Volume 9 Number 24 1981 Nucleic Acids Research

Albumin is encoded by 2 messenger RNAs in Xenopus laevis*

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Received 7 November 1981

ABSTRACT

A cDNA clone library was prepared from liver poly(A) RNA of non-estrogenized *Xenopus laevis*. Albumin coding sequences were screened by hybridization to a cDNA prepared from poly(A) RNA enriched by sucrose density gradient centrifugation, and by a sensitive solid-phase radio-immunoassay to detect clones that contain templates for albumin antigenic determinants. Nine clones were obtained by this approach, and all but one have the cDNA inserted in phase with the β-lactamase gene of pBR322. Mapping of these clones with restriction endonucleases yielded 2 distinct patterns, suggestive of heterogeneity in the coding sequences. This was confirmed by heteroduplex analyses of hybrids formed between clones representative of each of the 2 classes. Both classes of albumin cDNA clones were used to select mRNAs of the same size (2.3kb) that code for peptides that are indistinguishable by SDS gel electrophoresis. Examination of the organization of the albumin genes by blot hybridization of the cDNA clones to restriction fragments of *Xenopus* DNA failed to detect any differences at the genomic level. The considerable diversity of the albumin cDNAs is suggestive of a multiplicity of albumin genes, rather than differential processing of a common precursor RNA.

INTRODUCTION

The vitellogenic response of oviparous vertebrates to estrogen is a useful model system for the study of the regulation of gene expression by steroid hormones. Administration of the steroid to a female or a male animal results in the induction and accumulation of large quantities of vitellogenin mRNA and its subsequent translation and secretion of vitellogenin into the circulation (1,2). During this response the constitutive production of serum proteins, and more specifically serum albumin appears to be repressed by estrogen (3-6). In chickens the decrease in serum albumin is not very dramatic, although functional albumin mRNA has been shown to decrease 2-fold and remain repressed even after vitellogenin synthesis has ceased (3). In *Xenopus*, however, the response is much greater, resulting in the virtual cessation of albumin production both *in vivo* (4) and *in vitro* (5,6). Albumin synthesis might therefore provide a useful model for

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both constitutive gene expression and its repression by a specific hormonal stimulus.

To implement studies on the regulation of albumin gene expression, a double-stranded cDNA to albumin mRNA was cloned into the Pst I site of pBR322. In the course of mapping these cDNA clones with restriction endonucleases, 2 classes of cDNAs were found that had widely dissimilar restriction patterns. The heterogeneity of these cDNAs was confirmed by heteroduplex analyses. In the present report I have utilized these cloned cDNAs to examine some properties of the albumin mRNAs and structural aspects of their corresponding genes.

MATERIALS AND METHODS

Experimental Animals

Laboratory bred male *Xenopus laevis* were obtained from Nasco (Ft. Atkinson, Wis.) and kept in plexiglass aquaria at 20°C with a 12 hr light-dark cycle. They were fed frog brittle every 2 days, except that they were fasted for 4 days prior to killing. Animals were anesthesized by a 1 ml injection i.p. of 0.5 mg tricaine methane sulfonate (Finquel, Ayerst) in 0.9% (w/v) saline. The livers were removed, rinsed in saline, and quickly frozen in liquid nitrogen. The animals were killed by exsanguination and decapitation. The tissue was used the same day for preparation of RNA. The handling of the red sheep for antiserum production was performed by Bethyl Laboratories (Montgomery, Texas).

Isolation of Xenopus Serum Albumin

Serum from nonestrogenized *Xenopus* was dialyzed overnight against 50 mM Tris (pH 7.5) and applied to a DEAE-cellulose column. This was eluted with a linear gradient of 0 to 0.35 M KCl and the peak eluting at 0.14 M salt was collected. This material was concentrated and dialyzed against 20 mM sodium phosphate buffer (pH 7.1). The dialysate was then applied to an Affi-gel Blue column (Bio Rad), washed with 2 bed volumes of phosphate buffer and eluted with 1.4 M NaCl in phosphate buffer. The albumin preparation was then dialyzed against water, lyophilized, and dissolved in 50 mM ammonium bicarbonate buffer. This was applied to a column of Sephadex G-100 (Pharmacia) and the albumin monomer peak was lyophilized to dryness.

Preparation of Sheep Antialbumin Antibodies

100 ug doses of albumin were emulsified in 0.5 ml of H_2O with 0.5 ml of complete Freund's adjuvant and injected intramuscularly into red sheep at 3 week intervals. The first bleeding was performed 2 weeks after the third

injection and bleedings were done 2 weeks after each subsequent injection. The antiserum was clarified and delipidated and the IgG[1] fraction was obtained from 3 successive 33% ammonium sulfate precipitations (7). After extensive dialysis against borate buffered saline 3 ml of the antibody preparation was chromatographed on DEAE-Sephacel (1.6 x 20 cm column) in 17.5 mM sodium phosphate buffer, (pH 6.8). The resultant IgG preparation contained approximately 50% by weight specific antibodies to Xenopus albumin as determined by quantitative precipitation and double immunodiffusion (7). All protein assays utilized the Bradford procedure (8) with a standard of bovine gamma globulin.

Isolation of Xenopus Liver Poly(A) RNA

Total RNA was isolated from the livers of nonestrogenized *Xenopus laevis* by a modification of the guanidine hydrochloride procedure of Deeley et al. (9). It was necessary to treat the isolated nucleic acid with 20 ug/ml of proteinase K followed by 2 extractions with buffer-saturated phenol: chloroform to remove residual melanin contamination. High molecular weight RNA was separated from DNA and small RNAs by precipitation 3 times from sodium acetate and the poly(A) RNA was selected by 2 passages over oligo(dT) cellulose. The yield of poly(A) RNA was 652 ug from 15 g of tissue.

