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Xenopus laevis serum albumins are encoded in two closely related genes

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ABSTRACT

cDNA clones containing sequences complementary to Xenopus laevis albumin mRNA have been identified in a collection of cDNA clones made from poly(A)+ RNA prepared from male Xenopus laevis liver. Although all the albumin cDNA clones crosshybridise, restriction enzyme and heteroduplex analysis show that there are 2 closely related albumin mRNA sequences. The 2 albumin mRNAs are only mismatched by 8% but could be isolated by positive selection using stringent hybridization conditons. Oocytes injected with the 2 purified mRNAs, secreted either the 68,000 or 74,000 dalton albumin into the culture medium showing that the 2 albumins of X. laevis serum are encoded in the 2 closely related mRNAs. Measurements of the abundance of albumin mRNA show that the 2 albumin mRNAs together account for about 9% of total poly(A)+ RNA in male Xenopus laevis liver but the mRNA coding for the 74,000 dalton mRNA is about twice as abundant as that coding for the 68,000 dalton mRNA.

INTRODUCTION

Albumin, the most abundant serum protein in vertebrates, is made and secreted by the liver in which it normally accounts for about 10% of total protein synthesis. As albumin is synthesized in large quantities exclusively in one tissue, it is a useful protein for studying factors regulating tissue specific gene regulation. Moreover, in certain amphibian species, the levels of albumin have been reported to increase at metamorphosis (1) and to decrease in adults after estrogen treatment (2) and so it is also of interest as a hormonally and developmentally regulated protein. In Xenopus laevis, it has been claimed that there are two albumins of 68,000 and 74,000 daltons (3,4) whereas most other vertebrate species examined have only one albumin. It has been suggested that the gene coding for albumin was duplicated as part of a genome duplication which occurred about 30 million years ago (4) and that the resulting genes have subsequently diverged so that they now code for two proteins of quite distinct molecular weights which are expressed at different levels. As a first step towards characterising the mRNAs and genes coding for the two albumins and the

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differences that have evolved between them, we have isolated and characterised cDNA clones containing sequences complementary to Xenopus albumin mRNA. In this study we show that there are 2 closely related albumin mRNA sequences expressed in male Xenopus liver and that these 2 sequences code for the 68,000 and 72,000 dalton albumins of Xenopus serum. These findings are discussed in relation to the evolution of the X. laevis genome.

MATERIALS AND METHODS

α-^{32}P-dCTP (2000-3000 Ci/mmol), α-^{3}H-dCTP (19 Ci/mmol), α-^{3}H-dGTP (10 Ci/mmol) and L-^{35}S-methionine (1400 Ci/mmol) were obtained from The Radiochemical Centre, Amersham, UK. Non-radioactive deoxynucleotides, restriction enzymes, DNA polymerase and oligo-d(pT)$_{12-18}$ were from Boehringer Mannheim: AMV reverse transcriptase was a gift from J.W. Beard, Life Sciences; terminal transferase was from PL Biochemicals Ltd; low melting agarose was from Biorad; Sephacryl S200, poly (U)-sepharose 4B were from Pharmacia.

Preparation of RNA

Cytoplasmic RNA was isolated from the livers of male Xenopus laevis and fractionated on poly(U)-sepharose as described previously (5). Poly(A)+ RNA was fractionated by centrifugation through a 17 ml 5-20% sucrose gradient, containing 10 mM Tris pH 7.4, 10 mM NaCl, 1 mM EDTA and 0.2% SDS, for 21 h at 15° C and 25,000 rpm in a Beckman SW 27 rotor.

Preparation of cDNA clones

10 μg of poly(A)+ RNA from the 18 S region of the gradient containing albumin mRNA was transcribed in a total volume of 500 μl containing 1 mM dATP, dGTP and dTTP, 0.2 mM dCTP, 10 μCi α-^{32}P-dCTP, 50 mM Tris pH 8.3, 10 mM $MgCl_2$, 0.4 mM dithiothreitol (DTT), 100 mM KCl, 20 μg/ml oligo-d(pT)$_{12-18}$ and 4 mM sodium pyrophosphate. This mixture was incubated on ice for 10 min, 200 units AMV reverse transcriptase added and incubated for 1 h at 42° C. It was then heated in a boiling waterbath for 4 min, cooled rapidly on ice and centrifuged in a bench-top centrifuge at 4° C for 2 min.

