Isolation and characterization of a cDNA clone specific for avian vitellogenin II

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ABSTRACT

A clone for vitellogenin, a major avian, estrogen responsive egg yolk protein, was isolated from the cDNA library of estrogen-induced rooster liver. Two forms of plasma vitellogenin, vitellogenin I (VTG I) and vitellogenin II (VTG II), distinguishable on the basis of their unique partial proteolysis maps, have been characterized and their corresponding hepatic precursor forms identified. We have used this criterion to specifically characterize which vitellogenin protein had been cloned. Partial proteolysis maps of VTG I and VTG II standards, synthesized *in vivo*, were compared to maps of protein synthesized *in vitro* using RNA hybrid-selected by the vitellogenin plasmid. Eight major digest fragments were found common to the *in vitro* synthesized vitellogenin and the VTG II standard while no fragments were observed to correspond to the VTG I map. A restriction map of the VTG II cDNA clone permits comparison to previously described cDNA and genomic vitellogenin clones.

INTRODUCTION

Vitellogenin is the major yolk precursor protein synthesized by and secreted from the livers of oviparous vertebrates in response to estrogenic stimulation (1). The enormous increases in hepatic vitellogenin synthesis and vitellogenin mRNA following hormone treatment have focused attention on avian and amphibian vitellogenins as models for estrogen-regulated gene expression (1, 2). Recent studies indicate that vitellogenin is not a unique protein, but appears to be a small family of similar proteins encoded by several genes (3, 4). Vitellogenin cDNA and genomic clones have been isolated and characterized from both chicken (5,6,7) and *Xenopus* (4, 8). At the protein level, two major vitellogenins, VTG I and VTG II, have been purified from plasma of estrogen-stimulated roosters (3). Comparisons of amino acid compositions, peptide maps, and immunological properties indicate substantial differences in the structures of these proteins. Each avian vitellogenin contains over 100 moles of serine-phosphate as well as carbohydrate in covalent linkage (3, 9). Because of the large size and complexity of the vitellogenins, it has not

been possible to relate specific plasma vitellogenins to individual cloned vitellogenin DNA sequences.

In the absence of protein sequence data, the relationships between specific plasma vitellogenins and individual DNA clones may be established through analysis of cell-free translation products directed by hybrid-selected mRNA's (10). Such analysis requires comparison of the primary translation product with appropriate forms of VTG I and VTG II. In the case of the heavily modified vitellogenins, the appropriate standards for comparison are the nonglycosylated, nonphosphorylated forms of these proteins. We recently showed that newly synthesized vitellogenins exist almost exclusively as glycosylated, nonphosphorylated precursors in the hepatocyte (11). Specific intracellular precursors, pVTG I and pVTG II, were identified and readily distinguished by limited proteolysis mapping using a two dimensional technique (11). The nonglycosylated, nonphosphorylated vitellogenins, $^{NG}pVTG$ I and $^{NG}pVTG$ II, are made when hepatocytes are treated with tunicamycin. In the present study we have used $^{NG}pVTG$ I and $^{NG}pVTG$ II to identify a cDNA clone corresponding specifically to one of the two major avian vitellogenins. A restriction map of the identified cDNA clone permits us to relate this cDNA to previously described cDNA and genomic vitellogenin clones.

MATERIALS AND METHODS

Materials.

Guanidine-hydrochloride and 17β -estradiol were purchased from Sigma Chemical Company. Formamide (puriss) was purchased from Tridom/Fluka, Incorporated. Poly (U) Sephadex, rabbit reticulocyte lysate (lot #1c21), and exonuclease III were purchased from Bethesda Research Laboratories, Incorporated. AMV reverse transcriptase, (lot #G979) was a gift of Dr. J.W. Beard and the National Cancer Institute, USA. Calf thymus terminal transferase (lot #00H09) was purchased from Worthington Diagnostics. SI nuclease (lot #15) and *Staphylococcus aureus* V8 protease were purchased from Miles Laboratories, Incorporated. [α ³²P]dCTP (400Ci/mmol) and [γ ³⁵S] methionine (1160 Ci/mmol) were purchased from Amersham. Restriction enzymes, purchased from Bethesda Research Laboratories and New England Biolabs, were used according to manufacturers' directions.

