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Characterization of Vitellogenin from White Sturgeon, *Acipenser transmontanus*

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Abstract. Sturgeon are an ancient family (Acipenseridae) of fishes that lie close to the divergence of fish that eventually evolved into terrestrial animals and those that evolved into modern teleost species. Therefore, white sturgeon vitellogenin sequences fill a gap in the current understanding of the functional domains of this protein family. Vitellogenin cDNA was sequenced and used to investigate gene expression in white sturgeon, *Acipenser transmontanus*. Estrogen-induced vitellogenin mRNA was detected in the livers of both males and females and was also detected in undifferentiated gonads of estrogen-treated fish. Low levels of vitellogenin mRNA were also detected in the testis of both control and estrogen-treated males. The cDNA encoded a 186-kDa protein that was missing only six to seven of the amino-terminal amino acids. Comparisons to silver lamprey, *Xenopus,* and chicken vitellogenin sequences indicate that the overall structure of the yolk protein domains were highly conserved. There was considerable homology in three regions of the lipovitellin I domain. These conserved sequences are likely to be involved in vitellogenin receptor binding. The phosvitin domain of white sturgeon vitellogenin contained fewer and shorter serine repeats as predicted from yolk protein phosphate content of fish compared to *Xenopus* and chicken. However, the vitellogenin of white sturgeon had a lower serine content as compared with silver lamprey, indicating that an increased serine content is not strictly a function of evolutionary age.

Key words: Sturgeon — Vitellogenin — cDNA — Lipovitellin — Phosvitin

Introduction

Vitellogenins have a critical function as precursors for egg-yolk proteins that are sources of nutrients for oviparous species during early development. The organization of the vitellogenin genes and the relationship of the yolk proteins to their precursors have been extensively studied in *Xenopus laevis* and in the chicken, *Gallus domesticus*. The complete amino acid sequences of *Xenopus* and chicken vitellogenins have been determined from genomic DNA sequences (Gerber-Huber et al. 1987; van het Schip et al. 1987). More recently a complete cDNA sequence has been reported for silver lamprey, *Ichthyomyzon unicuspis* (Sharrock et al. 1992) vitellogenin. Fishes are the largest class of oviparous vertebrates and there has been little molecular analysis of their egg-yolk proteins. We have initiated studies of white sturgeon, *Acipenser transmontanus,* vitellogenin due to the sturgeon's potential for aquaculture and to their importance in fish evolution.

Sturgeon are living fossils and are unique among the 30,000 ray-finned species in existence today. They are descendants of the actinopterygians (the ray-finned bony fishes) whose evolutionary history resulted in three radiations. The most primitive was the chondrosteans, fol-

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lowed by holosteans, and finally teleosteans, which produced the greatest diversity of vertebrate species that ever evolved (Radinsky 1987). The extant sturgeon species made their first appearance about 70 million years ago and are somewhat modified forms of the primitive chondrosteans (Radinsky 1987). Sturgeon fill a prominent gap in the study of vertebrate vitellogenins between the most primitive vertebrates (silver lamprey) and the terrestrial vertebrates (amphibians and avians).

Vitellogenins and yolk proteins have been purified from several fish species (for review see Mommsen and Walsh 1988; Ng and Idler 1983) but the amino acid sequence of a teleost fish's vitellogenins and the relationship to their yolk proteins have not been determined. Partial-length cDNAs from rainbow trout (*Oncorhynchus mykiss;* Le Guellec et al. 1988) and tilapia (*Oreochromis aureus;* Ding et al. 1990) have been sequenced. Recently, a full-length cDNA sequence of a modern teleost, *Fundulus heteroclitus,* has been completed (G. LaFleur, personal communication). Cross-hybridization of the tilapia cDNA was demonstrated with species representing four families within the suborder Percoidei (Lee et al. 1992). Rainbow trout were used in hybridization experiments to demonstrate the memory effect of estrogen induction of vitellogenin gene transcription in teleosts (Le Guellec et al. 1988). The effects of various steroids on vitellogenin gene transcription in trout hepatocytes were directly assayed by cDNA hybridization (Vaillant et al. 1988).

Materials and Methods

RNA Isolation. Estrogen treatment of white sturgeon and the preparation of the RNA used for cDNA cloning and northern blot analysis are described in Bidwell et al. (1991). Total RNA was also isolated from the liver, heart, brain, spleen, and gonads of six estrogen-treated and six control 2-year-old white sturgeon. Liver samples were processed separately by individual and the other tissues were pooled in estrogen-treated and control groups. Total RNA was isolated from an additional group of two male and two female 4-year-old white sturgeon for northern blot analysis of tissue-specific vitellogenin mRNA expression. All tissues were processed separately by individual.