For the synthesis of the second strand, 500 μl of 1 mM dATP, dTTP, dGTP and dCTP, 50 mM Tris pH 7.3, 10 mM $MgCl_2$, 0.4 mM DTT and 4 mM sodium pyrophosphate were mixed with the supernatant of the first strand synthesis, 500 units of AMV reverse transcriptase added and incubated for 1 h at 42° C. EDTA (to 20 mM) and yeast tRNA (to 10 μg/ml) were added, the cDNA was extracted with an equal volume

of phenol and precipitated with ethanol. The precipitated cDNA was dissolved in 200 μl of 10 mM Tris pH 7.3 and passed over a 5 ml column of Sephacryl S200 equilibrated with S1 nuclease buffer (0.3 M NaCl, 30 mM sodium acetate pH 4.5, 3 mM $ZnSO_4$). Most of the cDNA (about 1 μg) eluted in the excluded volume and was therefore separated from the tRNA and small cDNA molecules; it was collected in a volume of 0.6 ml and digested with S1 nuclease, to remove single stranded cDNA, for 1 h at 37° C. Tris pH 8 (to 10 mM), EDTA to 10 mM and tRNA to 10 μg/ml were added and the DNA phenol extracted and ethanol precipitated.

The precipitate was dissolved in 50 μl 10 mM Tris pH 7.5, 1 mM EDTA and passed over a 5 ml column of Sephacryl S200, equilibrated with 100 mM potassium cacodylate, 2 mM $CoCl_2$ and 0.1 mM DTT, to remove tRNA and equilibrate the cDNA with the correct buffer for homopolymer tailing. The cDNA (about 600 ng) was collected in the excluded volume (300 μl). A 50 μl aliquot was tailed with about 30 dC residues by adding α-^{3}H-dCTP (to 0.25 mM), bovine serum albumin (to 100 μg/ml) and terminal transferase (to 100 units/ml) and incubating for 4 min at 37° C. Incorporation of ^{3}H-dCTP was measured by TCA precipitation of a 5 μl aliquot. An equimolar amount of Pst 1 digested pBR 322, similarly tailed with about 30 dG residues, was added and the mixture passed over a 5 ml S200 sephacryl column equilibrated with 150 mM NaCl, 10 mM Tris pH 7.5 and 1 mM EDTA. The tailed cDNA and plasmid which eluted together in the excluded volume, were annealed by heating at 70° C for 10 min then 60, 50, 40 and 30° C for 1 h each. Calcium treated (6) E. coli cells (HB101 strain) were then transformed by incubating the recombinant plasmid (0.2-25 ng) with about 5 x 10^7 cells in 10 mM Tris pH 7.3, 50 mM $CaCl_2$ and 10 mM $MgCl_2$ for 15 min on ice followed by 2 min at 37° C, 2 min at 42° C after addition of 1 ml of L-broth and then 37° C for 45 min. The cells were plated out onto agar plates containing L-broth and 15 μg/ml tetracycline and after 17 h at 37° C, individual colonies were picked into microtitre dishes containing L-broth and 15 μg/ml tetracycline and grown at 37° C for 6 h. Bacterial clones carrying a plasmid with a cDNA insert were identified by their sensitivity to 25 μg/ml ampicillin.

Colony Hybridization

Clones were grown and fixed for hybridization on Whatman 540 cellulose paper essentially as described by Grunstein and Wallis (7). Filters (5.5 x 7 cm) containing 48 colonies were prehybridized for 4 h at 37° C in 3 ml of 50% formamid,

5 x Denhardts (8) solution, 4 x SET (1 x SET is 0.15 M NaCl, 5 mM EDTA, 50 mM Tris pH 8), 0.2% SDS, 250 μg/ml yeast tRNA, 0.1% sodium pyrophosphate and 100 μg/ml denatured calf thymus DNA. Filters were then hybridized in 3 ml of the same solution containing 5 x 10^5 dpm of ^{32}P-labelled probe for 15 h at 37° C, washed twice for 1 h in 50% formamide, 4 x SSC (1 x SSC is 0.15 M NaCl, 0.015 M trisodium citrate), 0.1% SDS and twice for 30 min in 2 x SSC, 0.1% SDS, blotted dry and exposed to Ilford RS X-ray film against an Ilford fast tungstate intensifying screen.