Animals.

White leghorn roosters (8-12 weeks old) (SPAFAS, Norwich, Conn.) received an intramuscular injection of 17β estradiol (25mg/Kg body weight) in propylene glycol 3 days before killing.

RNA Isolation.

Total RNA from rooster liver was isolated by a modification of the guanidine-HCl method of Cox (12). Animals were sacrificed, and the livers were immediately frozen in liquid nitrogen and stored at -70°C . Frozen tissue (20g) was ground to a fine powder under liquid nitrogen with a mortar and pestle and quickly solubilized in 400ml guanidine extraction buffer (G-E) (7.5M guanidine-HCl, 20mM potassium acetate, pH7.0, 5mM EDTA, 5mM DTT) at -10°C with the aid of a polytron homogenizer (Brinkman). One half volume of chilled (-20°C) ethanol was added and the solution was kept at -20°C for two hours. The RNA was collected by centrifugation at 6.5K rpm, -10°C , for 30 minutes in a Beckman JS7.5 rotor and resuspended in fresh G-E buffer. The RNA was again precipitated with one half volume of ethanol as above, resuspended in 50mM Tris-HCl, pH9.0, 5mM EDTA, 100mM NaCl, 0.5% SDS (10ml) and extracted twice with an equal volume of phenol, chloroform, isoamylalcohol (1/1/.04). The aqueous phase was adjusted to pH7.0 and RNA was precipitated overnight with two volumes of ethanol at -20°C . The RNA was washed three times by dissolving the RNA in 10mM Tris-HCl, pH7.0, 5mM EDTA, 100mM NaCl and precipitating with two volumes of ethanol. The RNA was stored as an ethanol precipitate at -20°C . Typically 5-7.5mg of RNA was recovered per gram wet weight of liver tissue. The ratio of the absorbance at 260nm to 280nm was 2.0-2.1.

Synthesis of double stranded cDNA for cloning.

A poly A⁺ enriched fraction of RNA was isolated by chromatography on poly (U) Sephadex by the method of Haff and Bogorad (13) except two rounds of chromatography were performed. Formamide was deionized with a mixed bed resin (Bio Rad, AG501 X 8-D). Poly A⁺ RNA (24 μg) from the liver of an estrogen treated rooster was heated to 65°C for 15 minutes in distilled water and then incubated in 50mM Tris-HCl, pH8.3, 10mM MgCl₂, 140mM KCl, 60 $\mu\text{g}/\text{ml}$ oligo dT, 14mM β -mercaptoethanol, 0.8mM each of dATP, dCTP, dTTP, dGTP, and 50 μCi [α -³²P]dCTP with AMV reverse transcriptase (5 units) for 1.5 hours at 42°C in 0.4ml. EDTA (20mM) and carrier RNA (100 μg) was added and the reaction was extracted once with phenol, chloroform, isoamylalcohol (1/1/.04) and twice with ether. The reaction mixture was chromatographed on Sephadex G50 equilibrated with 10mM Tris-HCl, pH8.0, 5mM EDTA, 80mM NaCl, and the void volume containing cDNA was precipitated with ethanol. RNA was removed by alkaline hydrolysis (0.3N KOH, 12 hours, room temperature), adjusted to pH 7.0, and ethanol precipitated. Second strand synthesis was performed as the first strand except [α -³²P]dCTP, oligo dT, and RNA were omitted. The cDNA was deproteinized, chromatographed on Sephadex G50, and precipitated with ethanol

as above. The resulting DNA was treated with 50 units of S1 nuclease for 30 minutes at 37 °C in 0.1ml of 30mM sodium acetate, pH 4.5, 300mM NaCl, 4.5mM ZnCl₂ to eliminate the hairpin generated by reverse transcriptase and single stranded regions. The DNA was deproteinized as above and ethanol precipitated. Poly dC tails (30-35 per 3' end) were added to the double stranded DNA with terminal transferase by the method of Roychoudury and Wu (14). High molecular weight, tailed, double stranded DNA was isolated by chromatography on A15m resin from which the void volume was ethanol precipitated.

