84% of vLvH A–vLvL A and 12–18% of vLvH B or B′–vLvL B, respectively (Fig. 12). Thus, there is a possibility that the quantitative ratio of vLv A to vLv B in postvitellogenic oocytes regulates the buoyancy of spawned pelagic eggs by controlling the quantity of free amino acids present during oocyte hydration. The Lv A/Lv B ratio likely

also controls the proportional availability of different types of nutrients, free amino acids versus Lv, for use during early embryonic development. In fact, our recent experiments on purification and immunological measurement of native vLv A and vLv B in postvitellogenic oocytes demonstrated the constancy in quantitative ratio of vLv A:vLv B; an approximately 4:6 ratio is observed (Matsubara and Ohkubo, unpublished data).

Thorsen *et al.* (1996) elegantly demonstrated physiological mechanisms for adjusting egg buoyancy to neutral with respect to the specific gravity of surrounding seawater of the spawning habitat by comparing eggs from different populations of Atlantic cod, *Gadus morhua.* These cod spawn eggs in brackish water at a salinity of 1.43% and also in the marine environment with a salinity of 3.30%. Thorsen *et al.* suggested that one of the most important causes for the increased hydration of eggs spawned in brackish water was their higher content of free amino acids. The much higher ratio of free amino acids to protein content observed in brackish-water eggs led these investigators to formulate a hypothesis that an increased share of hydrolyzed protein contributes to the establishment of neutral egg buoyancy in low salinity water, and the ability to hydrolyze increased quantities of yolk protein may be a genetically acquired factor (Thorsen *et al.*, 1996). Although it is still uncertain whether the dual Vg system is involved in regulating specific buoyancy in other marine, pelagic egg spawners, our model for barfin flounder possibly explains acute regulation of free amino acid generation by controlling the ratio of Vg A versus Vg B accumulated by the oocytes through the period of vitellogenesis. Further investigations will be necessary to clarify mechanisms for controlled accumulation of the two forms of Vg into growing oocytes.

ACKNOWLEDGMENTS

The authors thank Dr. S. Adachi, Faculty of Fisheries, Hokkaido University, for useful advice. We also thank A. Nishiki, K. Watanabe, and the other staff of Japan Sea-Farming Association, Akkeshi Station, Hokkaido, for their kind help and supply of the barfin flounder. This work was supported by a grant (BDP-98-IV-2-13) from the Council Secretariat Ministry of Agriculture, Forestry, and Fisheries, Japan.

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Received for publication February 9, 1999
Revised May 17, 1999
Accepted June 6, 1999