Synthesis of ^{32}P-labelled Probes

High specific activity cDNA (5 x 10^7 cpm/μg) was made from 1 μg poly(A)+ RNA or purified albumin mRNA in 50 μl reaction volume using the method described above except that no non-radioactive dCTP was added and 50-100 μCi of α-^{32}P-dCTP were used. After the reaction the RNA was removed as described (9). Cloned cDNA was labelled by nick-translation to specific activities of about 10^8 cpm/μg (10).

Gel Electrophoresis

Fragments of cloned cDNA were isolated from a 1.5% low melting agarose gel containing 20 mM Tris pH 8, 10 mM EDTA (11). For the preparation of purified albumin mRNA, 10 μg of poly(A)+ RNA was similarly electrophoresed (buffer also contained 0.4% SDS). The RNA was extracted from 2 mm slices of the gel (11) and fractions containing albumin mRNA were identified by analysis of in vitro translation products on SDS-polyacrylamide gels. The position of marker 28 and 18S ribosomal RNA was determined by staining with ethidium bromide after removal of SDS from the gel by soaking in several changes of 10 mM Tris pH 7.

Translation of RNA in Xenopus Oocytes

Xenopus oocytes were injected with RNA dissolved in distilled water. Oocytes were put individually into microtitre wells in 100 μl Barth's saline (12) containing 20 mM HEPES and 10 μg/ml each of penicillin and streptomycin sulphate. Oocytes which had not leaked after an overnight incubation at 18° C were labelled in groups of 3-4 in 50 μl of Barth's saline containing HEPES, antibiotics and 400 μCi/ml of L-^{35}S-methionine for 24 h at 18° C. Aliquots of the oocyte medium were analysed on 15 cm long, 1 mm thick SDS-polyacrylamide gels (12% acrylamide, 0.06% bisacrylamide) with a 3% stacking gel as described previously (13). The gels were impregnated with PPO and then exposed to prefogged Ilford RS X-ray film (14). If required, albumin

was precipitated using antiserum raised against Xenopus albumins: medium from the incubated oocytes was incubated in a total volume of 100 µl in phosphate buffered saline (0.15 M NaCl, 4 mM KCl, 2 mM KH_2PO_4, 8 mM Na_2HPO_4) containing 1% triton X-100, 0.005% PMSF and 5 µl antiserum for 4 h at room temperature. 5 µl of 100 x diluted serum from male Xenopus laevis was then added and the immunoprecipitate allowed to form overnight at 4° C. The immunoprecipitate was sedimented, washed twice in 1 ml of PBS containing 1% triton X-100 and 20 mM methionine and then dissolved in sample buffer (13) for 5 min at 100° C before analysis by SDS-polyacrylamide gel electrophoresis.

Positive Selection of Albumin mRNA

Albumin mRNA was positively selected using a 1,050 bp Hpa II fragment containing the entire cDNA insert of clone pcXa 1 immobilized on a 1.2 cm diameter DBM filter (15). 50 µg poly(A)+ RNA was hybridized to this filter for 6 h either at 47° C in 0.5 ml of 70% formamide, 0.2% SDS, 1 mM EDTA, 10 mM PIPES pH 7, 0.3 M NaCl (stringent hybridisation conditions) or at 37° C in 50% formamide, 0.2% SDS, 10 mM PIPES pH 7, 1 mM EDTA and 0.3 M NaCl (non-stringent hybridisation conditions). The filters were then washed at 37° C for 45 min in 50 ml of 50% formamide, 0.2% SDS, 1 mM EDTA, 10 mM PIPES pH 7 and 0.3 M NaCl. The hybridized RNA was eluted by incubating for 10 min at 37° C in 300 µl of 90% formamide, 1 mM Tris pH 7.5, 0.1 mM EDTA, 10 µg/ml yeast tRNA then precipitated by addition of NaCl to 0.1 M and 3 volumes of ethanol.