Cloning of double stranded DNA.

Poly dC tailed, double stranded DNA (5 and 50ng) was annealed to a four fold weight excess of pBR322 which had been tailed with dG residues at the Pst I site. The mixtures were incubated in 10mM Tris-HCl, pH 7.5, 10mM EDTA, 300mM NaCl for ten minutes at 70 °C, one hour at 45 °C, 1.5 hours at 40 °C, and 1.5 hours at 37 °C, and used to transform bacteria by the procedure of Dagert and Ehrlich (15). This procedure routinely yielded 1-5 X 10⁶ transformants per µg of intact pBR322. Bacteria containing plasmids were selected by growth on LB agar plates with 10 µg/ml tetracycline. Bacteria containing recombinant plasmids were selected by their sensitivity to 100µg/ml ampicillin.

Preparation of Rot fractionated probe.

High specific activity cDNA suitable for use as a probe was synthesized essentially as described above except the unlabeled dCTP concentration was 0.008mM. Typically a specific activity of 1-4 X 10⁷ cpm/µg cDNA was obtained. To isolate a probe enriched for abundant, estrogen-induced sequences, a preparative Rot procedure similar to that employed by King *et al.* (16) was used. To eliminate non-abundant sequences 100ng of cDNA synthesized from estrogen-induced liver poly A⁺ RNA was hybridized with an excess of induced liver poly A⁺ RNA to a Rot value of 0.8 mole-sec./liter at 68 °C in 25mM TrisHCl, pH 8, 600mM NaCl, 100µg/ml denatured calf thymus DNA. Analytical hybridizations showed that at this Rot value a rapidly hybridizing class of sequences corresponding to 50% of the probe was selected. Unhybridized probe was eliminated by adjusting the mixture to 50mM sodium acetate, pH 4.5, 300mM NaCl, 6mM zinc acetate, 50µg/ml denatured calf thymus DNA, and incubating for one hour at 48 °C with 700 units of S1 nuclease. The remaining probe was deproteinized and subjected to alkaline hydrolysis as described above. To eliminate abundant sequences not induced by estrogen, the probe was then hybridized with liver poly A⁺ RNA from control roosters to a Rot of 8.0 mole-sec./liter. The reaction was adjusted to 20mM NaPO₄, pH7.0, and the cDNA bound to hydroxylapatite at 55 °C. The resin was washed with 50mM NaPO₄, pH7.0, and the single

stranded cDNA probe eluted with 140mM NaPO₄, pH 7.0. The resulting probe, corresponding to abundant mRNAs that were induced by estrogen, was used to screen the liver cDNA library by the method of Gergen *et al.* (17). Eleven colonies selected by this probe were further characterized.

Northern analysis.

Total liver RNA (12.5µg) from control and induced roosters was electrophoresed on 1.2% agarose gels with 10mM methyl mercury hydroxide (18) and transferred to diazobenzyloxymethylcellulose (DBM)-paper by the method of Alwine, *et al.* (10). Plasmids were isolated by the procedure of Birnboim and Doly (19), nick translated with [α -³²P]dCTP (20), and used to probe the DBM-paper bound RNA (10).

Hybrid-selection of mRNA.

