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Purification of Avian Vitellogenin III: Comparison with Vitellogenins I and II†

Sho-Ya Wang, David E. Smith, and David L. Williams*

ABSTRACT: Vitellogenin is an egg yolk precursor protein synthesized by livers of oviparous vertebrates in response to estrogenic stimulation. Previous studies have shown that chicken vitellogenin consists of two major species that differ in amino acid composition, peptide maps, and immunological properties. A third vitellogenin (VTG III) has now been isolated and characterized. VTG III differs from VTG I and VTG II in 11 amino acids. VTG III is also a phosphoprotein but contains only 44 mol of P in comparison to 116 mol of P for VTG I or VTG II. Partial proteolysis mapping shows major differences among VTG I, VTG II, and VTG III. Immunoblot analysis shows no reactivity between anti-VTG I and either VTG II or VTG III, no reactivity between anti-VTG II and either VTG I or VTG III, and no reactivity between anti-VTG III and either VTG I or VTG II. Radioimmunoassay also shows no significant reactivity between anti-VTG III and either VTG I or VTG II. We conclude that VTG III is a distinct vitellogenin most likely encoded by a third vitellogenin gene. Immunological analysis of pulse-labeled hepatocytes shows a newly synthesized intracellular form of VTG III, pVTG III. As is the case with the precursors to VTG I and VTG II, pVTG III has a greater electrophoretic mobility in sodium dodecyl sulfate-polyacrylamide gels than the respective fully phosphorylated plasma vitellogenin. Dephosphorylation of plasma VTG III increases its mobility to an apparent molecular weight of 180 000, which corresponds to the mobility of hepatocyte pVTG III. Thus, each vitellogenin has an immunologically distinct nonphosphorylated hepatocyte precursor.

Vitellogenin is an egg yolk precursor protein synthesized by livers of oviparous vertebrates in response to estrogenic stimulation (Bergink et al., 1974). After secretion from the liver and uptake into the developing oocyte, vitellogenin is cleaved into a family of lipovitellin polypeptides and at least two heavily phosphorylated phosvitins. These proteins function as transport mechanisms for the movement of lipid, phosphorus, and metals to the yolk and serve directly as nutrient sources in embryonic development. In addition to its important developmental role, vitellogenin has received considerable attention as a model for steroid-regulated gene expression (Bergink et al., 1974; Ryffel,

† From the Department of Pharmacological Sciences, Health Sciences Center, State University of New York at Stony Brook, Stony Brook, New York 11794. *Received July 6, 1983.* This work was supported by National Institutes of Health Grant AM18171. D.S. is a predoctoral trainee in Pharmacological Sciences (GM07518).

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1978; Tata & Smith, 1979). Recent studies indicate that vitellogenin is not a unique protein but consists of a small family of related proteins encoded by several genes (Wahli et al., 1979, 1981). Two avian vitellogenins, VTG I[1] and VTG II, have been purified from rooster plasma (Wang & Williams, 1980a). Comparisons of amino acid compositions, peptide maps, and immunological properties indicate substantial differences between the two major vitellogenins. Intracellular nonphosphorylated precursors to VTG I and VTG II also have been identified and distinguished by immunological properties and peptide maps (Wang & Williams, 1982). These results indicate that VTG I and VTG II are distinct gene products.

We report here the isolation and characterization of a third avian vitellogenin, VTG III, which accumulates in rooster plasma in response to estrogen. In comparison to VTG I and VTG II, VTG III is a minor species in laying hens or estrogen-stimulated roosters. VTG III is different from VTG I and VTG II in amino acid composition, phosphorus content, peptide fingerprint, and immunological properties. However, like VTG I and VTG II, the synthesis of VTG III is completely dependent upon estrogen. Furthermore, VTG III shows the characteristic memory or anamnestic response upon secondary stimulation with estrogen. These results indicate that VTG III is a distinct vitellogenin and suggest that avian vitellogenesis involves at least three vitellogenin genes.

Experimental Procedures

Hormone Treatment, Hepatocyte Preparation, and Radiolabeling. White leghorn roosters (0.6–1 kg, SPAFAS, Norwich, CT) were injected intramuscularly with diethylstilbestrol (50 mg/kg) in propylene glycol on day 0 and day 9. Control animals received the vehicle. Hepatocytes and liver slices were prepared and incubated in short-term culture with [^{3}H]leucine (100 μM, L-[4,5-^{3}H]leucine, 12 Ci/mmol, New England Nuclear) exactly as described by Wang & Williams (1982). In vivo labeling with [^{32}P]P_i was as described by Wang & Williams (1980a). Liver slices and washed hepatocytes were homogenized at 0–4 °C in 0.02 M sodium phosphate, pH 7.4, 0.15 M NaCl, 0.005 M ethylenediaminetetraacetic acid, 1% Triton X-100, and 100 μg/mL phenylmethanesulfonyl fluoride, and debris was removed by centrifugation for 5 min at 10000*g*. The extracts were used for immunoprecipitation and SDS–polyacrylamide gel electrophoresis as described below.