Rot Analysis

Rot analysis was essentially as described previously (9) except that the buffer was 0.36 M NaCl, 1 mM EDTA, 10 mM Tris pH 7.4. Hybridization was to the Hpa II fragment of clone pcXa 1, described above, nick translated to a specific activity of 10^8 cpm/µg.

Heteroduplexes

Heteroduplexes were formed between Bam H1 digested plasmids and spread for electron microscopy as described previously (16).

RESULTS

Identification of Albumin cDNA Clones

cDNA clones were constructed as described in the Materials and Methods from a fraction of poly(A)+ RNA from male Xenopus liver which had been enriched for albumin mRNA by sedimentation through a sucrose gradient.

201 clones were grown individually on cellulose paper for colony hybridization. This number of clones was analysed to increase the probability that all sequences were contained in this collection of clones and that we would be able to isolate clones containing reasonably long cDNA inserts. To illustrate how potential albumin clones were selected, autoradiographs obtained for 48 clones after hybridization to the various probes used for screening are shown in fig. 1. We first identified clones carrying sequences complementary to abundant liver specific RNA. The clones were screened with ^{32}P-labelled cDNAs made from total poly(A)+ RNA from male Xenopus liver ("liver cDNA") and a Xenopus kidney cell line which does not synthesize albumin ("kidney cDNA"). Four clones (3C, 4C, 4H and 6E) gave a strong hybridization signal with the "liver cDNA" indi-

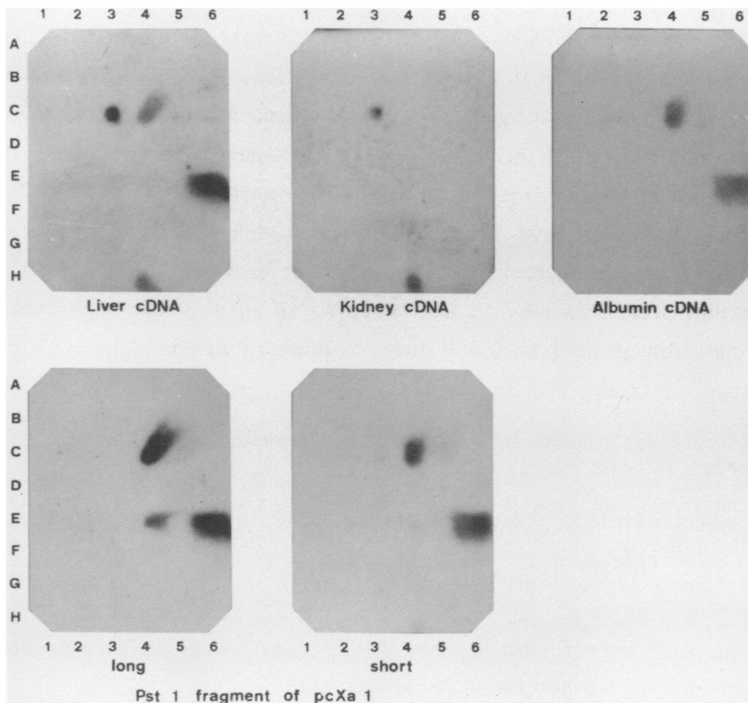

Fig. 1. Colony hybridization of 48 cDNA clones.
Autoradiographs of 48 clones grown and immobilised on Whatman 540 cellulose paper which had been hybridized to ^{32}P-cDNA made from total poly(A)+ RNA from male Xenopus liver (liver cDNA), ^{32}P-cDNA made from total poly(A)+ RNA from a Xenopus kidney cell line (kidney cDNA), ^{32}P-cDNA made from purified albumin mRNA (albumin cDNA) and the long and short Pst 1 fragments of clone pcXa 1 labelled by nick-translation.

cating that they are derived from abundant sequences in the poly(A)+ RNA. Although only strongly reacting clones are seen on this filter, clones giving weaker signals were found on other filters. Two clones (4C and 6E) which had hybridized with the "liver cDNA" did not hybridize to the "kidney cDNA" indicating that these clones contained a sequence which was abundant in "liver cDNA" but not in "kidney cDNA".