Selection of mRNA by hybridization to plasmid covalently bound to DBM-paper was done by the method of Alwine, *et al.* (10) with minor modifications. The plasmid (10µg) was randomly nicked by alkaline treatment (0.3N KOH, 100°C, 15 minutes), neutralized, ethanol precipitated, and lyophilized. The DNA pellet was solubilized in a small volume of distilled water, heat denatured (85°C, 1 minute), brought to 80% DMSO and 80 mM sodium acetate, pH 4.0, and immediately applied to activated DBM-paper discs (1.5cm diameter). After incubation overnight at 25°C, filters were washed by the procedure described by Alwine, *et al.* (10) to remove non-covalently bound DNA. Inclusion of trace quantities of nick translated plasmid indicated that 50% of the DNA remains bound to the filter after washing. Filter-bound plasmid was hybridized with 10µg of estrogen-induced poly A⁺ liver RNA in 0.05ml of hybridization buffer (70% deionized formamide, 10mM HEPES, pH 6.8, 5mM EDTA, 300mM NaCl, 0.2% SDS) for 3 hours at 47°C. The papers were washed twice with hybridization buffer at 47°C and three times with 1 X SSC, 0.5% SDS. RNA selectively bound to the filters was removed by incubating three times in 0.05ml elution buffer (90% deionized formamide, 10mM HEPES, pH 6.5, 1mM EDTA, 0.1% SDS, 25µg/ml wheat germ tRNA) for 15 minutes at 47°C. The three eluates were pooled, ethanol precipitated, washed three times with 70% ethanol, lyophilized, and resuspended in distilled water for cell-free translations.

The RNA selected by one filter, or 0.5µg total poly A⁺ RNA, was incubated with a rabbit reticulocyte lysate mixture as described by the manufacturer with 70µCi [³⁵S]methionine for 1.5 hours at 30°C. The reaction was stopped by chilling on ice and centrifuged at 10,000g for 10 minutes. The supernatant was analysed on 5% and 10% SDS-polyacrylamide gels by the method of Laemmli (21).

Peptide mapping.

For partial proteolysis mapping (22), cell free translation products were run on an SDS-5% polyacrylamide gel. The vitellogenin region of the gel was excised and incubated with V8 protease as previously described (11). The gel section was loaded onto a second slab gel at 90° to the first dimension and run into an SDS-10% polyacrylamide gel. Gel sections containing radio-labeled ^{NG}pVTG I and ^{NG}pVTG II were treated in the same fashion and run on the same second dimension slab gel as the translation products. Radioactive peptides were visualized by fluorography (23). The ^{NG}pVTG standards were prepared by incubating liver slices with [³⁵S]-methionine (300μCi/ml) for 1 hour as described (11) except that tunicamycin (50μg/ml) was added 30 minutes prior to the radiolabeled amino acid.

Sequence analysis.

Restriction fragments to be sequenced were 3' end labeled as described by Smith and Calvo (24) and sequenced by the method of Maxam and Gilbert (25).

Immunological studies.

The characterization and use of antibodies against the mixture of VTG I and VTG II (anti-VTG M) have been described (11).

RESULTS

Construction of an estrogen-induced cDNA library.

Electrophoresis of estrogen-induced and control liver poly A+ RNA on methyl mercury agarose gels (figure 1) shows a broad size spectrum of discrete RNA species. The bands designated vitellogenin (lane 1) and apolipoprotein II (apo II) (lane 1) were tentatively identified on the basis of their size (vitellogenin, 6,600 bases, ref. 26, apo II, 700 bases, ref. 27,) relative abundance, and occurrence in RNA from estrogen-induced (lane 1) but not control (lane 2) livers. Chicken serum albumin was tentatively identified on the basis of its size (2,600 bases, ref. 28,) relative abundance, and presence in RNA from both control (lane 2) and induced (lane 1) liver.

Five μg of cDNA were synthesized from 24μg of poly A+ RNA from estrogen-induced liver. Upon second strand synthesis the percent of double stranded character, as judged by SI nuclease resistance, increased from 3% to 50%. Following addition of poly dC residues to the SI nuclease-treated double stranded DNA, the material was chromatographed on an A15m column to eliminate low molecular weight material. DNA eluting in the void volume was annealed to Pst I cut, poly dG tailed pBR322. 55ng of cDNA yielded 3,100 transformants (tetracycline resistant) 95% of which contained inserts in the ampicillin gene.