Vitellogenin Purification. VTG I and VTG II were purified by DEAE-cellulose chromatography and preparative SDS–4% polyacrylamide gel electrophoresis as previously described (Wang & Williams, 1980a). VTG III elutes from DEAE-cellulose at 0.03–0.06 M NaCl. These fractions are enriched in VTG III but are contaminated with 10–20% VTG I and traces of VTG II. The VTG III enriched fraction was dialyzed against sterile water, lyophilized, and subjected to preparative SDS–4% polyacrylamide gel electrophoresis (Wang & Williams, 1980a). The gel eluate was assayed by analytical SDS–5% polyacrylamide slab gel electrophoresis, and appropriate fractions were dialyzed against sterile water and chromatographed on Sephadex G-100 equilibrated with water. This step was necessary to remove nondialyzable nonprotein contaminants prior to amino acid, phosphorus, and protein measurements (Wang & Williams, 1980a). VTG III was hydrolyzed in constant-boiling HCl containing 0.1% phenol for 22 h at 110 °C. Triplicate samples of the hydrolysate were run on a single-column analyzer while another portion of the hydrolysate was used for phosphorus determination (Wang & Williams, 1980a) with a commercial phosphorus standard (Sigma Chemical Co., St. Louis, MO). Serine was corrected for destruction as described by Richardson et al. (1978). Egg yolk granules, α-lipovitellin, and β-lipovitellin were purified as described previously (Wallace, 1965; Wang & Williams, 1980a).

Immunological Procedures. Rabbit antisera to the unresolved mixture of plasma vitellogenins (anti-VTG M) and to VTG II (anti-VTG II) have been described previously (Wang & Williams, 1980a, 1982). Antiserum against VTG I was prepared in the same fashion. To prepare antiserum against small quantities of VTG III, an SDS–polyacrylamide gel band containing approximately 20 μg of VTG III was lyophilized, ground to a powder, suspended in Freund's complete adjuvant, and injected at multiple subcutaneous sites in a New Zealand white rabbit. A booster injection was given on day 14 in the same fashion. Approximately 20 μg of VTG III eluted from a gel was used for additional booster injections on days 38 and 57, and the rabbit was bled on day 64. Antiserum was adjusted to 0.02% NaN_3 and stored at −70 °C. Prior to use, antiserum was adjusted to 100 μg/mL phenylmethanesulfonyl fluoride and centrifuged at 10000*g* for 5 min.

Radiolabeled cell extracts were analyzed with a double-antibody procedure using one of the primary antisera described above and goat anti-rabbit γ-globulin as the second antibody (Blue et al., 1980). Washed immunoprecipitates were dissolved in electrophoresis sample buffer, boiled, and analyzed by SDS–5% polyacrylamide gel electrophoresis (Williams, 1979; system A) in the buffer system of Laemmli (1970). Radioactive proteins were visualized by fluorography (Bonner & Laskey, 1974). Protein molecular weights were estimated by comparison to mobilities of standard proteins as previously described (Williams, 1979).

For immunoblotting, plasma from an estrogen-treated rooster was electrophoresed on an SDS–5% polyacrylamide slab gel, and the proteins were transferred to nitrocellulose (Howe & Hershey, 1981). Transfer required 10–14 h at 200 mA at 4 °C. The following procedure of antiserum exposure and washes was done on a rocking platform. The blot was incubated for 90 min at 40 °C in excess 0.2 M NaCl, 0.01 M Tris-HCl, pH 7.4, and 3% bovine serum albumin (NTB) and for an additional 90 min at 37 °C in NTB containing primary antiserum at an appropriate dilution (1:100 to 1:2000). Incubations were performed with 0.1 mL/cm^2 antiserum solution in sealed plastic bags. The blot was rinsed once in NTB for 10 min and in NTB containing 0.05% NP-40 for 20 min. Subsequent washes were in 0.2 M NaCl, 0.01 M Tris-HCl, pH 7.4, and 0.05% NP-40 for 20 min and in 0.2 M NaCl and 0.01 M Tris-HCl, pH 7.4, for 10 min a 23 °C. The blot was incubated with 0.05 μCi/mL ^{125}I-labeled protein A (89.2 μCi/μg, New England Nuclear) in NTB (0.1 mL/cm^2) for 60 min at 23 °C and washed as above. The blot was then dried between two sheets of Whatman 3MM filter paper and exposed to Kodak XR film at −70 °C.

For radioimmunoassay, VTG III was iodinated with ^{125}I by the chloramine T method (Greenwood et al., 1963) to a specific activity of 0.05 μCi/μg. Assays were performed with an antiserum dilution sufficient to precipitate approximately 70% of the ^{125}I-labeled VTG III. Samples containing [^{125}I]VTG III, competitor, 0.3 μL of anti-VTG III, and 2 μL of preimmune rabbit serum were incubated for 20 h at 4 °C in a final volume of 50 μL of 0.02 M sodium phosphate, pH 7.4, 0.15 M NaCl, 1% Triton X-100, and 1% sodium deoxycholate.

[1] Abbreviations: VTG, vitellogenin; pVTG, nonphosphorylated vitellogenin precursor; SDS, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane.