Complementary DNA Synthesis. Complementary DNA was made by the Gubler and Hoffman (1983) method using a commercial kit (Life Technologies Inc., Gaithersburg, MD) and the murine Moloney leukemia virus reverse transcriptase. T4 DNA polymerase was used to make the cDNA blunt ended. *Eco*RI linkers (λgt11 linkers: Boehringer Mannheim Biochemicals, Indianapolis, IN) were ligated to the cDNA to produce cohesive ends. The excess linkers were removed using a P30 Biospin column (BioRad Laboratories, Hercules, CA) and the linker ends were phosphorylated in 66 mM Tris-HCl pH 7.6, 10 mM $MgCl_2$, 10 mM DTT, 1 mM ATP, 1 mM spermidine-HCl (Wu et al. 1987). Sixty units of T4 polynucleotide kinase (United States Biochemicals Inc., Cleveland, OH) were added for each microgram of cDNA. The kinase reactions were incubated for 30 min at 37°C, extracted with phenol:chloroform:isoamyl alcohol (25:24:1), and ethanol precipitated.

Vitellogenin cDNA Fractionation. In order to increase the proportion of full-length vitellogenin cDNA over partial cDNAs for the screening process, high-molecular-weight cDNA was isolated by electrophoresis through 0.8% low-melting-point agarose (NuSeive GTG, FMC Bioproducts, Rockland, ME). DNA in the size range of 5–7 kb was excised and placed in a preweighed microcentrifuge tube. An "in gel" ligation technique was used to ligate the cDNA to an *Eco*RI site of Bluescript KS+ (Stratagene, La Jolla, CA). Ten microliters of melted agarose was added to 20 ng of vector and adjusted to 66 mM Tris-HCl, pH 7.6, 10 mM $MgCl_2$, 10 mM DTT, 0.4 mM ATP, 1 mM spermidine HCl, 200 mg/ml BSA, in 20 μl. One unit of T4 DNA ligase was added and the reaction was incubated at 14°C overnight (Wu et al. 1987) and *E. coli* strain DH5α transformed by the method of Hanahan (1983).

Northern Blot Analysis. Formaldehyde agarose gel electrophoresis was used for separation of total RNA for northern blotting. Each RNA sample was denatured in 50 mM HEPES pH 7.8 (hydroxyethylpiperazine-ethanesulfonic acid), 1 mM EDTA, 50% formamide, and 2% formaldehyde by heating at 65°C for 5 min. A denaturing gel of 50 mM HEPES pH 7.8, 1 mM EDTA, and 6% formaldehyde was electrophoresed for 6 h at 45 mV. After electrophoresis, the gel was soaked for 30 min in 10 × SSC (1.5 M NaCl, 0.15 M sodium citrate). Northern blots were prepared by transfer of RNA to Nytran (Schleicher & Schuell, Keene, NH) by capillary action and vacuum baked at 80°C as described for Southern blotting by Manniatis et al. (1982).

Prehybridization was carried out for 3–5 h in hybridization solution (HY) consisting of 50% formamide, 0.9 M NaCl, 0.09 M sodium citrate, 0.05% Ficoll, 0.05% polyvinylpyrolidone, 0.05% BSA, 0.5% SDS, 0.1% sodium pyrophosphate, 10 mM EDTA, and 100 μg/ml sonicated calf thymus DNA. The cDNA probes were labeled with [α-^{32}P]dCTP using the random priming method of Feinberg and Vogelstein (1983, 1984). Unincorporated nucleotide was removed using a P30 Biospin column (BioRad Laboratories) and 5×10^5 cpm of probe was added per ml of HY. Northern blots were hybridized 18 h at 42°C and then washed once in 2 × SSC, 0.5% SDS at room temperature for 30 min, followed by two washes in 1 × SSC, 0.1% SDS at 65°C for 20 min and a final wash in 0.2 × SSC, 0.1% SDS at 65°C for 20 min. The membranes were allowed to air dry and then exposed to Kodak XAR-5 film for 12–48 h with intensifying screens.

DNA Sequencing. The complete sequence of the plasmid pSLVG 1019 was determined by sequencing both strands of the cDNA using a set of directional deletions from both ends of the insert. The nested deletions were created using the Exonuclease III/Mung Bean nuclease method described by Steggles (1989). Fresh alkali lysate minipreps were prepared from DH5α cells containing SLVG 1019 by the method of Birnboim and Doly (1979). For deletions of the Bluescript (+) strand, the plasmid was cut with *Xba*I and *Sac*I. For deletions of the (−) strand the plasmid was cut at the *Xho*I site and the 5′ overhang filled in with α-phosphorothioate dNTPs. The plasmid was then cut at the *Cla*I site. The religated plasmids were transformed into DH5α cells by the method of Hanahan (1983). Supercoiled plasmid DNA was isolated from several transformed colonies of each deletion time point using the rapid boiling technique of Holmes and Quigley (1981). The plasmids were prepared for sequencing by denaturing the supercoiled DNA in 0.2 N NaOH for 5 min at room temperature and then neutralizing with ammonium acetate (Wang 1988). The denatured plasmids were concentrated by precipitation with ethanol. Denatured double-stranded DNA was sequenced using Sequenase 2.0 and the manufacturer's protocol (United States Biochemicals Inc.).