FIGURE-1 Agarose gel electrophoresis and Northern analysis of specific rooster liver mRNAs.

left: Poly A⁺ RNA (10 μ g) from estrogen-induced (E-lane 1) and control (C-lane 2) rooster liver was electrophoresed in 1.2% agarose gels in the presence of 10mM methyl mercury hydroxide and stained with ethidium bromide. mRNAs for vitellogenin, apolipoprotein II, and chicken serum albumin as well as the mobilities for 28S ribosomal RNA and 18S ribosomal RNA are indicated. right: Total cell liver RNA (12.5 μ g) from estrogen-induced (E-lanes 4,5) and control (C-lanes 3,6) rooster livers were electrophoresed in 1.2% agarose gels in the presence of 10mM methyl mercury hydroxide, transferred to DBM-paper, and hybridized with nick translated plasmids. Lanes 3-6 indicate results of hybridizations with putative vitellogenin (lanes 3 and 4) and apolipoprotein - II (lanes 5 and 6) plasmids used as probes. Mobilities of 28S ribosomal RNA and 18S ribosomal RNA are indicated.

Preliminary screening of a liver cDNA library.

King *et al.* (16) have shown that probes specific for vitellogenin and apo-II, two major estrogen-inducible proteins in the rooster liver, can be prepared by a series of Rot fractionations of high specific activity cDNA made against estrogen-induced liver RNA. We employed a similar scheme to isolate such a probe and used it to screen our estrogen-induced liver cDNA library by the method of Gergen *et al.* (17). A set of eleven clones giving a strong positive signal with the estrogen-specific probe was selected for further study. Plasmid from each was isolated, nick translated, and used to probe total liver RNA, isolated from estrogen-induced and control roosters, that had

been electrophoresed through 1.2% agarose, 10mM methyl mercury hydroxide, gels and then transferred to DBM-paper (figure 1). Two of the eleven hybridized to RNA species of the size expected for vitellogenin mRNA. The RNAs were present in estrogen-induced RNA (lane 4) but not in control RNA (lane 3). The nine remaining clones hybridized to a low molecular weight species tentatively identified as apo II on the basis of its size and occurrence in estrogen-induced (lane 5) RNA but not in control RNA (lane 6).

Identification of a cDNA clone for vitellogenin II.

Figure 2 shows the SDS-5% polyacrylamide gel profile of cell-free products synthesized from RNA selected by hybridization to pBR322 (lane 1) and the plasmid carrying the putative vitellogenin sequences (lanes 2 and 3). The only bands observed with RNA selected by pBR322 correspond to endogenous products of the reticulocyte lysate. RNA selected by the putative vitellogenin plasmid yields a broad size spectrum of discrete products up to a band (designated a) that migrates slightly slower than the pVTG II standard and slightly faster than the pVTG I standard (lane 4). This product has an apparent molecular weight of approximately 183,000. The broad size spectrum of translation products (lanes 2 and 3) is similar to that reported by Weinring *et al.* (29) for translations of vitellogenin mRNA. The polypeptide designated band a and the spectrum of products between band a and band b were precipitated by antibody specific to plasma VTG I and VTG II (11) (data not shown). When RNA from control rooster liver was hybridized with the putative vitellogenin cDNA clone, subsequent translation of the filter eluate did not show polypeptide a or the spectrum of products between bands a and b (data not shown). These results indicate that the putative vitellogenin cDNA plasmid hybridizes selectively to an estrogen-induced mRNA that codes for a vitellogenin polypeptide.