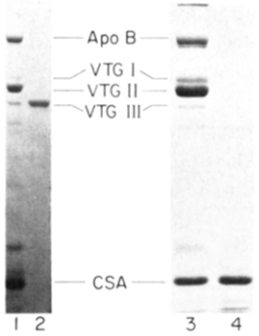

FIGURE 1: Occurrence of vitellogenin III. Purified VTG III and fresh plasma samples were run on SDS–5% polyacrylamide slab gels that were stained with Coomassie blue: (lanes 1 and 3) 0.5 μL of rooster plasma 3 days after treatment with diethylstilbestrol; (lane 2) 10 μg of purified VTG III; (lane 4) 0.5 μL of plasma from a control rooster. Protein bands labeled apo B and CSA are apolipoprotein B and plasma albumin, respectively.

Goat anti-rabbit γ-globulin (8 μL) was added for 30 min, and the precipitate was collected by centrifugation for 4 min at 10000*g*, washed 2 times with 1 mL of the above buffer, and counted in an Isodyne 1185 gamma counter (Searle). Data are expressed as the fraction (B/B_0) of [^{125}I]VTG III precipitated in the presence and absence of competitor times 100.

Miscellaneous Procedures. Partial proteolysis mapping (Cleveland et al., 1977) of purified VTG I, VTG II, and VTG III was performed with *Staphylococcus aureus* V8 protease exactly as described by Wang & Williams (1980a). Partial proteolysis mapping of radiolabeled cellular vitellogenins was performed in situ after electrophoresis is an SDS–5% polyacrylamide gel as described by Wang & Williams (1982). Peptides were separated in a second-dimension SDS–10% polyacrylamide gel. Radioactive peptides were visualized by fluorography. Plasma vitellogenins were dephosphorylated with alkaline phosphatase as described by Wang & Williams (1982). Buffers and glassware used in the purification and analysis of the vitellogenins were sterilized by autoclaving. Phenylmethanesulfonyl fluoride was added to solutions immediately before use from a freshly made stock in dimethyl sulfoxide.

Results

Occurrence of VTG III. Analysis of plasma from an estrogen-treated rooster by SDS–5% polyacrylamide gel electrophoresis (Figure 1, lane 3) shows the three major estrogen-regulated proteins previously described, apolipoprotein B, VTG I, and VTG II (Wang & Williams, 1980a; Williams, 1979). VTG III migrates faster than VTG II and is present in plasma from estrogen-treated (lane 3) but not control (lane 4) roosters. VTG III shows an apparent molecular weight of 210000 in this gel system. Two preliminary experiments showed that VTG III shares characteristics similar to those of VTG I and VTG II.

First, to determine whether VTG III is a phosphoprotein, an estrogen-treated rooster was injected intraperitoneally with 1 mCi of [^{32}P]P_i 18 h prior to the analysis of plasma on an SDS–5% polyacrylamide gel. After being stained with Coomassie blue, the gel was scanned by densitometry (Wang & Williams, 1983). An autoradiogram of the gel was also prepared and scanned by densitometry. The results showed that VTG III contained 21 and 13% of the [^{32}P]P_i present in VTG I and VTG II, respectively (data not shown). The ratio of [^{32}P]P_i to Coomassie blue stain was 20% of the ratios for either VTG I or VTG II. Thus, VTG III is a phosphoprotein but contains less phosphorus per mole than VTG I or VTG II.

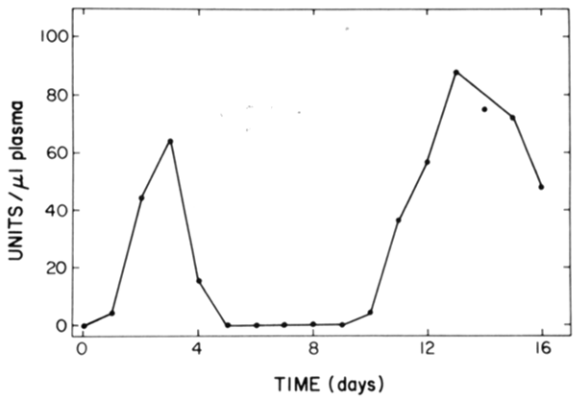

FIGURE 2: Primary and secondary induction of VTG III. Plasma samples were prepared daily after the administration of diethylstilbestrol on day 0 and day 9. After preliminary runs were performed to determine the dilutions required in order that the VTG III responses were within the linear range, appropriate dilutions from each time point were analyzed on the same slab gel. The gel was stained with Coomassie blue and scanned at 580 nm, and VTG III peak areas (units) were determined by weighing the peaks cut from the densitometric tracings.

Second, the accumulation of VTG III was monitored after primary and secondary stimulation with estrogen. Figure 2 shows that VTG III reached a maximum at 3 days after primary stimulation and declined to the preinduction level by 5 days. Upon secondary stimulation with estrogen at day 9, VTG III showed an enhanced accumulation (Figure 2) as is characteristically observed for vitellogenin (Bergink et al., 1973, 1974). Thus, VTG III also shows the memory or anamnestic response to estrogen. These results suggest that VTG III is a previously unrecognized vitellogenin. In comparison to VTG I and VTG II, VTG III is a minor species. When the primary response is maximal (Figure 2), densitometric scans of stained gels show that VTG I, VTG II, and VTG III are present in a ratio of 0.33:1.0:0.08, respectively.