Sequence Analysis. The sequence data was assembled and analyzed using the Genetics Computer Group Sequence sequence analysis software version 7.2 (Madison, WI). The silver lamprey vitellogenin cDNA sequence (Accession No. M88749; Sharrock et al. 1992), *Xenopus* vitellogenin A2 gene sequence (Accession No. Y00345; Gerber-Huber et al. 1987), and chicken vitellogenin II gene sequence (Accession No. M18060; van het Schip et al. 1987) were retrieved from the Genbank

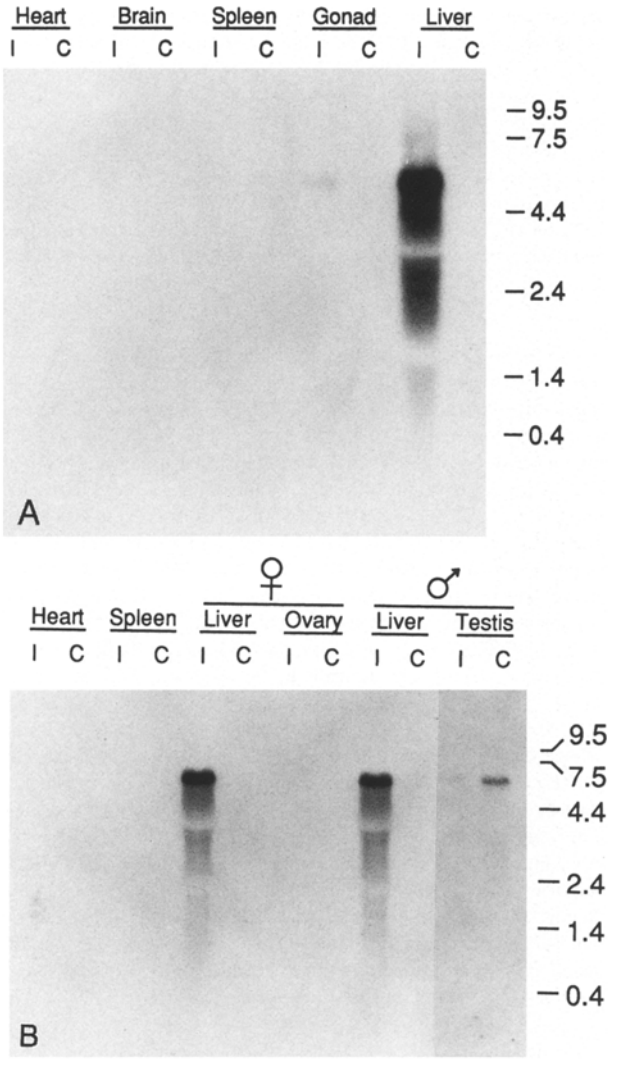

Fig. 1. Northern blot analysis of tissues from estrogen-treated and control white sturgeon. An autoradiograph of a northern blot of 10 μg of total RNA from the indicated tissues (*I* = estrogen-treated, *C* = control) from **(A)** 2-year-old white sturgeon and **(B)** 4-year-old white sturgeon.

data base and the protein sequence was created using the GCG TRANSLATE program. The amino acid sequences were compared using the GCG COMPARE and DOTPLOT program with a four-word comparison window as well as the multiple sequence alignment program PILEUP. Calculation of amino acid similarity and identity were determined with a gap weight of 3.0 and a gap length weight of 0.1.

Results

Vitellogenin mRNA Expression

Excision of high-molecular-weight cDNA from low-melting-point agarose and "in gel" ligations were used due to limiting amounts of cDNA. One hundred twenty recombinants were screened and three clones were isolated that contained 5.5-kb inserts with identical restriction enzyme patterns. The entire 5.5-kb insert of SLVG 1019 was used as a probe for a northern blot and was found to hybridize to the 5.7-kb vitellogenin mRNA (Bidwell et al. 1991) from livers of estrogen-treated white sturgeon but did not hybridize to RNA from control liver or to RNA from spleen or brain from estrogen-treated and control white sturgeon (Fig. 1A,B). The vitellogenin cDNA probe also detected the induction of vitellogenin mRNA in undifferentiated gonad of estrogen-treated 2-year-old white sturgeon (Fig. 1A). In 4-year-old sexually differentiated white sturgeon, no vitellogenin mRNA was detected in the ovary, but after longer exposure of the northern blot, low levels were detected in the testes of both estrogen-treated and control males (Fig. 1B). The hybridization signal in the control testis was more prominent than in the testis of estrogen-treated fish.