We have previously shown that vitellogenin consists of two distinct highly phosphorylated, glycoproteins, VTG I and VTG II (3,11). To fully characterize a vitellogenin cDNA clone it was necessary to perform experiments which would assign it specifically to one of these proteins. Since the two vitellogenins appear to exhibit some immunological cross reactivity (11) it was unlikely that immunological techniques would provide adequate discrimination to conclusively identify a cell-free translation product as a specific vitellogenin. We have shown, however, that the nonphosphorylated forms of VTG I (pVTG I) and VTG II (pVTG II) can be readily distinguished on the basis of their partial proteolysis maps (11) as performed by the method of Cleveland, *et al.* (22). Our strategy was to compare the partial proteolysis maps obtained

FIGURE-2 SDS-(5%)polyacrylamide gel electrophoresis of the in vitro translation products of RNA selected by the putative vitellogenin plasmid. lane 1: Proteins synthesized in vitro with RNA hybrid selected by pBR322. lanes 2 and 3: Proteins synthesized in vitro with RNA hybrid selected by the putative vitellogenin plasmid (plasmid was bound to two separate filters and the experiment performed in duplicate). The full length translation product (a.) is shown and the star indicates an endogenous reticulocyte lysate labeled protein. lane 4: Proteins synthesized in vivo from tunicamycin treated, [35S]methionine labeled, liver tissue blocks. The positions of the nonglycosylated, nonphosphorylated vitellogenins (NGpVTG I and NGpVTG II) are indicated. The phosphorylated, nonglycosylated form of vitellogenin II (NGpVTG II) is also indicated.

with the protein synthesized in vitro from RNA selected by hybridization to the putative vitellogenin clone to the maps of appropriate VTG I and VTG II standards. It has been shown that newly synthesized pVTG I and pVTG II migrate on SDS-polyacrylamide gels with molecular weights of 200,000 and 190,000 respectively (11).

When synthesized in the presence of tunicamycin, a specific inhibitor of core glycosylation (30, 31), ^{NG}pVTG I and ^{NG}pVTG II have slightly greater electrophoretic mobilities, corresponding to molecular weights of 190,000 and

180,000, respectively (32). These vitellogenins are not labeled when hepatocytes are incubated with [^3H]-glucosamine, indicating that core glycosylation does not occur in the presence of tunicamycin (data not shown). Since the nonglycosylated, nonphosphorylated vitellogenins should most closely resemble the in vitro translation products, we have used [^{35}S]-pVTG I and [^{35}S]-pVTG II as standards for the partial proteolysis mapping of the cell-free translation products. Figure 3A shows the results of an experiment in which the 183,000 dalton vitellogenin cell-free product (band a in Figure 2) was excised from the gel and subjected to partial proteolysis mapping in parallel with pVTG I and pVTG II standards. After digestion with V8 protease, the resultant peptides were resolved on an SDS-10% polyacrylamide gel. The digest peptides from the hybrid-selected, cell-free product closely resemble the digest products of pVTG II. Most of the major V8 digest products of pVTG II can be identified in the cell-free products: eight prominent peptides common to both digests are indicated by lines between the two digests. We have observed the same set of digest peptides in other experiments which yielded more complete digestion of the 183,000 dalton cell-free product. Note that none of the major digest peptides of pVTG I are present in the digest of the cell-free product: five prominent pVTG I peptides are designated by stars to the left of the in vivo standards. These data indicate that the 183,000 dalton vitellogenin cell-free product corresponds to plasma VTG II and not VTG I.

Previous studies have shown that cell-free translation of vitellogenin mRNA yields extensive quantities of incomplete translation products except at very high KCl concentrations which drastically reduce initiation efficiency (29). Such incomplete products may result from premature termination, translational pauses, or mRNA degradation (29, 34). In agreement with Felber et al. (34), we have noted a greater proportion of incomplete products in translations of hybrid-selected vitellogenin mRNA as opposed to translations of total RNA suggesting some degradation of the 7000 nucleotide vitellogenin mRNA during isolation. Since the translation products in the gel region between bands a and b (Figure 2, lanes 2 and 3) were precipitated by antibody specific to plasma vitellogenin, it seemed likely that these polypeptides were incomplete VTG II polypeptides. This was confirmed by a partial proteolysis map of the translation products in the entire gel region between bands a and b. As shown in Figure 3B, these products yielded the same digest peptides as the 183,000 dalton VTG II translation product and the pVTG II standard. Digest peptides corresponding to pVTG I were not observed. Experiments in