In the second screening procedure, the clones were screened with ^{32}P-cDNA made from electrophoretically purified albumin mRNA ("albumin cDNA"). Only the clones which had hybridized to the "liver cDNA" but not the "kidney cDNA" (clones 4C and 6E in fig. 3) hybridized significantly and so these two screening procedures identified the same clones as potential albumin clones.

We do not know the identity of the sequences contained in the clones which hybridized strongly to both the "liver cDNA" and "kidney cDNA", however it is possible that these clones contained sequences complementary to either another abundant poly(A)+ RNA common to both the liver and the kidney cell line or to 18 S ribosomal RNA as this would probably be an abundant contaminant of the poly(A)+ RNA used for cloning. The electrophoretically purified albumin mRNA migrated about half way between the 18 and 28 S ribosomal RNAs and so unlike the RNA enriched for albumin mRNA by centrifugation through a sucrose gradient, should not be contaminated by 18 S ribosomal RNA.

Six of the clones which hybridized most strongly to the "albumin cDNA" were grown in small cultures and the size of the cDNA insert in the plasmid estimated by electrophoresis of a crude preparation of the supercoiled plasmid (17). The plasmid containing the longest cDNA insert (950 bp, designated pcXa 1) was isolated and shown to contain sequences complementary to albumin mRNA (see text relating to fig. 3).

Because impure probes were used for the original screenings, it was desirable to unambiguously identify the clones which contained only albumin or related sequences by screening with a cloned albumin probe.

To eliminate cross-hybridization of the pBR 322 vector DNA in clone pcXA 1 with the vector DNA in the other clones, a pure cDNA insert was prepared from pcXa 1. As the cDNA was cloned in the Pst 1 site, the simplest way to prepare the pure cDNA insert is to excise it with Pst 1. pcXa 1 contains one Pst 1 site in the cDNA (see fig. 2) and so two fragments (330 and 660 bp) were isolated. Both fragments were nick-translated and hybridized individually to the clones immobilized on the cellulose filters. An example of the hybridization obtained

with one filter containing 48 clones is shown in fig. 1 and a summary of the hybridization to all the clones is shown in table 1. 62 clones had previously hybridized to the "albumin cDNA" and their degree of hybridization was classified as strong, intermediate or weak. All 28 clones which gave strong or intermediate hybridization signals with the "albumin cDNA", also hybridized to the long (660 bp), short (330 bp) or both Pst 1 fragments of clone pcXa 1. This shows that all these clones contain sequences complementary to albumin mRNA and verifies that these clones gave the strongest hybridization signal with the "albumin cDNA" because they contain the most abundant sequences. Two examples of clones hybridizing to both the "albumin cDNA" and the Pst 1 fragments of clone pcXa 1 are shown in fig. 1 (clones 4C and 6E). Twelve clones which had not hybridized to the "albumin cDNA", hybridized to the long but not the short Pst 1 fragment of pcXa 1 (for instance clone 4E, which is pcXa 5 in fig. 2). The simplest explanation of this data is that because the "albumin cDNA" was short (200-500 nucleotides as it was synthesized in the absence of non-radioactive dCTP) it only hybridizes to clones containing sequences derived from the 3' end of the albumin mRNA whereas the clones which hybridized to the long Pst 1 fragment but not to the "albumin cDNA" or short Pst 1 fragment are derived from the middle of the mRNA. Further analysis of these clones (see below) has shown this interpretation to be correct and this colony screening procedure was therefore extremely useful in predicting the region of the albumin mRNA sequence contained in the cDNA clones.

Table 1. Hybridization behaviour of the 201 cDNA clones prepared from poly (A)+ RNA of male Xenopus liver with "albumin cDNA" and the Pst 1 fragments of clone pcXa 1.