In Vitro Translation Assay and Immunoprecipitation

RNA was translated in 10 ul of a micrococcal nuclease-treated reticulocyte lysate containing [^{35}S]methionine by the method of Pelham and Jackson (10). Samples to be examined directly by electrophoresis received H_2O to 25 ul and 25 ul of 2 X loading buffer (125 mM Tris [pH 6.8], 6% SDS, 10% 2-mercaptoethanol, 20% glycerol). These were heated at 100°C for 10 min and 10 ul samples were then applied to a discontinuous 8.7% SDS-polyacrylamide gel (1 x 100 x 140 mm) (11). Immunoprecipitation of *in vitro* translation products was performed by the method of Sefton et al. (12) using both sheep and rabbit antialbumin antibodies. The gels were processed for fluorography by the method of Bonner and Laskey (13) using DMSO-PPO. They were dried onto Whatman 3MM paper and exposed to Kodak X-Omat R (XR-5) film for 17 hr at 25°C.

Sucrose Density Gradient Centrifugation

The poly(A) RNA was enriched for albumin coding sequences by centrifugation on an isokinetic gradient of 10 to 30% sucrose in 10 mM sodium acetate (pH 5.2), 1 mM EDTA, 0.1% SDS in a SW-41 rotor for 15 hr at 153,000 x g max, 20°C. 0.4 ml fractions were collected from the bottom, adjusted to 10 ug of yeast tRNA, and 0.2 M sodium acetate (pH 5.2), and precipitated from ethanol. The RNA was collected, dissolved in 50 ul of H_2O and translated in the retic-

ulocyte lysate as described. This usually gave a 2-fold enrichment for albumin coding material.

Synthesis and Tailing of a Double Stranded cDNA

cDNA to an albumin enriched mRNA population was prepared by the method of Monahan et al. (14) in which the concentrations of each of the 4 deoxyribonucleotide triphosphates is 1 mM and the reaction is performed at 46°C. Single stranded regions were removed with S1 nuclease and the cDNA was tailed at 37°C with dCMP in a 200 ul reaction containing 200 mM potassium cacodylate (pH 7.2), 1 mM $CoCl_2$, 1 mM 2-mercaptoethanol, 10 uCi α-[^{32}P]dCTP, 1 nM dCTP and 25 units of terminal transferase. This DNA was used to transform a log phase culture of *E. coli* 294 that had been permeablized with Ca^{2+}. The transformed cells were plated on L-agar containing 12.5 ug/ml of tetracycline. All procedures were performed in a P1 laboratory under P1 + EK1 conditions.

Selection of Clones Bearing Albumin cDNA

Tet^rAmp^s colonies were adsorbed onto nitrocellulose filters and screened by a modified Grunstein and Hogness procedure (15) using a [^{32}P] cDNA prepared from enriched albumin mRNA. In order to detect the production of albumin antigenic determinants, the positive colonies were then screened by a solid phase immunological procedure utilizing polyvinyl sheets onto which the antibody was adsorbed (16). For this assay a purified IgG preparation of sheep antialbumin was radioiodinated by the Bolton and Hunter procedure (17) to a specific activity of 4.6 x 10^6 cpm/ug.

Growth, Isolation and Restriction Digestion of Recombinant Plasmids

Cultures bearing recombinant plasmids were grown in M-9 medium by the procedure of Norgard, et al. (18) in which the plasmid DNA was amplified by addition of 1 mg/ml of uridine during log phase growth followed by the addition of 100 mg of chloramphenicol/L. Plasmids were isolated by a clear lysate procedure (19) and purified by cesium chloride density gradient centrifugation (20). The recombinant plasmids were then digested with restriction endonucleases following the protocols given by the suppliers.

Positive Translation Assay for Cloned Albumin cDNA

As a final screening procedure, cloned DNA containing the putative albumin cDNA was coupled to DBM paper and used to select specific mRNAs (21). 100 ug of plasmid DNA was coupled to DBM paper and 10 ug of poly(A) RNA was hybridized to this in 20 mM PIPES (pH 6.4), 5 mM EDTA, 400 mM NaCl, 0.2% SDS, 50% formamide at 42°C overnight. The filters were washed 4 times at 60°C in 20 mM PIPES (pH 6.4), 5 mM EDTA, 200 mM NaCl, 0.1% SDS and the RNA

was eluted with 1 mM EDTA (pH 7) by heating for 2 min at 100°C. It was precipitated twice from ethanol with carrier tRNA. The final pellet was dissolved in 10 ul of sterile H_2O and 1 ul was translated in the reticulocyte lysate. The product of this step was identified by immunoprecipitation.

Analysis of Nucleic Acids by Gel Electrophoresis

Multiple restriction sites were mapped by the method of Smith and Birnsteil (22). DNA restriction fragments were electrophoresed in 1 or 1.5% agarose or 5% polyacrylamide gels in 40 mM Tris, 20 mM sodium acetate, 2 mM EDTA buffer (pH 7.8). They were stained for 0.5 hr in 0.5 ug/ml of ethidium bromide and photographed. RNA was electrophoresed in 1.5% agarose gels in a buffer containing 50 mM boric acid, 5 mM sodium borate, 10 mM sodium sulfate, 0.1 mM EDTA (pH 8.2) and 5 mM methylmercury hydroxide. The RNA was transferred to DBM paper by the procedure of Alwine et al. (23) and these filters were hybridized overnight with 5 x 10^4 cpm/cm^2 of nick translated (24) cloned DNA in 50% formamide, 5 X SSC, 0.5 mg/ml yeast tRNA, 0.2% SDS and 1 X Denhardt's solution. They were then washed in 2 X SSC + 0.1% SDS at 25°C and in 0.1 X SSC + 0.1% SDS at 50°C. Autoradiography was performed with Kodak X-Omat R film.

Xenopus DNA was isolated from the blood, liver and lung of a single animal by gentle homogenization in the presence of 0.5% SDS followed by digestion with boiled RNAase A and proteinase K and a series of extractions with buffer-saturated phenol. The DNA was wound out onto a glass rod under cold ethanol and the procedure of enzymatic digestion and phenol extraction was repeated. After the DNA was wound out a second time it was dried *in vacuo* and unwound into sterile water. The purity of the DNA was determined by the ratios of its absorbance at 260, 280 and 230 nm.