FIGURE-3 Two dimensional SDS-(10%)polyacrylamide gel electrophoresis fingerprint maps of V8 protease digested products.
 A, left: Protein standards synthesized in vivo (see text). Positions of the nonglycosylated, nonphosphorylated vitellogenin I (NGpVTG I) and vitellogenin II (NGpVTG II) in the first dimension electrophoresis are shown at the top. right: Complete in vitro translation product from RNA hybrid selected with the putative vitellogenin plasmid. B: Total proteins synthesized in vitro with RNA hybrid selected with the putative vitellogenin plasmid. Migration of the complete translation product (a, Mr= 183,000d) and a smaller incomplete product (b, Mr= 55,000d) in the first dimension of electrophoresis are indicated at the top.
 Arrows at the bottom indicate direction of electrophoresis in the first dimension, bars indicate peptides common between NGpVTG II and in vitro translation products, and stars(*) indicate peptides unique to pVTG I.
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which more extensive V8 digestion of cell-free products was performed did not alter the resulting proteolysis map.

The restriction map shown in Figure 4A was generated from the VTG II cDNA plasmid. The insert region is about 1,450 nucleotides long. This map closely resembles that determined by Cozens, et al. (5) for a vitellogenin cDNA clone, pVT3.

FIGURE-4 Restriction digest analysis of vitellogenin II (A), and apolipoprotein II (B). cDNA plasmid inserts. Abbreviations: A, Ava I: Hd, Hind III: He, Hae III: P, Pst I: Pv, Pvu II.

Identification of a cDNA clone for Apolipoprotein II.

The putative apo II clone was digested with a variety of restriction enzymes to generate the restriction map shown in Figure 4B. The resulting map is in agreement with that published by Weinringa *et al.* (35). To confirm the identity of the apo II cDNA clone two regions of the 5' end were sequenced. Three prime end labeling (24) of the Pvu II site and the Hinf I site enabled sequence information to be derived from the indicated regions by the method of Maxam and Gilbert (25). The nucleotide sequences that were determined were in complete agreement with that published by Weinringa, *et al.* (35) (data not shown). The cDNA insert, 620 nucleotides long, contains the entire coding region of the mRNA (see Figure 4B).

Identification of vitellogenin cDNA clones with a plus/minus screening technique.

The identification of vitellogenin and apo II cDNA clones by the procedure of King *et al.* (16) requires the purification of a probe specific for estrogen-induced, abundant mRNAs. Since vitellogenin and apo II mRNAs are not present in liver RNA from control animals to an appreciable extent, we tested whether total cDNA prepared from estrogen-induced and control liver RNAs could serve as plus and minus probes to identify clones corresponding to estrogen-induced mRNAs. Screening of a random selection of 312 colonies in parallel with the two cDNA probes yielded 39 colonies that were detected with cDNA from estrogen-induced RNA but not with cDNA from control RNA. When these colonies were screened with the Ava I-Pvu II fragment from the VTG II cDNA clone (Figure 4A), 18 colonies carrying vitellogenin cDNA were identified. All 18 vitellogenin cDNA clones were contained in the subset of 39 colonies detected with the plus/minus technique.

DISCUSSION

In the present report we describe the isolation and characterization of a

cDNA clone corresponding to plasma VTG II. A cDNA probe for estrogen-induced liver mRNAs was prepared as described by King *et al.* (16) and used to identify a subset of colonies carrying sequences for vitellogenin and apo II. Two of eleven colonies obtained in this fashion were identified as vitellogenin by Northern analysis (Figure 1) and hybrid-selection, translation techniques (Figure 2). The other nine colonies were identified as apo II by Northern analysis (Figure 1), and one such colony was subject to partial sequence analysis to confirm the identity. Both the nucleotide sequence and restriction map (Figure 4B) are identical to those reported by Wieringa *et al.* (35). As was observed by King *et al.* (16), approximately 10% of the liver cDNA clones were reactive with the Rot-enriched probe for estrogen-induced mRNAs. When the cDNA library was screened with a plus/minus technique using cDNA synthesized from unfractionated RNAs isolated from estrogen-stimulated (plus) and control (minus) rooster liver, a subset of clones corresponding to estrogen-induced mRNAs was also identified. This procedure efficiently detected the VTG II cDNA clones in the library. This procedure is considerably simpler than that described by King *et al.* (16) since it does not involve the preparative fractionation of the cDNA probe to enrich for sequences corresponding to abundant estrogen-induced mRNAs. In essence, with the plus/minus technique the hybridization kinetics select those clones corresponding to abundant mRNAs while the plus and minus probes distinguish those clones corresponding to estrogen-induced mRNAs.