Purification and Characterization of VTG III. Although VTG III is present in fresh plasma (Figure 1, lane 3), it was not recovered in the >0.06 M NaCl eluate from DEAE-cellulose as previously described (see Figure 1, lane 1, Wang & Williams, 1980a). Analysis of early column fractions showed that VTG III eluted at 0.03–0.06 M NaCl (data not shown). These fractions also contained 10–20% contamination with VTG I. Purification of VTG III from VTG I was achieved by preparative SDS–polyacrylamide gel electrophoresis (Wang & Williams, 1980a). The final preparation showed no contamination with VTG I or VTG II (Figure 1, lane 2) and no change in electrophoretic mobility as compared to that of VTG III in fresh plasma (Figure 1, lane 1).

As shown in Table I, the amino acid composition of VTG III is very different than the composition of VTG I or VTG II. VTG III contains less serine, lysine, histidine, and arginine and more glutamic acid, proline, alanine, leucine, and tyrosine as compared to VTG I or VTG II. Other differences between VTG III and VTG I (methionine, isoleucine) or VTG III and VTG II (glycine, phenylalanine) are also evident. Table I also shows that VTG III contains 0.76% phosphorus by weight in comparison to 2% phosphorus for VTG I or VTG II. On the basis of a polypeptide molecular weight of 180000, VTG III contains 44 mol of phosphorus/mol of protein compared to 116 mol for VTG I or VTG II (Wang & Williams, 1982).

Partial proteolysis mapping of purified VTG I, VTG II, and VTG III was performed with V8 protease from *S. aureus* (Cleveland et al., 1977). Resolution of the digests on an SDS–10% polyacrylamide gel shows few similarities among the peptide profiles of VTG I, VTG II, and VTG III (Figure

Table I: Amino Acid and Phosphorus Content of Vitellogenin III

	VTG III[a]	VTG I[b]	VTG II[b]
Asp	8.6 ± 0.2	9.1 ± 0.4	9.0 ± 0.4
Thr	5.1 ± 0.1	4.8 ± 0.3	4.6 ± 0.1
Ser	8.7 ± 0.3	15.8 ± 0.5[c]	13.8 ± 0.3[c]
Glu	12.2 ± 0.1	10.5 ± 0.4[c]	10.5 ± 0.4[c]
Pro	6.8 ± 0.5	4.4 ± 0.3[c]	4.6 ± 0.4[c]
Gly	5.7 ± 0.1	5.1 ± 0.5	4.9 ± 0.2[c]
Ala	8.3 ± 0.1	7.5 ± 0.3[c]	7.4 ± 0.3[c]
Val	6.5 ± 0.1	6.8 ± 0.5	6.0 ± 0.5
Met	2.6 ± 0.1	1.6 ± 0.1[c]	2.3 ± 0.5
Ile	5.1 ± 0.1	4.3 ± 0.2[c]	5.2 ± 0.4
Leu	9.5 ± 0.1	6.9 ± 0.3[c]	8.1 ± 0.4[c]
Tyr	3.5 ± 0.1	2.3 ± 0.2[c]	2.8 ± 0.3[c]
Phe	3.2 ± 0.2	2.7 ± 0.2	2.5 ± 0.3[c]
Lys	7.6 ± 0.1	8.4 ± 0.4[d]	8.1 ± 0.2[d]
His	2.7 ± 0.4	3.4 ± 0.1[d]	3.2 ± 0.1[d]
Arg	5.0 ± 0.1	6.3 ± 0.4[c]	6.9 ± 0.2[c]
P[e]	0.76	2.0	2.0
P/mol[f]	44	116	116

[a] Values shown are mole percent ± SD from triplicate determinations of VTG III. Trp and Cys were not determined. [b] Values for VTG I and VTG II are from Wang & Williams (1980a) but corrected for serine destruction as described by Wang & Williams (1980b). [c] Statistical comparison of these residues between VTG III and VTG I or VTG II shows these residues to differ at a probability level of less than 0.005. Comparisons were made with a t test with arcsin transformation of percentages. [d] Statistical comparisons of these residues between VTG III and VTG I or VTG II shows these residues to differ at a probability level of less than 0.01. Comparisons were made with a t test with arcsin transformation of percentages. [e] Values are percent phosphorus by weight calculated as grams of phosphorus/(grams phosphate + grams amino acid). Values for VTG I and VTG II are from Wang & Williams (1980a). [f] Values are moles of phosphorus/mole of protein on the basis of molecular weights of 180 000 for VTG I, VTG II, and VTG III. Note the discussion of vitellogenin polypeptide molecular weights in the text.

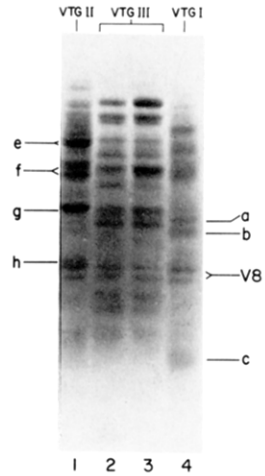

FIGURE 3: Limited proteolysis mapping of VTG I, VTG II, and VTG III. VTG II (lane 1), VTG III (lanes 2 and 3), and VTG I (lane 4) were digested with V8 protease for 1 h as described under Experimental Procedures. Samples containing 20 μg of protein were analyzed by SDS–10% polyacrylamide slab gel electrophoresis. The gel was stained with Coomassie blue. The doublet labeled V8 is the protease used for digestion. The bands labeled a–h correspond to major intermediate or core peptides of VTG I or VTG II (Wang & Williams, 1980a). Peptides b–h are also found in digests of the yolk lipovitellins.