Hybridization to "albumin cDNA"	Hybridization to the Pst 1 fragments of clone pcXa 1		
	Long fragment only	Short fragment only	Both fragments
Strong (11)	0	1	10
Intermediate (17)	2	8	7
Weak (34)	11	5	1
No hybridization (139)	12	0	0

Characterisation of cDNA Clones

Eight clones were mapped with restriction enzymes (fig. 2). Surprisingly, the restriction maps of plasmids containing sequences from the same part of the mRNA (eg. pcXa 1 and 12) were not identical. In fact 2 distinct groups of sequences could be clearly identified (pcXa 1-5 and pcXa 11-13) and only 4 out of the 12 restriction sites mapped were common to both groups. The restriction mapping, therefore, provided the first evidence that there are 2 distinct but related albumin mRNAs in Xenopus liver. Of the 8 clones mapped in sufficient detail to be assigned to one of the two groups, 5 belonged to group 1 and 3 to group 2.

To confirm the restriction map, recombinant plasmids were also mapped from R-loops and heteroduplexes. All the recombinant plasmids tested formed R-loops with a 2,300 nucleotide long poly(A)-containing liver RNA and the part of the mRNA sequence contained in the recombinant plasmid was determined from the region of the RNA forming the R-loop. Heteroduplexes between plasmids of the

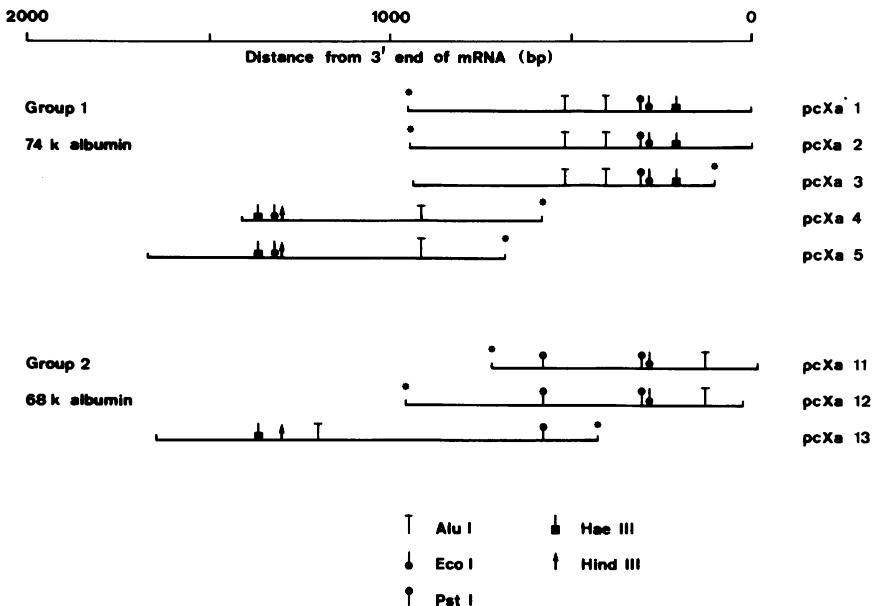

Fig. 2. Restriction maps of the cDNA inserts of 8 recombinant plasmids containing cDNA complementary to X. laevis albumin mRNA.
The cDNA did not contain any Bam HI, Hpa II or Hha I sites. The asterisks show the end of the cloned cDNA which is closest to the single ECo I site in pBR 322.

same or the different groups which contained sequences from the same region of the mRNA were always paired except for a 200-250 bp. region between the clones pcXa 1 or 2 and pcXa 11 or 12 (see text relating to fig. 4).

Together, these experiments showed that 1700 nucleotides of the 2,300 nucleotide long albumin mRNA were contained in several overlapping clones.

Identification of the Translational Products of the 2 Albumin mRNAs

Oocytes injected with 50 ng of poly(A)+ RNA from male liver and then incubated in ^{35}S-methionine secrete 2 labelled proteins (fig. 3, track 2) which comigrate with the 2 albumins of 74,000 and 68,000 daltons which are the predominant proteins secreted by male Xenopus liver during short term organ culture (fig. 3, track 1). They are both precipitated by antiserum raised against Xenopus albumin (fig. 3, track 8) and we therefore conclude that oocytes injected with liver poly(A)+ liver RNA secrete proteins with the same mobility as albumins secreted from the liver. We have therefore used this in vitro translational system to identify the proteins encoded in the cloned cDNA.