For analysis of the albumin genes the DNA was digested overnight with the appropriate restriction endonucleases and electrophoresed on a 0.8% agarose gel. The restriction fragments were transferred to a nitrocellulose sheet by the procedure of Southern (25) and were hybridized overnight at 68°C with 10^6 cpm/cm^2 nick-translated cloned DNA in 2X Denhardt's solution + 0.5% SDS and 1 mM EDTA. The filters were washed 3 times (2h/wash) at 68°C with 1X SSC + 0.5% SDS. They were air dried and autoradiographed at -70°C.

Electron Microscopy of Nucleic Acid Hybrids

Plasmids were linearized by digestion with *Bam* HI and equivalent amounts were mixed at a concentration of 1.5 μg/ml in 10 mM Tris, 1 mM EDTA (pH 7.6). They were denatured by heating at 60°C for 10 min in 80% (v/v) recrystallized formamide. 200 mM Tris, 20 mM EDTA (pH 8.5) was added to reduce the formamide concentration to 66% and the plasmids were annealed at 25°C for 2-3 h. fd

phage and SV40 form II DNAs were then added as single- and double-stranded size markers followed by cytochrome c to a concentration of 0.3 mg/ml. The DNA was picked up from a 10% formamide hypophase on parlodian-coated 200 mesh copper grids, stained with uranyl acetate, rinsed with isopentane and rotary shadowed with gold-palladium in an Edwards evaporator. Grids were examined in a Zeiss EM 10A electron microscope and molecules were photographed at a magnification of 9800X.

Enzymes

All restriction endonucleases except Hind III were obtained from Bethesda Research Laboratories, as was DNA polymerase and terminal transferase. Hind III and S1 were obtained from Boehringer-Mannheim Biochemicals. AMV reverse transcriptase was the kind gift of Dr. J. Beard of Life Sciences, Inc. Deoxyribonuclease I was purchased from Worthington.

Chemicals and Isotopes

Deoxyribonucleotide triphosphates, and obligo(dT) were obtained from P-L Biochemicals. Guanidine hydrochloride was purchased from BRL. Oligo(dT) cellulose (type 3) was purchased from Collaborative Research. Agarose (ME) was obtained from Sea Kem. Tricaine methanesulfonate was obtained from Ayerst. [^{32}P]dCTP and dTTP (2000-3000 Ci/mmol) and [^{3}H]dCTP (25 Ci/mmol) were purchased from New England Nuclear. [^{125}I] Bolton-Hunter reagent was purchased from Amersham. Autoradiography was performed on Kodak X-Omat R film (XR-5) and enhancement was obtained with Dupont Cronex Lighting Plus screens. All other reagents were of the highest purity available.

RESULTS

Characterization of Xenopus Serum Albumin

This study was initiated by a characterization of Xenopus serum albumin so that monospecific antibodies to this protein could be used for the identification of its corresponding mRNA. Albumin was isolated by chromatography of normal male serum on DEAE-cellulose followed by affinity chromatography on Affigel blue, a resin containing Cibacron Blue that exhibits a specific hydrophobic interaction with albumins. The protein so selected was then isolated from a Sephadex G-100 column so that the monomeric form was predominant. Albumin is a protein whose essential structural features have been highly conserved throughout evolution so it was useful to compare the amino acid composition of the Xenopus protein with those from bovine and human sera to confirm the identity of the amphibian protein. These data are presented in Table I.

TABLE I

Comparison of the Amino Acid Composition of Xenopus Serum Albumin with Bovine and Human Serum Albumins

Amino Acid	Xenopus	Number of Residues Bovine	Human
Cys	33	35	35
Asp + Asn	70	53	53
Thr	33	34	27
Ser	22	28	24
Glu + Gln	98	78	88
Pro	34	28	24
Gly	14	15	12
Ala	37	46	63
Val	25	36	39
Ile	23	14	8
Leu	57	61	61
Tyr	15	19	18
Phe	27	26	31
His	35	17	16
Lys	58	59	59
Arg	17	23	24
Met	11	4	6

The amino acid analysis of Xenopus serum albumin yielded a minimum molecular weight of 6471 with Met as the least abundant amino acid. The number of Met residues [11] was determined by dividing the known molecular weight (72,000) of albumin by the minimum molecular weight. The quantities of the remaining amino acid residues were determined by the relative abundance ratios with respect to Met to yield a theoretical molecular weight of 71,500. The values for bovine and human serum albumins were obtained from the data of Brown (26). Xenopus albumin has 16 disulfide bonds as compared to 17 in the mammalian proteins (data not shown).

The features to note about these data are the similarities in the content of Cys, Pro, Leu, Tyr and Phe between the three proteins. Pro and Cys play essential roles in the maintenance of the three domain structure of albumin (26) and Leu, Tyr and Phe are highly conserved in their location and number in the two mammalian proteins. The complete amino acid sequence of XSA would be necessary for a definitive comparison between these proteins, however, these data and the physical properties of our preparation strongly suggest that it is indeed albumin. In agreement with literature reports (27) we have found this protein to have a relative molecular mass of 72,000 which is larger than its mammalian counterpart (69,000).

Isolation and Preliminary Characterization of Albumin mRNA Activity

RNA was isolated from *Xenopus* liver by a modified guanidine hydrochloride extraction procedure and the poly(A) RNA was selected on oligo(dT) cellulose. To determine the presence of mRNA coding for albumin peptides, the poly(A) RNA was translated in a micrococcal nuclease-treated reticulocyte lysate and the primary translation products were analyzed by immunoprecipitation and SDS gel electrophoresis. For these studies we employed 2 antisera that were raised independently against XSA. The first was a rabbit antiserum prepared in Dr. Robin Wallace's laboratory, and the second was a sheep antiserum prepared in our own laboratory. The RNA samples were translated according to the procedure described in Methods along with a sample of poly(A) RNA obtained from HeLa cells during the late stage of adenovirus infection. The latter served as an internal standard both for the efficiency of translation and for the size of the translation product (along with a series of stained standards).

The data in Figure 1 show that a major band with a relative molecular mass of 75,000 was specifically immunoprecipitated with both the rabbit and sheep antisera to XSA. When the mobility of this peptide was compared to that of XSA (72,000) it was found to migrate slower under all conditions examined.