Table 1. Amino acid composition of the four vertebrate vitellogenins

Amino acid	Mole percent of each amino acid			
	Sturgeon[a]	Chicken	*Xenopus*	Silver lamprey
A	8.825	7.289	8.080	8.722
C	1.491	1.944	1.716	1.426
D	3.220	4.374	3.818	3.840
E	6.202	5.508	6.419	4.937
F	2.922	2.754	3.597	2.962
G	4.413	4.698	4.870	4.937
H	2.385	2.484	2.933	2.578
I	6.202	5.508	5.202	4.443
K	7.513	6.695	7.692	6.747
L	8.468	7.883	7.692	8.832
M	2.922	2.484	2.601	2.414
N	4.830	4.104	4.759	3.182
P	3.936	4.806	4.151	4.608
Q	6.500	4.158	5.811	5.760
R	5.247	5.832	4.981	5.595
S	10.853	13.877	11.068	14.427
T	5.784	4.968	4.870	4.334
V	5.546	6.695	6.143	6.912
W	0.477	0.972	0.719	0.768
Y	2.266	2.970	2.878	2.578
Residues	1,677	1,852	1,807	1,823
Size (kDa)	186[b]	205	202	200

[a] Based on the cDNA sequence of pSLVG 1019
[b] The sturgeon sequence is missing 6–7 amino terminal amino acids

Vitellogenin cDNA Sequence Analysis

Plasmid SLVG 1019 has a 5,455-bp cDNA insert (accession No. U00455) that contains an open reading frame of 1,677 amino acids with a predicted molecular weight of 186 kDa (Table 1). The amino acid composition was typical for vertebrate vitellogenins containing all 20 amino acids with a high serine content and a lower amount of aromatic and glycine residues (Byrne et al. 1989). The white sturgeon vitellogenin serine content