The isolated vitellogenin cDNA clone was further characterized as corresponding specifically to plasma VTG II by partial proteolysis mapping of the translation product directed by mRNA selected via hybridization to the cDNA plasmid (Figure 3). The partial proteolysis maps of the 183,000 dalton band (Figure 3A) and the incomplete translation products (Figure 3B) show peptides which correspond specifically to ^{NG}pVTG II and not ^{NG}pVTG I. The peptide maps of ^{NG}pVTG I and ^{NG}pVTG II are sufficiently distinct that this identification can be made with reasonable certainty.

The molecular weight of the avian vitellogenin polypeptide has been estimated by SDS-polyacrylamide gel electrophoresis in the range of approximately 235,000 to 260,000 (1, 3, 9, 36). Recent studies, however, indicate that the vitellogenin phosphates cause a significant retardation in electrophoretic mobility in SDS-polyacrylamide gels thereby leading to overestimates of the molecular weight (11). Analysis of the nonphosphorylated precursor to VTG II yielded a polypeptide weight of 190,000 (11) while the nonphosphorylated, nonglycosylated form of VTG II yielded a molecular weight of 180,000 on

SDS-polyacrylamide gels (Figure 2, lane 4). Gel chromatography in 7M guanidine-HCl of VTG II or the nonphosphorylated precursor to VTG II also indicated a molecular weight of approximately 180,000 for the vitellogenin polypeptide (11). The largest cell-free translation product directed by hybrid-selected vitellogenin mRNA (Figure 2, lanes 2 and 3, band a) has an apparent molecular weight of 183,000 and reproducibly migrates slightly slower than the nonphosphorylated, nonglycosylated VTG II precursor. This slight difference in mobility might be due to a signal peptide for vitellogenin secretion, although the complex behavior of vitellogenin in SDS gels cautions that other features of vitellogenin might be responsible. Molecular weight estimates of the avian vitellogenin polypeptide made in vitro range from 170,000 to 240,000 (29, 37, 38). Much of this variation is likely due to different conditions of electrophoresis as well as difficulties in the accurate assessment of proteins in this molecular weight range. In addition to the present results, reports from two laboratories also indicate that the cell-free translation products of avian (37) and amphibian (34) vitellogenin mRNA's have significantly greater mobilities in SDS-polyacrylamide gels than plasma vitellogenin. These differences appear to be due to the marked influence of the post-translational modifications upon the mobility of vitellogenin in SDS-polyacrylamide gels. (11, 32). Interestingly, estimates of the size of vitellogenin mRNA range from 4800 to over 7000 nucleotides depending upon the technique and the calibration standards employed. (39, 40). The difficulties in these analyses suggest that the complete nucleotide sequence of vitellogenin mRNA will be required in order to understand the structure of the vitellogenin polypeptides. The present identification of a cDNA clone corresponding to avian VTG II will aid in this effort.

The restriction map of the VTG II cDNA clone (Figure 4A) closely resembles the pVT3 cDNA clone described by Cozens et al. (5), suggesting that the pVT3 cDNA clone corresponds to plasma VTG II. It should be noted, however, that the limited restriction data available may be inadequate to distinguish between VTG I and VTG II clones. More detailed comparisons will be required to conclusively establish the relationship between the VTG II clone and previously described vitellogenin cDNA and genomic clones.

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