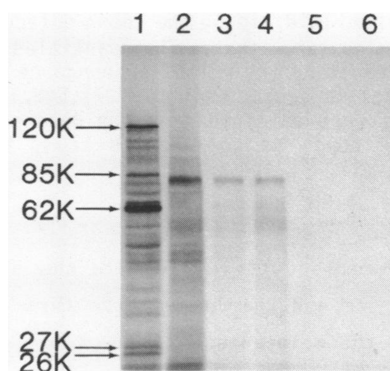

Figure 1. Analysis of *in vitro* translation products of poly(A) RNA. 1 ug samples of late adenovirus poly(A) RNA (lane 1) or *Xenopus* poly(A) RNA (lane 2) were translated for 1 hr at 37°C in a 10 ul reticulocyte lysate containing [^{35}S]methionine. The translation products were immunoprecipitated with sheep antialbumin (lane 3) rabbit antialbumin (lane 4), preimmune sheep serum (lane 5) and preimmune rabbit serum (lane 6). The mobilities of the molecular weight standards were determined after staining and the gel was then impregnated with PPO in DMSO and processed for fluorography.

The difference of approximately 3000 in relative molecular mass is equivalent to 26 amino acids and most likely reflects the synthesis of a precursor peptide in vitro. The "prepro" peptide of rat serum albumin contains 24 amino acids (28), and in the absence of sequence data on the Xenopus protein we term the primary translation product "preproalbumin".

To approximate the size of albumin mRNA and to enrich for this species, poly(A) RNA was centrifuged on a 10 to 30% sucrose density gradient and the resultant fractions were translated in vitro. Messenger activity for this protein had a sedimentation rate of 17S. Prior to this step albumin immunoprecipitable material accounted for 17% of the TCA-precipitable peptides produced by in vitro translation. Subsequent translation assays showed the fraction recovered from the gradient now encoded 40% of the TCA-precipitable peptides, a 2-fold enrichment.

Synthesis of a Double Stranded cDNA and Cloning in E. Coli

A double stranded cDNA to the preparation enriched for albumin mRNA was synthesized by standard procedures using AMV reverse transcriptase. The conditions used (46°C, 1 mM dXTPs) tend to give lower overall yields of cDNA, however the preparation so obtained has a higher proportion of full length transcripts. The single stranded regions were removed with S1 nuclease to yield the preparation used for cloning. Electrophoresis of the cDNA after denaturation with glyoxal (29) indicated that the hair-pin loop had successfully been cleaved and there was a substantial recovery of material of >2000 bp, which should roughly correspond to the size of albumin mRNA (data not shown).

An average of 20 residues of dCMP were added onto the 3' ends of the double stranded cDNA using terminal transferase. 750 ng of the resultant preparation was annealed with 3.2 ug of the plasmid pBR322 that had been exhaustively digested with the restriction endonuclease Pst I and tailed on the 3' ends with dGMP. This was then used to transform a log phase culture of E. coli (294). Approximately 4000 colonies were obtained, 95% of which were Tet^r Amp^s.

900 colonies were picked at random and screened for serum albumin related sequences by a modified Grunstein-Hogness procedure (16). To screen these, a [^{32}P]cDNA probe was prepared from the enriched mRNA preparation and hybridized under conditions of low stringency (6 X SSC) followed by washes of high stringency (1 X SSC). The 82 positive colonies detected by this procedure were selected for further screening.

Immunological Screening for Clones Producing Albumin Antigenic Determinants

With 82 putative albumin clones there was a good probability that some of these might have the cDNA inserted in an orientation such that albumin sequences might be expressed by the bacteria. To examine this possibility these clones were screened by the solid phase immunoassay of Broome and Gilbert (17). The clones were grown overnight on tetracycline containing agar and lysed with $HCCl_3$ vapor. A set of albumin standards was then applied to the agar surface and the colonies were overlaid with a polyvinyl chloride plastic sheet containing sheep antialbumin. After 3 hr at 4°C the sheets were washed and incubated overnight with the same IgG preparation that had been radioiodinated to a high specific activity. Nine positive clones were obtained (Figure 2). The sensitivity of antibody reaction was 1 pg of albumin. The clones are denoted by the prefix pXlA (for *Xenopus laevis* albumin).

Positive Selection of Albumin mRNAs with Cloned DNA

The final identification of the albumin clones was accomplished by a procedure in which albumin mRNA was selected from poly(A) RNA on DBM filters containing immobilized plasmid DNA. The analysis of the *in vitro* translation products from these mRNAs by SDS gel electrophoresis is shown in Figure 3. No activity was recovered on a filter containing the control pBR322 DNA, and all 6 cloned probes retrieved albumin mRNA. The absolute identity of these peptides was determined in a parallel experiment by immunoprecipitation of the translation products as in Figure 1.

Restriction Analysis of Albumin cDNA Clones

Up to this point there was no reason to presume that albumin was encoded by by more than one species of mRNA in *Xenopus*. However, in the process of mapping the cDNA clones with restriction endonucleases it became apparent that there were 2 classes of restriction patterns. These are shown in Figure 4, with fine structure maps for representatives of each class that comprise the full length of the cDNAs, (pXlA 1, 23 and 14) and partial maps for some smaller clones of the class I cDNAs (pXlA 6 and 61). It is clear from these data that there are virtually no overlapping restriction sites between the class I and class II cDNAs. The albumin cDNA clones are also unusual in that many restriction enzymes do not have sites within the coding sequences. Single sites were found for *Hind* III (class I and II), *Eco* RI (class II), *Hha* I (class I and II) and *Pst* I (class I). Both classes have 2 *Hae* III sites and the class I clones have 2 *Eco* RI sites. Additional sites for *Mbo* II and *Ava* II have not been mapped to date. There were no sites for *Bam* HI, *Hpa* I & II, *Taq* I, *Sma* I, *Bgl* I & II, *Xba* I, *Kpn* I, *Xho* I, *Sst* I & II, *Ava* I, *Sal* I, *Sac* I, *Pvu* I & II, *Bcl*