To identify the proteins encoded by the 2 related mRNAs, cloned cDNA of both groups was immobilised on DBM filters and hybridised to total poly(A)+ RNA from male Xenopus liver at two different stringencies. The RNA which hybridised to the filters was then injected into Xenopus oocytes and after incubation in ^{35}S-methionine, the labelled secreted proteins were analysed on SDS polyacrylamide gels.

The proteins secreted by oocytes injected with RNA, selected by the clone pcXa 11 under the non stringent hybridization conditions originally used to identify the clones by colony hybridization (fig. 1), are shown in fig. 3, track 3. Two proteins which comigrated with the 2 albumins were secreted and could be precipitated by albumin antiserum (fig.3, track 9). That both proteins are secreted by oocytes injected with mRNA selected by one clone implies that either one mRNA codes for both proteins or that the mRNAs coding for the two albumins are sufficiently related to be selected by one clone. To distinguish between these two possibilities, we injected RNA which had been selected under more stringent hybridization conditions either by clone pcXa 1 or pcXa 12. Only the 74K albumin was secreted by oocytes injected with RNA positively selected by pcXa 1 (track 4) whereas only the 68K albumin was secreted by oocytes injected with RNA selected by pcXa 12 (track 5); both these translational products were precipitated by albumin antiserum (track 10, 74K albumin;

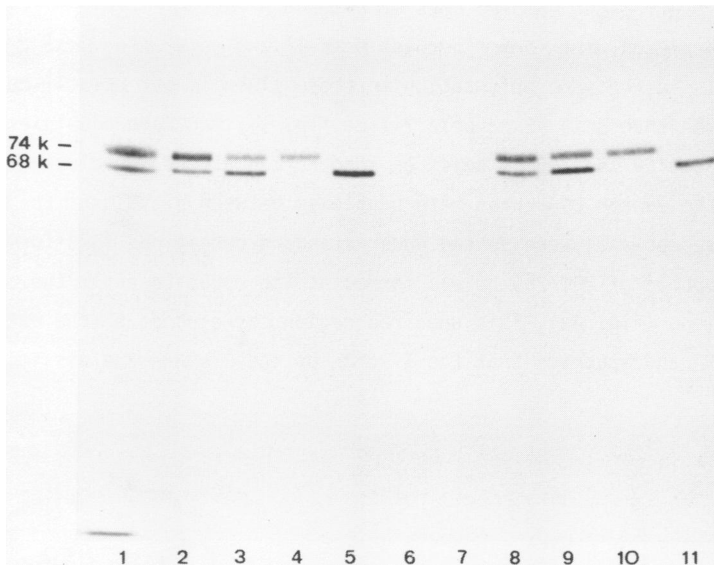

Fig. 3. Proteins secreted from X. laevis oocytes injected with positively selected albumin mRNA.

Oocytes injected with RNA selected by clone pcXa 12 (tracks 3,5) or pcXa 1 (track 4) under nonstringent (track 3) or stringent hybridization conditions (tracks 4,5), 50 ng poly(A)+ RNA from male Xenopus liver (track 2), RNA selected by pBR 322 (track 6) or water (track 7) were incubated in ^{35}S-methionine and the secreted proteins analysed on an SDS polyacrylamide gel followed by fluorography. Tracks 8-11 are immunoprecipitates of tracks 2-5. Track 1 shows the proteins secreted by an organ culture of male Xenopus liver and the 2 albumins of 74 and 68K are marked. 3,000 cpm of acid insoluble radioactivity were loaded onto each track except tracks 6 and 7 which were loaded with 1,000 cpm which was approximately 3 times the volume of labelling medium loaded onto the other tracks.

track 11, 68K albumin). Albumin was not secreted by oocytes injected with RNA positively selected by pBR 322 (track 6) or by oocytes injected with water (track 7). These experiments therefore establish that the 2 albumins of Xenopus laevis are encoded by the closely related mRNAs and that these 2 mRNAs may be isolated separately using stringent hybridization conditions.