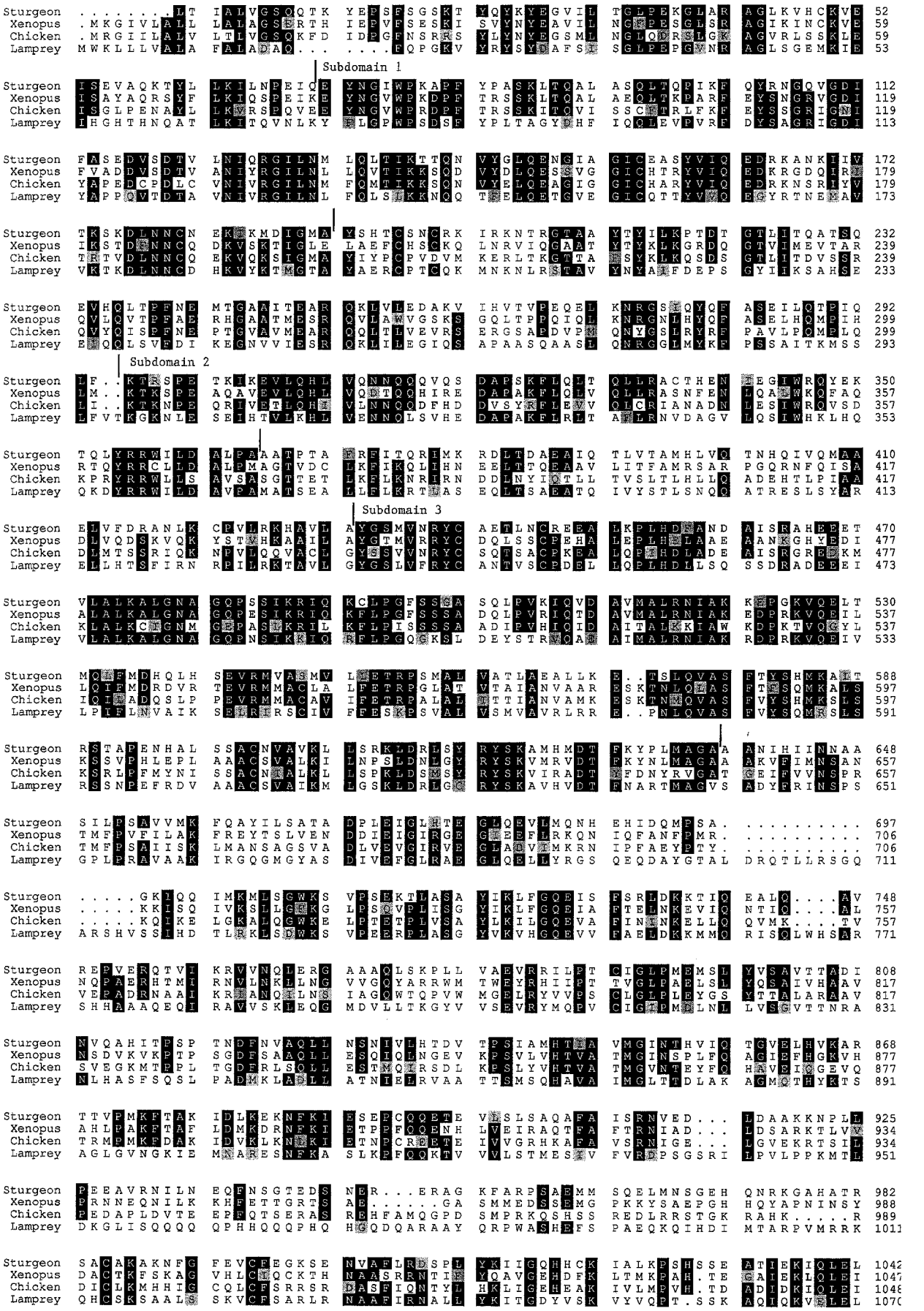

Fig. 2. Amino acid sequence alignments of white sturgeon, *Xenopus,* chicken, and silver lamprey vitellogenins. Identical residues are indicated in *black reverse print* and conservative amino acid changes are indicated by *gray shading.*

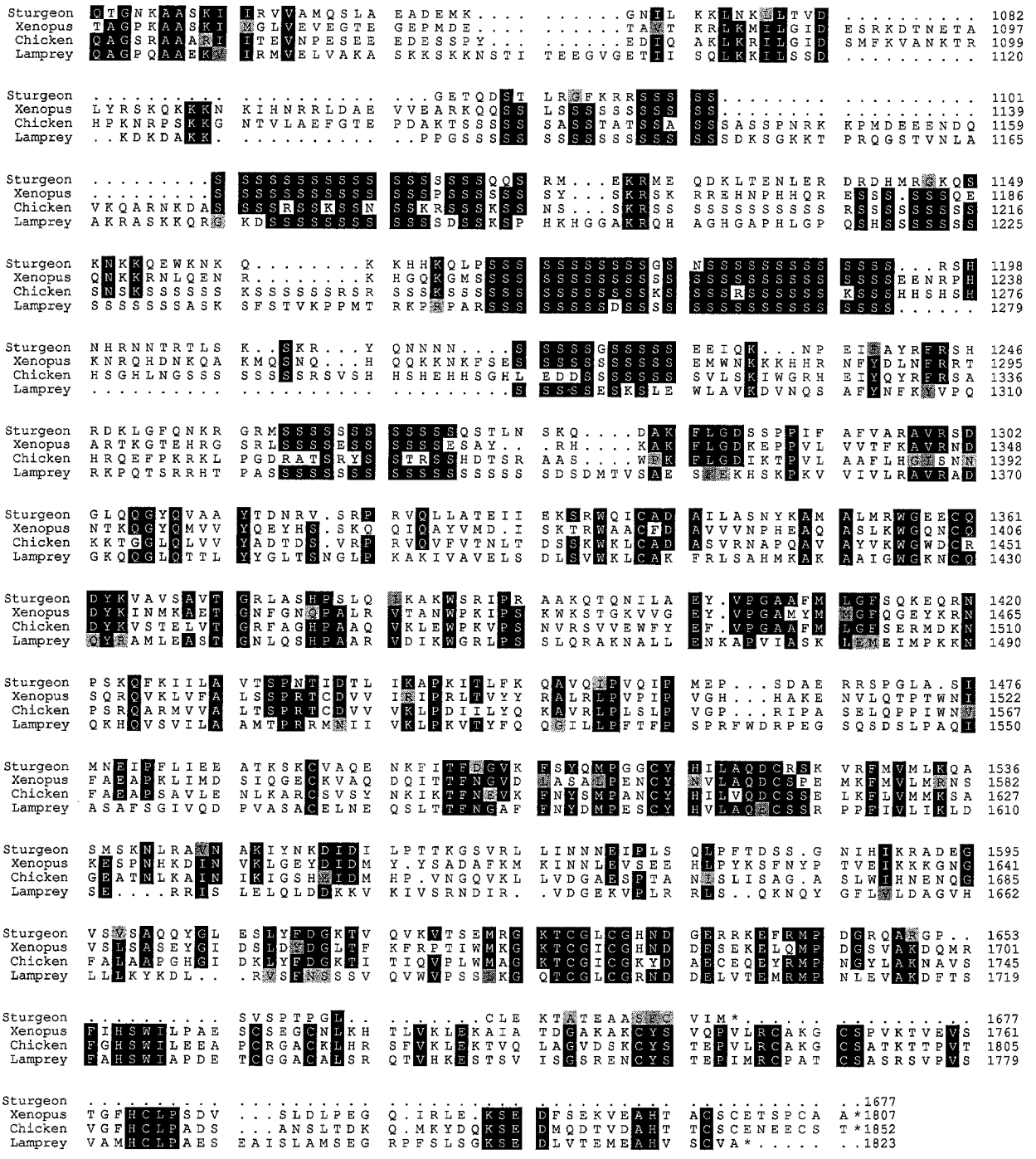

Fig. 2. Continued.

was nearly equal to *Xenopus* but was about 4% less than chicken and silver lamprey.