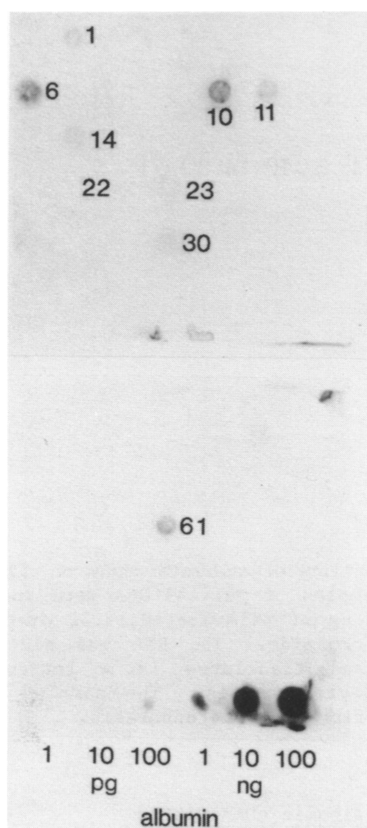

Figure 2. Immunological screening for clones producing albumin antigenic determinants. 82 clones selected by hybridization to [^{32}P] cDNA were grown on Tet agar, lysed with $HCCl_3$ vapor and overlaid with PVC sheets containing sheep antialbumin. The PVC sheets were washed with preimmune serum in PBS and incubated overnight with [^{125}I] antialbumin. Standards of 1 pg to 100 ng of albumin were applied to the agar prior to immunoadsorption.

I and Bst EII.

The lengths of the class I clones constitute virtually the complete cDNA for its corresponding mRNA as does the single class II clone pXlA 14 (see below). Furthermore, all of the clones were inserted in the same orientation in the plasmid with respect to the β-lactamase gene except for pXlA 6, which was inverted. The expression of albumin antigenic determinants by this clone was most likely due to read-through from the very strong P4 promoter of pBR322 (30).

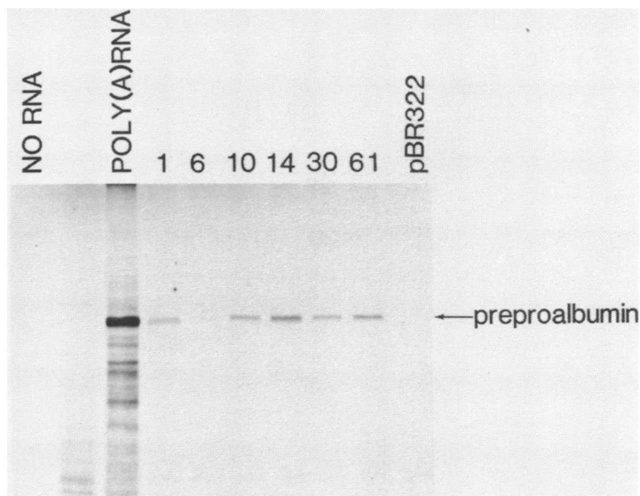

Figure 3. Positive selection of albumin mRNA on filters containing cloned DNA. 10 ug samples of poly(A) RNA were incubated with DBM filters containing 100 ug of pXlA 1,6,10,14,30 or 61 or pBR322 for 16 h at 42°C in 50% formamide. The RNA was eluted at 100°C with 1 mM EDTA (pH 7) and translated in a reticulocyte lysate followed by SDS gel electrophoresis. The samples shown were not immunoprecipitated prior to electrophoresis.

Heteroduplex Analysis of Albumin cDNA Clones

To verify that the class I and class II cDNA clones were indeed heterogeneous, we prepared heteroduplexes between the 2 classes and examined the hybrids by electron microscopy. Hybrids formed between the class I cDNA clones (such as pXlA 1 x 23) demonstrated homology, as was expected from the restriction maps (data not shown). However, heteroduplexes formed between the 2 classes showed substantial mismatching. Figure 5 shows a heteroduplex formed between the class I clone pXlA 1 and the full-length class II clone pXlA 14. It is clear that the majority of the cDNAs did not hybridize, as evidenced by the the presence of a single large bubble that corresponds to the sizes of the cloned inserts as determined by gel electrophoresis. These data therefore confirm the classification of the cDNA clones into 2 groups and underscore the differences between their coding sequences.

Analysis of Albumin mRNAs on Denaturing Gels

In light of the differences between the restriction maps of the class I and class II cDNA clones, albumin mRNA was examined for size heterogeneity by

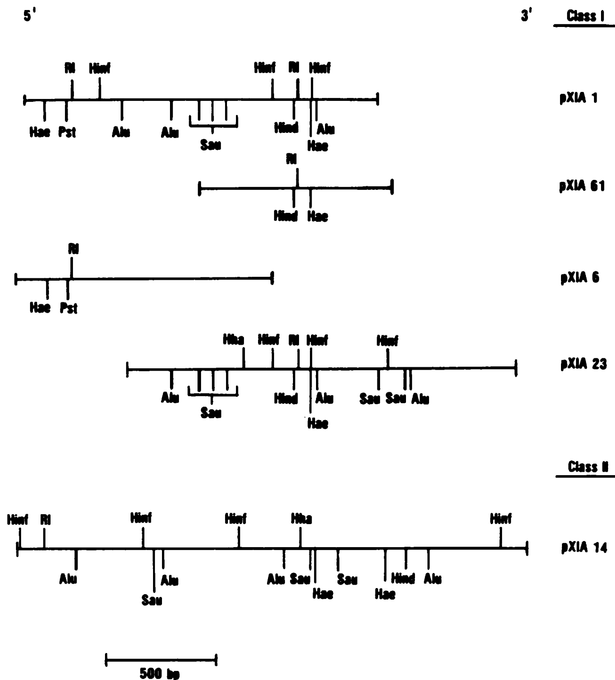

Figure 4. Restriction maps of albumin cDNA clones. The representative class I clones pXlA 1 and 23 and the full-length class II clone pXlA 14 were mapped in detail. Together, pXlA 1 and 23 constitute the full length of albumin mRNA. The terminal bars represent the Pst insertion sites, which were reconstituted in all cases. The enzymes mapped are Pst I, Eco RI, Hinf I, Alu I, Sau 3A, Hind III, Hae III and Hha I.