3). Several peptides of VTG III may show mobilities similar to VTG I or VTG II peptides, but the overall VTG III fingerprint is quite dissimilar.

Immunological Comparison of VTG I, VTG II, and VTG III. Immunoblot analysis was used to examine the cross-reactivity of VTG I, VTG II, and VTG III. Fresh plasma was

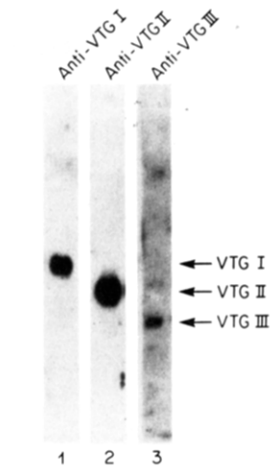

FIGURE 4: Immunoblot analysis of VTG I, VTG II, and VTG III. Fresh plasma samples from a diethylstilbestrol-stimulated rooster were resolved on an SDS–5% polyacrylamide slab gel, and the proteins were transferred to a nitrocellulose sheet as described under Experimental Procedures. Strips corresponding to individual gel lanes were cut from the blot and incubated with anti-VTG I (lane 1), anti-VTG II (lane 2), or anti-VTG III (lane 3). After being washed, the strips were incubated with ^{125}I-labeled protein A, washed, and exposed to X-ray film.

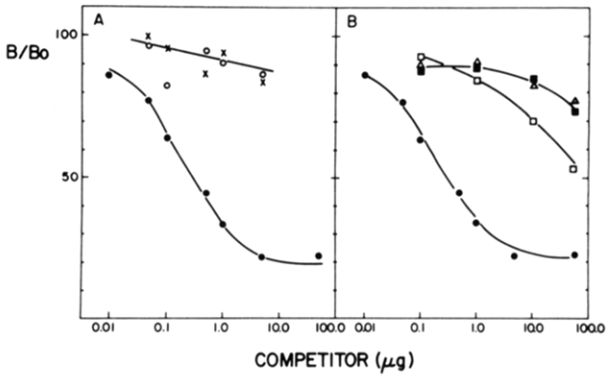

FIGURE 5: Comparison of VTG I, VTG II, and VTG III by radioimmunoassay. (Panel A) Anti-VTG III and [^{125}I]VTG III were incubated with increasing amounts of VTG I (○), VTG II (×), or VTG III (●) as described under Experimental Procedures. (Panel B) Anti-VTG III and [^{125}I]VTG III were incubated with increasing quantities of VTG III (●), yolk granule protein (■), β-lipovitellin (△) or α-lipovitellin (□). Radioactivity in the immunoprecipitate (B/B_0) is expressed as a percent of the value obtained in the absence of competitor.

resolved on an SDS–5% polyacrylamide gel, and the proteins were transferred to nitrocellulose. When the blot was probed with anti-VTG I (Figure 4, lane 1), VTG I showed strong reactivity, but no reaction was seen with VTG II or VTG III. Similarly, anti-VTG II reacted strongly with VTG II but showed no reaction with VTG I or VTG III (lane 2). With anti-VTG III as the probe (lane 3), VTG III was detected, but no reaction was seen with VTG I or VTG II. Identical results were obtained with plasma from a laying hen (data not shown). Therefore, the three antisera show remarkable specificity for their respective vitellogenin antigens.

The reactivity between anti-VTG III and VTG I and VTG II was also examined via radioimmunoassay. As shown in Figure 5A, increasing amounts of VTG III displaced up to 80% of [^{125}I]VTG III from the antibody. In contrast, equivalent amounts of VTG I and VTG II decreased the antibody binding of [^{125}I]VTG III by only 10–15%. Bovine albumin (10 μg) and chicken very low density lipoprotein (16 μg) also decreased the antibody binding of [^{125}I]VTG III by 8–10% in this assay (data not shown), suggesting that this minor inhibition may

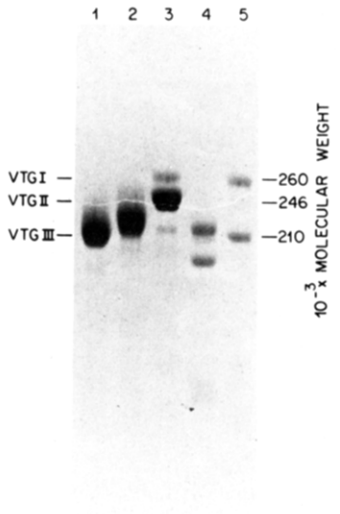

FIGURE 6: Dephosphorylation of VTG III. Vitellogenin samples enriched in VTG I and VTG III (lanes 4 and 5) or VTG II (lanes 1–3) were analyzed by SDS–5% polyacrylamide gel electrophoresis before (lanes 3 and 5) or after dephosphorylation with alkaline phosphatase for 30 min (lane 2), 1 h (lane 1), or 5 h (lane 4). In this experiment, treatment of VTG II with alkaline phosphatase for up to 4 h did not increase the electrophoretic mobility more than the 1-h treatment. Comparison on the same gel (not shown) with dephosphorylated VTG II samples previously described (Wang & Williams, 1982) showed that the VTG II sample in lane 1 has the limiting mobility produced by exhaustive dephosphorylation.