A multiple sequence alignment of the four derived amino acid sequences (Fig. 2) shows that the proteins can be aligned along their entire length, indicating the overall structure of white sturgeon vitellogenin is similar to chicken, *Xenopus,* and silver lamprey vitellogenins. The serine-rich phosvitin domain lies between the lipovitellin I domain at the amino terminus and the lipovitellin II domain at the carboxyl terminus. The white sturgeon sequence was missing six to seven of the amino-terminal residues that are part of the signal peptide and the white sturgeon lipovitellin I domain was 43 amino acid residues shorter than the silver lamprey and 33 residues shorter than both *Xenopus* and chicken. The first 948 residues of the lipovitellin I domain of chicken and *Xenopus* align with one insertion of two amino acids relative to white sturgeon and silver lamprey. The lamprey lipovitellin I domain contains one amino terminal gap (residue 117 relative to white sturgeon) in the first 697 residues and five inserted sequences of 3–16 amino acids relative to white sturgeon *Xenopus,* and chicken in residues 698 – 1,082.

White sturgeon vitellogenin had greater amino acid

similarity and identity to *Xenopus* than chicken or lamprey. The most highly conserved single amino acids between the four species were the 12 cysteine residues found in the lipovitellin domains as well as the positively charged lysine, arginine, and histidine residues. The similarity and identity of each of the three egg yolk protein domains were determined separately. The lipovitellin I domain was more conserved than lipovitellin II, and the phosvitin domain was the least-conserved domain. The white sturgeon lipovitellin I domain (residues 1–1,082 relative to sturgeon) had 67–66% similarity and 48–43% identity to *Xenopus* and chicken lipovitellin I. Silver lamprey lipovitellin I had 54% similarity and 33% identity to the white sturgeon lipovitellin I domain.

Dot-plot analysis of white sturgeon amino acid sequence vs chicken and *Xenopus* (Fig. 3A,B) indicates three prominent subdomains of highly conserved sequences of four to 28 amino acids in the lipovitellin I domain where three of four consecutive amino acids are identical. In the silver lamprey/white sturgeon comparison (Fig. 3C), the first two subdomains are less conserved, but major regions of the third subdomain are present. In the *Xenopus*/chicken dot-plot comparison (data not shown), the first and third subdomain are prominent and the second is missing. These subdomains had higher similarity and identity than the entire lipovitellin I domain and their adjacent sequences. The conserved sequences almost invariably have positively charged residues with two or more hydrophobic residues as described by Gerber-Huber et al. (1987). The first and third subdomains (Fig. 2; residues 72–192 and 432 – 637 relative to sturgeon) contained 12 and 17 conserved sequences, respectively, and they had 11 and 24 positively charged residues, respectively, conserved across three of the four species. The second subdomain (Fig. 2; 295–364 relative to sturgeon) was smaller with only five conserved sequences and eight conserved positively charged residues, respectively. The first and third subdomains had 77% similarity and 60–59% identity between white sturgeon and *Xenopus* and chicken, respectively. In the second subdomain, white sturgeon was 74% similar and 56% identical to *Xenopus* and somewhat lower for chicken and silver lamprey with 63% similarity and 49% identity for both. The sequences adjacent to these highly conserved subdomains had lower similarities of 50–60% and identities of 44–28%.

The third subdomain contains the motif LALKALGNA (Fig. 2; 472–479), which closely matches a highly conserved consensus sequence found in nematodes as