electrophoresis in a methylmercury hydroxide gel (Figure 6). Samples of total and poly(A) liver RNA were electrophoresed and blot-transferred onto DBM paper, followed by hybridization to the class I clones pXlA 1,6, or 61, and the class II clone pXlA 14 that were radiolabeled by nick-translation. In all cases, the albumin clones hybridized to a single band with a length of 2300 nucleotides. These data indicate that there is no size heterogeneity detectable in the albumin mRNA population, and the class II clone pXlA 14 is a full length copy of the mRNA. Taken together, the class I clones pXlA 1,6 and 61 span 83% of the cDNA, lacking only the terminal 400 nucleotides of the 3' end. These terminal nucleotides are present in pXlA 23, which contains the full 3' half of the cDNA. The size of the albumin mRNAs corresponds favorably with the 2045 nucleotides that are required to code for the primary

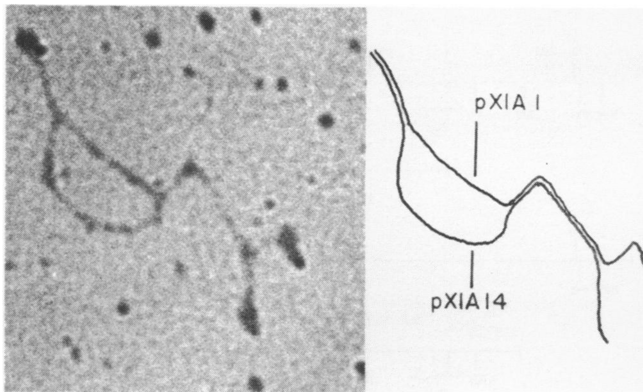

Figure 5. Heteroduplex analysis between class I and class II clones. Hybrids were formed between pXlA 1 (class I) and pXlA 14 (class II) according to the procedure outlined in Methods. Similar results were obtained if the class I clone pXlA 23 was used.

translation product, leaving 250 untranslated bases. The lengths of the poly(A) tails on albumin mRNA have not been determined.

Blot Hybridization of Xenopus DNA With Albumin cDNA Clones

Southern blot hybridization was performed with these clones on restriction fragments of *Xenopus* DNA to determine if the different restriction maps of the class I and class II cDNAs were reflected in the structural organization of the albumin genes. DNA was isolated from a single animal of the laboratory-bred strain of *Xenopus* and it was digested overnight with *Taq* I and *Bam* HI, which have no restriction sites within the cDNAs, and *Eco* RI and *Hind* III, which both cleave the cDNAs. After electrophoresis these fragments were transferred onto nitrocellulose and replicate filters were hybridized with the class I clones pXlA 1,6,61 or with the class II clone pXlA 14 (Figure 7).

In the case of the class I clones, *Bam* HI released the albumin gene intact as a large fragment of approximately 23 Kb. *Taq* I digestion produced a series of fragments, which suggests that the *Taq* sites are contained within the intervening sequences. The hybridization of the different class I cDNA probes gave many similar *Taq* fragments, however pXlA 6 failed to hybridize to fragments of 6 Kb and 4.5 Kb that were apparent when pXlA 1 was used as the probe. These difference are accounted for by the absence of sequences from the 3' half of the cDNA in pXlA 6 (Figure 4). Differences of a similar nature were detected when the pattern obtained with the 3' probe pXlA 61 was

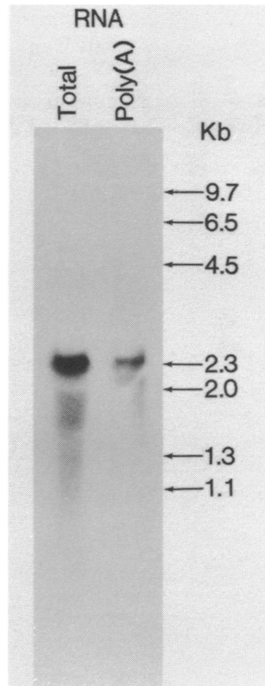

Figure 6. Analysis of albumin mRNA on denaturing gels. 100 ug of total liver RNA or 1 ug of poly(A) RNA were electrophoresed on a 1.5% agarose gel containing 5 mM methylmercury hydroxide. The RNA was transferred to DBM paper and hybridized to the same nick translated probes as used in Figure 5. In this figure the probe was the class II clone pXlA 14.

compared to those for pXlA 1 and 6. In each case, the failure of a particular class I probe to detect any given DNA fragment can be explained by the relative representation of the coding sequence in the cloned cDNA. The same holds true for the hybridization of the class I clones to Hind III and Eco RI fragments.

When the full-length class II probe pXlA 14 was hybridized to the nitrocellulose filters, the patterns of restriction fragments obtained were virtually identical to those obtained with the class I probes. In this case, however, all of the restriction fragments identified with the separate class I probes were visualized. An unusual feature of all of these filters is the very strong hybridization to a 3.5 Kb Taq fragment. The significance of this observation is not known. In all, these data indicate that the very different