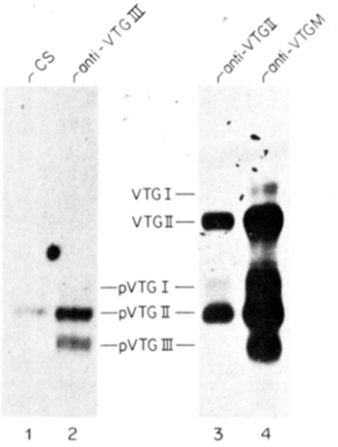

FIGURE 7: Identification of intracellular pVTG III. [^{3}H]Leucine-labeled hepatocyte extracts (lanes 1 and 2) or liver slice extracts (lanes 3 and 4) were reacted with preimmune rabbit serum (CS, lane 1), anti-VTG III (lane 2), anti-VTG II (lane 3), or anti-VTG M (lane 4 as described under Experimental Procedures. The immunoprecipitates were analyzed by SDS–5% polyacrylamide gel electrophoresis and fluorography.

be a nonspecific effect. When total egg yolk protein or purified β-lipovitellin was examined (Figure 5B), the antibody binding of [^{125}I]VTG III was reduced by approximately 25%. In contrast, α-lipovitellin was consistently more effective, yielding 45–50% inhibition of [^{125}I]VTG III binding. Higher concentrations of the lipovitellins could not be examined because of their limited solubilities.

Dephosphorylation of VTG III. Previous studies have shown that the vitellogenin phosphates markedly reduce the electrophoretic mobility in SDS–polyacrylamide gels (Wang & Williams, 1982). Removal of VTG II phosphates increases the mobility such that dephosphorylated VTG II runs with approximately the same mobility of the nonphosphorylated hepatocyte precursor to VTG II, pVTG II (Wang & Williams, 1982). Similar results were obtained with VTG I. Figure 6 (lane 5) shows the gel profile of vitellogenin from a leading fraction of the DEAE-cellulose eluate. This fraction contains similar amounts of VTG I (apparent M_r 260 000) and VTG III (apparent M_r 210 000) and a barely detectable amount of VTG II (apparent M_r 246 000). Treatment with alkaline phosphatase (Figure 6, lane 4) shifts the mobilities of VTG I and VTG III to apparent M_r 215 000 and 180 000, respectively. Note that the faint band of dephosphorylated VTG II runs slightly faster than dephosphorylated VTG I (lane 4) at a position overlapping the mobility of untreated VTG III (lane 5). The overlap in the mobilities of untreated VTG III and dephosphorylated VTG II is also evident by comparison to a sample enriched in VTG II (lane 3) that was dephosphorylated (lane 1). Thus, VTG III shows a mobility shift upon dephosphorylation as occurs with VTG I and VTG II. The magnitude of the shift is less for VTG III than for VTG I or VTG II as may be anticipated from the lower phosphate content of VTG III. Note that alkaline phosphatase removes 75–80% of the vitellogenin phosphates (Wang & Williams, 1982).

Identification of Intracellular pVTG III. Hepatocyte VTG I and VTG II primarily exist as the nonphosphorylated precursors. pVTG I and pVTG II show gel mobilities similar to the respective dephosphorylated VTG I and VTG II (Wang & Williams, 1982). From the mobilities of VTG III and dephosphorylated VTG III (Figure 6), it may be predicted that intracellular VTG III will have approximately the mobility of pVTG II while the nonphosphorylated precursor to VTG III, pVTG III, will run faster than pVTG II. To identify an intracellular precursor to VTG III, tissue slices were labeled in vitro with [^{3}H]leucine, and an extract was immunoprecipitated with antibody against bulk vitellogenin, anti-VTG M. Analysis of the immunoprecipitate (Figure 7, lane 4) shows the previously identified forms of intracellular vitellogenin, VTG I, VTG II, pVTG I, and pVTG II (Wang & Williams, 1982). In addition, a band, designated pVTG III, is seen at the position of dephosphorylated plasma VTG III. Note that the fluorogram is overexposed to show the minor species. When the tissue extract was reacted with anti-VTG II, pVTG III was not precipitated (lane 3). To ensure that pVTG III is an intracellular protein, isolated hepatocytes were incubated with [^{3}H]leucine. After washing of the cells, a cell extract was reacted with appropriate antisera. The results show that pVTG III is precipitated by anti-VTG III (Figure 7, lane 2) but not by preimmune serum (lane 1). Furthermore, pVTG III was not precipitated by anti-VTG I (data not shown). These results indicate that pVTG III is an intracellular vitellogenin specifically related to plasma VTG III but not to VTG I or VTG II. Note that the anti-VTG III immunoprecipitate (lane 2) also contains a band with the mobility of pVTG II or VTG III (see above for a discussion of the overlapping mobilities of pVTG II and VTG III). This band might represent fully phosphorylated intracellular VTG III. However, with the long fluorographic exposure (43 days) required to detect pVTG III (lane 2), the preimmune serum immunoprecipitate (lane 1) also shows a nonspecifically adsorbed protein with the mobility of VTG III. The comigration of these species precludes further characterization of the protein(s) at the position of VTG III.