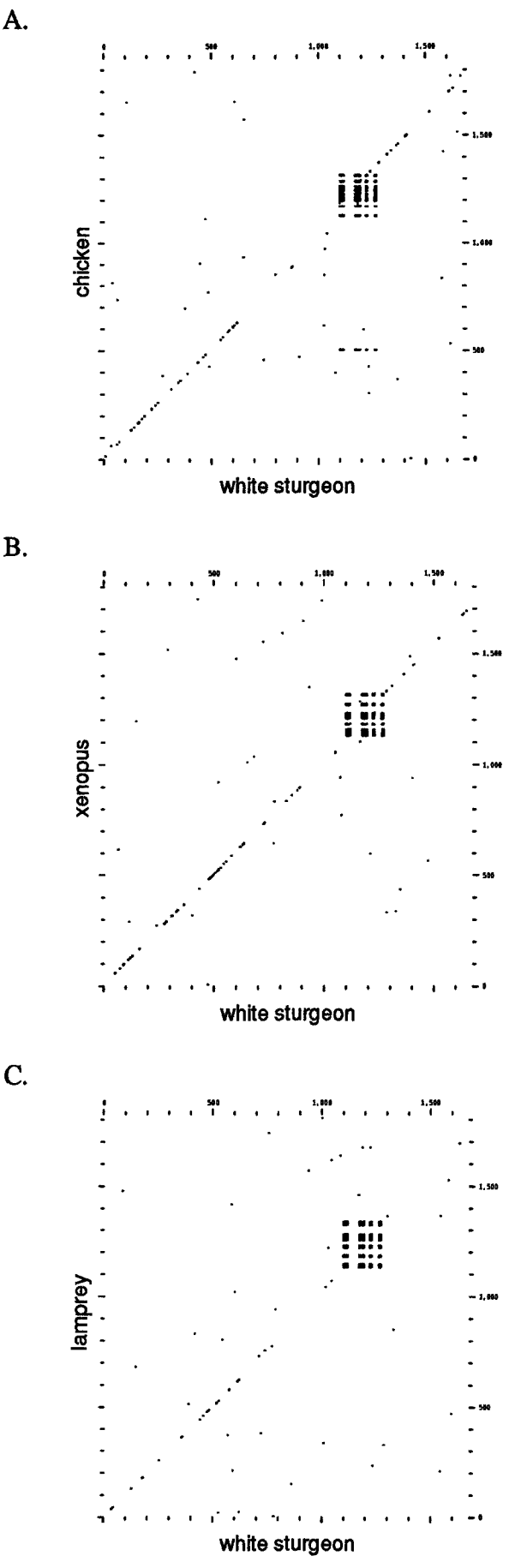

Fig. 3. Dot-plot comparisons of the vitellogenin amino acid sequence of white sturgeon with chicken, *Xenopus,* and silver lamprey. The *dots* in the matrix indicate regions where three of four amino acids are identical between the two vitellogenin sequences **(A)** chicken vs white sturgeon, **(B)** *Xenopus* vs white sturgeon, **(C)** silver lamprey vs white sturgeon.

well as vertebrates (Spieth et al. 1991). That motif initiates a conserved sequence of 28 amino acids in white sturgeon, *Xenopus,* and chicken and 24 amino acids in silver lamprey. Since the third subdomain is present in all four species and contains a conserved sequence from invertebrates, it is likely to have a major functional role in vitellogenesis.

The squares that extend both horizontally and vertically from the diagonal of the dotplots (Fig. 3) indicate the serine repeat regions that are present in the phosvitin domain of all four species. The phosvitin domain of white sturgeon vitellogenin encoded four serine repeats of 23, 11, 13, and 12 residues. It was the least conserved with 62–57% similarity and 48–46% identity between white sturgeon and *Xenopus* and silver lamprey, respectively. The chicken phosvitin domain was the least conserved with white sturgeon phosvitin with 49% similarity and 37% identity. The codon usage of the repeats was similar to chicken, *Xenopus,* and silver lamprey in that the first repeat uses primarily TCN codons and the last three repeats use mostly ACN codons. White sturgeon, silver lamprey, and *Xenopus* do not have a large number of arginine, lysine, and asparagine residues interspersed in the serine repeats as found in the chicken serine repeats (van het Schip et al. 1987).

The lipovitellin II domains were less conserved than lipovitellin I with 61–62% similarity and 38–39% identity between white sturgeon and *Xenopus* and chicken, respectively. Silver lamprey lipovitellin II was the least conserved with 54% similarity and 33% identity with white sturgeon. There were only two conserved stretches of amino acid sequence (Fig. 2; 1,501–1,525 and 1,608–1,647) between the four vitellogenins in the lipovitellin II domain. There are three potential N-linked glycosylation sites. Two are in the phosvitin domain where there were multiple asparagine residues immediately preceding a serine repeat (residues 1,217–1,220). The third site lies in the lipovitellin II domain at residues 1,420–1,422.