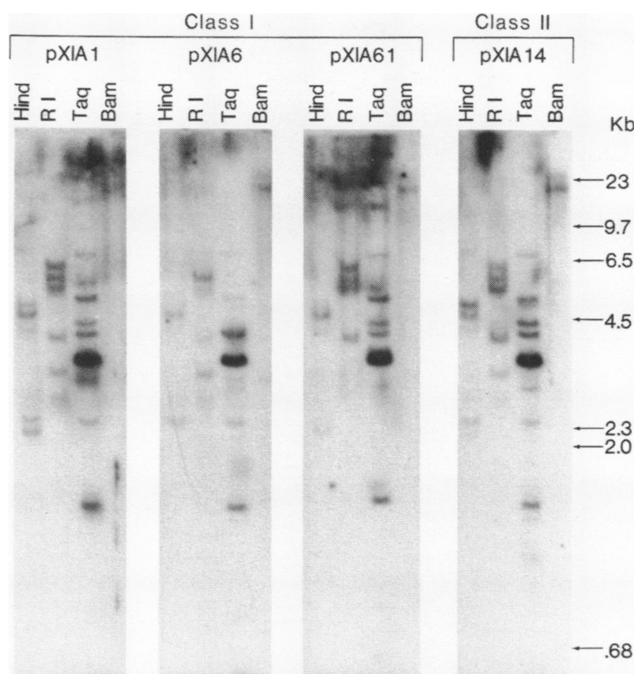

Figure 7. Southern blot hybridization of Xenopus DNA to albumin cDNAs. Xenopus DNA was digested with Eco RI and Hind III (which both have sites within the coding sequences) and Taq I and Bam HI (which have no sites within the coding sequences). The DNA samples were electrophoresed on a 0.8% agarose gel and transferred to nitrocellulose filters. These were hybridized to each of the indicated cDNA clones that were radiolabeled by nick translation.

albumin mRNAs are transcribed from the same gene and are radically altered during processing, or more likely, are encoded by 2 related genes.

DISCUSSION

There have been a number of reports in the literature dealing with Xenopus albumin, however a definitive identification of this protein has been lacking. Prior to any attempts at cloning nucleic acids that encode this protein it was essential to isolate and characterize the pure peptide and prepare monospecific antibodies to it. For this purpose we defined albumin by its size, abundance, hydrophobicity, and chromatographic behavior on Cibacron Blue-Sepharose. The purified protein has a relative molecular mass of 72,000 and an amino acid composition similar to that of bovine

and human serum albumins (Table I). The most notable features are the similar numbers of Cys, Pro, Leu, Tyr and Phe residues between the different albumins. These amino acids play critical roles in maintaining the 3 domain structure (26) of albumin and would likely be conserved. Furthermore XSA has the Cys residues organized in 16 disulfide bonds; HSA and BSA each have 17 disulfides. This purified protein was then used to raise a high titre monospecific antiserum for use in characterizing sequences that encode albumin antigenic determinants.

The isolation of liver poly(A) RNA and the preparation of the cDNA clones was straightforward. These clones were screened for Amp^s Tet^r phenotype, hybridization to a ^{32}P-cDNA prepared from an enriched poly(A) RNA preparation, and by a sensitive solid-phase radioimunoassay to detect the production of albumin antigenic determinants. In all, 9 clones were selected in this manner, all but one of which have the cDNA inserted in the same orientation with respect to the β-lactamase gene of pBR322. This is similar to the results obtained by Chang et al. (31) with dihydrofolate reductase and may be the result of read-through from the very strong P4 promoter of pBR322 (30).

When these clones were mapped with restriction endonuleases they were found to fall into 2 groups, termed here class I and class II. The members within each class had overlapping restriction patterns, however there were virtually no similarities in the restriction maps between the 2 classes. For the purpose of clarity, I have concentrated on 3 of these clones, pXlA 1, 23, and 14. Clones 1 and 23 together constitute the full length of the class I cDNA and represent the 5' and 3' ends, respectively. Clone 14 is a full length copy of the class II cDNA.

To determine if the heterogeneity in restriction maps was due to heterogeneity in the coding sequences, hybrids were formed between the representative cDNA clones and these were examined by electron microscopy. These experiments confirmed the observations based on restriction mapping and demonstrated that the cDNAs share little homology. Together, the restriction mapping and heteroduplex analyses indicate that the sequences that encode *Xenopus* albumin are widely divergent.

In the present study, I examined albumin mRNA and its *in vitro* translation products by electrophoresis under denaturing conditions and was unable to detect any size heterogeneity. Total and poly(A) RNA from *Xenopus* liver displayed a single band of 2.3 kb when DBM blots of methylmercury hydroxide gels were hybridized to nick translated probes from either the class I or class II cDNAs. Furthermore, no size heterogeneity was detectable for mRNAs that were first

isolated on DBM-filters containing the cloned cDNAs (data not shown). In addition, the data in Figure 3 show that the translation products of these mRNAs have identical mobilities on SDS gels.

Some aspects of the structural organization of the albumin genes were probed by Southern blot hybridization of Xenopus DNA restriction fragments to both class I and class II cDNA clones. In these experiments, the DNA was digested with Eco RI and Hind III, which both have sites within the cDNAs, and Taq I and Bam HI, which do not. Hybridization of the nitrocellulose filters to the various class I probes shown in Figure 7 resulted in overlapping patterns that reflected the relative representation of the mRNA sequence that was present in the respective cDNA clones. When the full-length class II cDNA clone pXlA 14 was the probe, the pattern obtained was identical to that observed with the class I probes.

There are several possible interpretations for these data. First, there is but one albumin gene and the different mRNAs that encode the protein are the product of differential processing of the primary transcript, as has been observed for immunoglobulin μ mRNA (32,33). I feel this is unlikely because the heteroduplexes formed between the class I and class II cDNA clones show very little homology, and one would expect these to be similar based on results obtained with other genes. Second, there are 2 albumin genes with enough homology between their sequences that the hybridization conditions employed did not differentiate between them. Third, there are 2 albumin genes that are similar in their structural organization and this was demonstrated by similar hybridization patterns obtained with the class I and class II cDNA probes. At present I cannot differentiate between the latter 2 possibilities; however, this is under investigation and should be clarified upon isolation of the genes from a DNA library. Finally, the observation of 2 mRNAs for albumin supports an earlier report by Bisbee et al. (34) that Xenopus laevis has 2 albumin peptides and it lends support to the notion proposed by these authors that the genome of Xenopus is duplicated.