Partial proteolysis mapping was used to compare the intracellular vitellogenins. Tissue slices were labeled with [^{3}H]leucine, and an anti-VTG M immunoprecipitate was resolved on an SDS–5% polyacrylamide gel. After in situ digestion with V8 protease, the resultant peptides were analyzed in a second-dimension SDS–10% polyacrylamide gel. As shown in Figure 8, the peptide pattern derived from pVTG

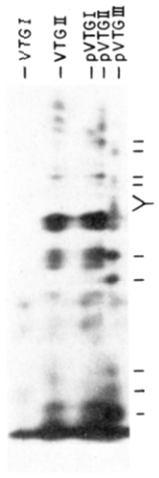

FIGURE 8: Limited proteolysis mapping of intracellular vitellogenins. After in vitro incubation of a liver slice with [^{3}H]leucine, tissue extract was immunoprecipitated with anti-VTG M, and the immunoprecipitate was run on an SDS–5% polyacrylamide gel. The vitellogenins were then digested with V8 protease in situ, and the digest was run into an SDS–10% polyacrylamide gel at 90° to the direction of the first electrophoresis (Wang & Williams, 1982). Radioactive peptides were visualized by fluorography. Lines indicate peptides unique to the pVTG III digest.

III is clearly distinct from the patterns for the other vitellogenins. Ten major peptides unique to the pVTG III digest are indicated in Figure 8.

Previous studies have shown that the pVTG III band is not labeled with [^{32}P]P_i but is labeled with [^{3}H]glucosamine when hepatocytes are incubated with these precursors (Wang & Williams, 1982). Furthermore, pVTG III is not found in proteins secreted by hepatocytes (Wang & Williams, 1982). Thus, by analogy to the properties exhibited by pVTG I and pVTG II (Wang & Williams, 1982) and on the basis of the immunological relationship to plasma VTG III, pVTG III appears to be the nonphosphorylated precursor to plasma VTG III.

Discussion

The vitellogenins are a family of liver proteins that serve as precursors to egg yolk lipovitellin and phosvitin polypeptides. In previous studies, we characterized plasma VTG I and VTG II and identified nonphosphorylated precursors to these phosphoproteins in the avian hepatocyte (Wang & Williams, 1980a, 1982). In this study we characterize a third species of plasma vitellogenin. Like VTG I and VTG II, VTG III is an estrogen-induced phosphoprotein. VTG III also shows the characteristic memory response to secondary estrogen stimulation (Figure 2). Furthermore, VTG III is immunologically related to purified yolk lipovitellin (Figure 5B). By these criteria, VTG III is a vitellogenin. However, comparison of amino acid compositions shows that VTG III differs from VTG I and VTG II in 11 amino acids (Table I). VTG III also contains far less phosphorus than VTG I or VTG II. In addition, polyclonal antiserum raised against VTG III shows no cross-reactivity with VTG I or VTG II when tested by immunoblot analysis (Figure 4) or radioimmunoassay (Figure 5A). Antisera raised against VTG I and VTG II also fail to react with VTG III (Figure 4). Furthermore, the V8 protease fingerprint of plasma VTG III is different than the fingerprint of VTG I or VTG II (Figure 3). These differences indicate that VTG III is a distinct gene product. We have observed that the three vitellogenins are invariably present in plasma from individual estrogen-stimulated roosters and laying hens from an inbred flock. As a result, the three vitellogenins are not allelic variants but most likely represent products of three genes within the haploid chromosome set.

Hepatocyte VTG I and VTG II exist primarily as the nonphosphorylated precursors, pVTG I and pVTG II (Wang & Williams, 1982). A newly synthesized form of hepatic VTG III, pVTG III, was identified by reactivity with anti-VTG III and lack of reactivity with anti-VTG I and anti-VTG-II (Figure 7). Intracellular pVTG III yields a unique peptide fingerprint upon partial proteolysis mapping (Figure 8) and shows the same gel mobility as dephosphorylated plasma VTG III with an apparent of M_r of 180000. pVTG III is not labeled with [^{32}P]P_i but is labeled with [^{3}H]glucosamine when hepatocytes are incubated with these precursors (Wang & Williams, 1982). These results suggest that pVTG III is the glycosylated, nonphosphorylated precursor to plasma VTG III although additional studies are needed to test this proposed precursor–product relationship in detail. The three nonphosphorylated vitellogenins show gel mobilities corresponding to apparent molecular weights of 200000 (pVTG I), 190000 (pVTG II), and 180000 (pVTG III). When hepatocytes are incubated with tunicamycin, a specific inhibitor of core glycosylation (Kuo & Lampen, 1974; Struck & Lennarz, 1977), the mobilities of pVTG I and pVTG II shift to apparent molecular weights of 190000 and 180000 (Protter et al., 1982). We have noted a similar tunicamycin-dependent shift for pVTG III to an apparent molecular weight of 170000 (unpublished results). Thus, in the absence of posttranslational glycosylation and phosphorylation, the three vitellogenins show apparent polypeptide molecular weights of 190000 (VTG I), 180000 (VTG II), and 170000 (VTG III). These results suggest that the three vitellogenins do, in fact, differ in polypeptide length although the differences are much less than estimated from the gel mobilities of the fully phosphorylated plasma vitellogenins.