Discussion

Estrogen treatment of oviparous vertebrates results in very high levels of expression of vitellogenin mRNA in liver cells (Baker and Shapiro 1977; Le Guellec et al. 1988). As would be expected, estrogen implants resulted in high levels of vitellogenin mRNA expression in the livers of white sturgeon. Expression of vitellogenin mRNA in the undifferentiated gonad and the differentiated testis was an unexpected result. The moderate level of expression of vitellogenin in the estrogen-stimulated undifferentiated gonad may be a nonspecific effect on an estrogen-responsive tissue. Our results show that vitellogenin mRNA was not found in livers of control males but was expressed in the control testis. Reduced levels were seen in the testis of estrogen-treated males. Vitellogenin expression was very low and the differences observed may be normal variation. Alternately, the presence of large amounts of serum vitellogenin in the estrogen-treated males may have down-regulated vitellogenin gene expression in the testis. Vitellogenin has been detected in the testis of tilapia (Ding et al. 1989) although it was not determined if the testis was the site of synthesis. Vitellogenin had previously been referred to as female-specific protein; it had not been detected in untreated male vertebrates. In sea urchin, *Strongylocentrus purpuratus,* vitellogenin was synthesized in the intestines and gonads of both sexes and accounted for an estimated 50% of the coelomic fluid (Shyu et al. 1986). Because such a large amount of vitellogenin *Xenopus* and chicken was present in these males, it was speculated that vitellogenin may have some other function in sea urchin. These results in white sturgeon add support to speculation that vitellogenin may have other functions, such as being a carrier for the uptake of hormones, vitamins, and other biomolecules for spermatocytes in males as well as for oocyte growth in females (Ding et al. 1989). Amino acid sequence analysis of invertebrate and vertebrate vitellogenin has shown regions of similarity to human apolipoprotein B (Baker 1988; Byrne et al. 1989). The biological similarity of apolipoprotein B and vitellogenin—i.e., binding of hydrophobic molecules, cell-specific uptake, and estrogen regulation of chicken apolipoprotein B (Kirchgessner et al. 1987)—was further evidence that the two proteins may have had a common ancestor or vitellogenin may have functioned as a serum transport protein in addition to its role as a yolk protein precursor (Baker 1988).

Comparisons of the derived amino acid sequences for *Xenopus,* chicken, and nematode vitellogenin have shown considerable homology and suggest that these vertebrate and invertebrate yolk precursor proteins were derived from a common ancestor (Nardelli et al. 1987b). The vitellogenin protein was considered to be under less evolutionary constraints than enzymes and therefore should have more divergent sequences. However, the process of vitellogenesis requires significant modification by the liver, binding to the oocyte receptor, and proteolytic processing by the oocyte, so common protein sequence elements are expected (Nardelli et al. 1987a). Comparison of the four vertebrate vitellogenin amino acid sequences shows that the sturgeon vitellogenin does contain sequence elements found in other vitellogenins and adds support for their functional role in the production of mature yolk proteins.

The sequence similarity was strongest at the amino terminal in the lipovitellin I domain of vitellogenin. Fine structure analysis of *Xenopus* and chicken vitellogenin sequences showed that the lipovitellin domains were the most conserved (Nardelli et al. 1987a), the phosvitin domain was the least conserved, and this domain was ab-

sent in the nematode (Nardelli et al. 1987b). Studies of the oocyte receptors in chicken and *Xenopus* have shown that the lipovitellin I domain of vitellogenin binds to the receptor (Stifani et al. 1990). A common sequence element was the positively charged amino acids and their arrangement with hydrophobic residues. These charged residues were more concentrated in the lipovitellin I domain. Gerber-Huber et al. (1987) suggested that these sequences allowed for hydrophobic interactions between vitellogenin monomers, while leaving charged residues and the phosvitin domain exposed as hydrophilic loops to maintain the solubility of the protein in the serum. Chemical modification of lysine and arginine residues of locust, *Locusta migratoria,* vitellogenin reduces binding to the oocyte receptor (Roehrkasten and Ferenz 1992). As described in Results, the third subdomain of lipovitellin I contained a conserved sequence found in all four species and had the greatest number of positively charged residues. This information, combined with receptor binding studies, suggests that these regions of high homology and especially subdomain 3 are likely to be involved in vitellogenin/receptor binding.

The phosvitin domain was the most divergent, which was consistent with previous analysis (Nardelli et al. 1987a; Wahli 1988). Studies on serum vitellogenin and egg-yolk proteins in several species have led to the observation that phosphoserine content increases from lower to higher vertebrates (Wiley and Wallace 1981; de Vlaming et al. 1980; Wahli 1988). They hypothesized that phosvitins in fish would have shorter and fewer serine repeats. The white sturgeon phosvitin sequence contains four repeats of serine residues ranging from 23 to 12 serines, which supports their hypothesis. *Xenopus* and chicken phosvitin sequences contain much longer serine repeats. *Xenopus* phosvitin has 5 repeats of 34 to 6 serine residues (Gerber-Huber et al. 1987) and chicken phosvitin contains 4 repeats with 86 to 9 serines (van het Schip et al. 1987). However, the silver lamprey, a more primitive vertebrate than white sturgeon, has 5 repeats with 33 to 18 serine residues and has a higher overall mole percent serine (Sharrock et al. 1992). Therefore, the phosvitin domain of vitellogenin which has been most rapidly evolving (Wahli 1988) has undergone both contraction and expansion from lower to higher vertebrates.

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