ACKNOWLEDGEMENTS

I wish to thank Dr. James H. Clark of the Department of Cell Biology for his encouragement and support of this research which was initiated while I was a postdoctoral fellow in his laboratory. I am also grateful to Drs. Robin Wallace, Steven Wiley and Lee Opresko of the Oak Ridge National Laboratory for their gifts of Xenopus serum albumin and rabbit antiserum, and their continued help in the analysis of this protein. Dr. Fred Hartman and Mr. Claude Stringer, also of the Oak Ridge Laboratory performed the amino acid analysis. Special thanks go to Drs. Eugene Lai, Charles Lawrence and Jeff Nordstrom of the Department of Cell Biology for their advice and support throughout this work and to Dr. Bill Ruyechan of the Department of Biochemistry, USUHS,

for his help with the heteroduplex analysis. The technical assistance of Mr. Richard C. Pendleton is gratefully acknowledged.

* Supported by grants CA-05856 to D. R. Schoenberg and CA-20605 to J. H. Clark from the National Cancer Institute, USA, and grant C07525 from the Uniformed Services University.

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1 The abbreviations used are: XSA, *Xenopus* serum albumin; bp, base pairs; SDS, sodium dodecyl sulfate; IgG, immunoglobulin G; SSC, 0.15M NaCl, 0.015 M sodium citrate (pH 7.0); Denhardt's solution, 3 x SSC, 0.02% Ficoll 400, 0.02% polyvinylpyrrolidone, 0.02% bovine serum albumin; PBS, phosphate-buffered saline.

NOTE: Subsequent to the original submission of this manuscript, a report appeared from Westley et al. [(1981) Nucl. Acid Res. 9,3557-3574] on the preparation of cDNA clones to *Xenopus* albumin. There are considerable differences between the restriction maps presented in that paper and those presented in this manuscript.

REFERENCES

1. Ryffel, G.U. (1978) Mol. Cell. Endocrinol. 12, 237-246
2. Tata, J.R. and Smith, D.F. and Smith, D.F. (1979) Recent Prog. Hor. Res. 35, 47-90 3. Williams, D.L., Wang, S. and Klett, H. (1978) Proc.
3. Williams, D.L., Wang, S. and Klett, H. (1978) Proc. Natl. Acad. Sci. USA 75, 5974-5978
4. Farmer, S.R., Henshaw, E.C., Berridge, M.V. and Tata, J.R. (1978) Nature 273, 401-403
5. Green, C.D. and Tata, J.R. (1976) Cell 7, 131-139
6. Wangh, L.J., Osborne, J.A., Hentschel, C.C. and Tilly, R. (1979) Dev. Biol. 70, 479-499
7. Garvey, J.S., Cremer, N.E. and Sussdorf, D.H. (1977) Methods in Immunology, 3rd Ed, pp. 218-226, W.A. Benjamin, Reading, Mass.
8. Bradford, M. (1976) Anal. Biochem. 72, 248-254
9. Deeley, R.G., Gordon, J.I., Burns, A.T.H., Mullinix, K.P., Bina-Stein, M. and Goldberger, R.F. (1977) J. Biol. Chem. 252, 8310-8319
10. Pelham, H.R.B. and Jackson, R.J. (1976) Eur. J. Biochem. 67, 247-256
11. Laemmli, U. (1970) Nature 227, 680-685
12. Sefton, B.M., Beemon, K. and Hunter, T. (1978) J. Virol. 28, 957-971
13. Bonner, W.M. and Laskey, R.A. (1974) Eur. J. Biochem. 46, 83-88
14. Monahan, J.J., McReynolds, L.A. and O'Malley, B.W. (1976) J. Biol. Chem. 251, 7355-7362
15. Grunstein, M. and Hogness, D. (1975) Proc. Natl. Acad. Sci. USA 72, 3961-3965
16. Broome, S. and Gilbert, W. (1978) Proc. Natl. Acad. Sci. USA 75, 2746-2749
17. Bolton, A.E. and Hunter, W.M. (1973) Biochem. J. 133, 529-539
18. Norgard, M.V., Emigholz, K. and Monahan, J.J. (1979) J. Bacteriol. 138, 270-272
19. Clewell, D.B. and Helinski, D.R. (1970) Biochemistry 9, 4428-4440
20. Radloff, R., Bauer, W. and Vinograd, J. (1967) Proc. Natl. Acad. Sci. USA 57, 1514-1521
21. Stark, G.R. and Williams, J.G. (1979) Nucl. Acid Res. 6, 195-203
22. Smith, H.O. and Birnsteil, M.L. (1976) Nucl. Acid Res. 3, 2387-2398

23. Alwine, J.C., Kemp, D.J. and Stark, G.R. (1977) Proc. Natl. Acad. Sci. USA 74, 5350-5354
24. Mackey, J.K., Brackmann, K.H., Green, M.R. and Green, M. (1977) Biochemistry 16, 4478-4483
25. Southern, E.M. (1975) J. Mol. Biol. 98, 503-517
26. Brown, J.R. (1977) In Albumin Structure, Function and Uses, Rosenoer, V.M., Oratz, M. and Rothschild, M.A., Eds., pp. 27-51, Pergamon Press, New York
27. Berridge, M.V., Farmer, S.R., Green, C.D., Henshaw, E.C. and Tata, J.R. (1976) Eur. J. Biochem. 62, 161-171
28. Strauss, A.W., Bennett, C.A., Donohue, A.M., Rodkey, J.A., Boime, I. and Alberts, A.W. (1978) J. Biol. Chem. 253, 6270-6274
29. McMaster, G.K. and Carmichael, G.G. (1977) Proc. Natl. Acad. Sci. USA 74, 4835-4838
30. Stuber, D. and Bujard, H. (1981) Proc. Natl. Acad. Sci. USA 78, 167-171
31. Chang, A.C.Y., Nunberg, J.H., Kaufmann, R.J., Erlich, H.A., Schimke, R.T. and Cohen, S.W. (1978) Nature 275, 617-624
32. Rogers, J., Early, P., Carter, C., Calame, K., Bond, M., Hood, L. and Wall, R. (1980) Cell 20, 303-312
33. Early, P., Rogers, J., Davis, M., Calame, K., Bond, M., Wall, R. and Hood, L. (1980) Cell 20, 313-319
34. Bisbee, C.A., Baker, M.A., Wilson, A.C., Hadji-Azimi, I. and Fischberg, M. (1977) Science 195, 785-787