As judged by radioimmunoassay, α-lipovitellin shows considerably greater reactivity with anti-VTG III than does β-lipovitellin or total yolk granule protein (Figure 5B). This result suggests that cross-reactivity is due to a yolk polypeptide(s) enriched in α-lipovitellin but not β-lipovitellin. The large quantitative difference between the VTG III and α-lipovitellin curves (Figure 5B), however, may indicate that the reactive polypeptide(s) is (are) a minor α-lipovitellin component. Studies in progress, in fact, show that anti-VTG III reacts with one α-lipovitellin polypeptide that is not detected by anti-VTG I or anti-VTG II (unpublished experiments). With regard to the yolk phosvitins, it is clear that VTG III (44 mol of P, Table I) does not contain sufficient phosphorus to serve as precursor to either the M_r 34000 (104 mol of P) or the M_r 28000 (85 mol of P) phosvitin (Clark, 1970). If VTG III is cleaved in the yolk to produce a phosphoserine-rich polypeptide, this polypeptide must be considerably smaller or contain much less phosphorus per mole than either of the two known chicken phosvitins. Interestingly, Wiley & Wallace (1981) recently identified in *Xenopus* yolk two low molecular weight (M_r 13000 and 19000) phosphopeptides containing 29 and 47 mol of phosphorus. These phosphopeptides do not derive simply from yolk phosvitin but most likely do arise from the plasma vitellogenins.

The vitellogenins function as transport proteins for the movement of metals and lipid to the yolk and also serve directly as nutrient sources in embryonic development. One would anticipate that the three vitellogenins would possess structural similarities related to common functions such as posttranslational processing in the hepatocyte, uptake into the oocyte,

site-specific cleavage to yolk polypeptides, and the formation of insoluble yolk granules. It is not surprising that the partial proteolysis maps of the three vitellogenins are very different since this technique may be particularly sensitive to minor changes in primary sequence (Cleveland et al., 1977). Perhaps more surprising is the absence of immunological cross-reactivity among the vitellogenins with very sensitive techniques such as radioimmunoassay (Figure 5) and immunoblotting (Figure 4). With the immunoblotting procedure, we have seen no cross-reactivity among the vitellogenins with antiserum dilutions that give maximum reactivity against the antigen. Since these antisera are polyclonal, there can be little doubt that there are major immunological differences among the vitellogenins. The absence of detectable cross-reactivity might also reflect that each antiserum was raised against an antigen–SDS complex such that the antibodies are directed against primary sequence and, therefore, do not detect features of secondary structure that might be functionally important and common to the three vitellogenins. Interestingly, comparison of two *Drosphila* yolk protein genes (Hung & Wensink, 1983) has shown a much greater conservation in predicted secondary structure than in primary sequence.

Comparisons of amino acid compositions and phosphorus contents suggest that VTG I and VTG II are similar while VTG III is very different. VTG I and VTG II differ in only four amino acids while VTG III differs from VTG I and VTG II in 11 amino acids (Table I). In addition, VTG I and VTG II each contain 116 mol of phosphorus while VTG III contains 44 mol of phosphorus. These differences may indicate that VTG I and VTG II are products of genes that have diverged from each other more recently than they have diverged from VTG III. *Drosophila* also has three steroid-regulated yolk protein genes, two of which are similar and closely linked while a third is quite dissimilar and distantly located in the genome (Barnett et al., 1980; Hung & Wensink, 1983). In *Xenopus*, analysis of cloned vitellogenin DNA sequences shows there to be two multigene groups (A and B) with regard to nucleotide sequence homology (Wahli et al., 1979, 1981). The A and B groups may result from an ancestral gene duplication whereas the two genes within each group may have arisen via a recent genomic duplication event that is unique to *Xenopus laevis* (Wahli et al., 1982). Elucidation of the genetic basis for the multiple chicken vitellogenins will require similar analyses of the cloned vitellogenin genes.

The estrogenic inductions of VTG I and VTG II are not tightly coupled in a quantitative sense or in terms of the temporal response characteristics (Wang & Williams, 1983). It is not known whether VTG III production is temporally coupled to VTG I or VTG II expression or is independent of both. Furthermore, VTG III is a minor species that is present at levels of 25 and 8% compared to plasma VTG I and VTG II, respectively. The vitellogenins also show major differences in hepatic synthesis rates (Wang & Williams, 1982, 1983; Protter et al., 1982). These results show that there are different limits for the synthesis of each vitellogenin under conditions of maximum hormonal stimulation in the rooster. Expression of the three vitellogenins also differs in the laying hen. These differences, therefore, reflect the physiological situation and presumably have functional significance. At present, little is known about the roles of individual vitellogenins in yolk formation or the hepatic mechanism that operate to maintain the synthesis of the three vitellogenins in the appropriate ratio. Further analysis of avian vitellogenesis and vitellogenin gene expression should provide insight into these questions.

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