Sequence Relatedness of the 2 Albumin mRNAs

The differences between the 2 albumin mRNAs were examined in more detail by

forming heteroduplexes between plasmids of the 2 groups and then spreading them for electron microscopy using 2 different conditions of stringency and by measuring the melting temperatures of heteroduplexes.

Heteroduplexes were formed between pcXa 11 and pcXa 12 or pcXa 2 (fig. 4). The cDNA is in the same orientation in these plasmids but pcXa 11 contains 200 bp less cDNA than pcXa 12 or pcXa 2 (see fig. 1); and heteroduplexes can thus be orientated by the 200 bp deletion loop formed at the junctions of the cloned cDNA and the vector DNA. When heteroduplexes between plasmids of the two groups (pcXa 11 and pcXa 2) were spread under standard conditions (40% formamide, 2 M urea), a bubble of 200-250 bp was formed at the opposite end of the cDNA insert from the loop (fig. 4a). This unpaired region corresponds to the extreme 3' end of the mRNA and suggests that the 3' ends of the 2 mRNAs are dissimilar. Under

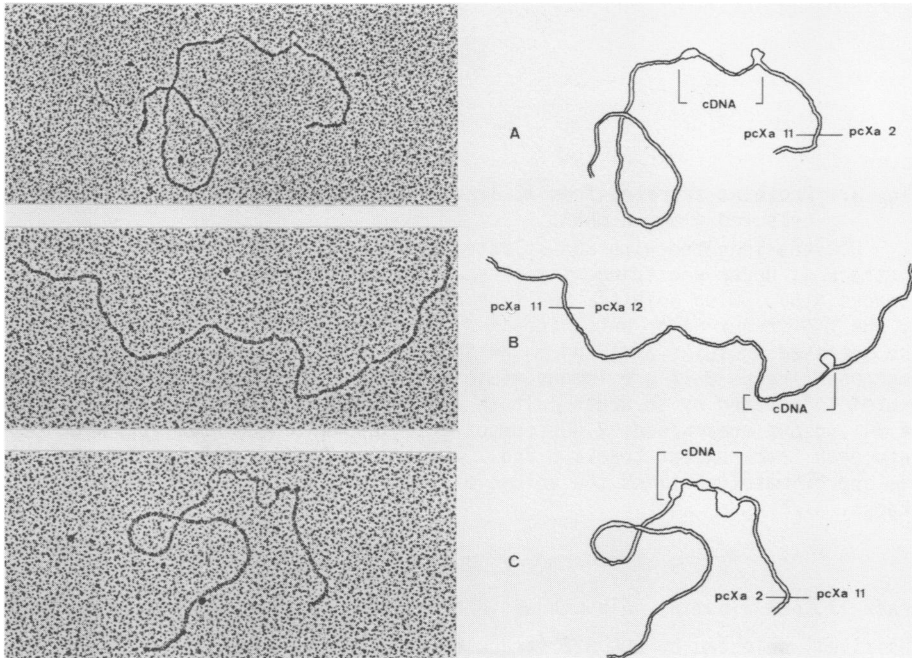

Fig. 4. Heteroduplexes between Bam H 1 digested plasmids containing albumin cDNA.

Heteroduplexes were formed between pcXa 11 and pcXa 2 and spread from 40% formamide and 2 M urea (a) or between pcXa 11 and pcXa 12 (b) or pcXa 11 and pcXa 2 (fig. 3c) and spread from 56% formamide and 2.8 M urea as described (16). The line drawings show the parts of the molecules derived from the plasmid and the cDNA.

the same spreading conditions, heteroduplexes formed between plasmids of the 2 groups which contain the more 5'RNA sequences (e.g. pcXa 4 and 13) are always completely paired (apart from the loops formed by length differences). The 2 albumin mRNAs are therefore probably more dissimilar at their 3' ends than in any region up to 1,500 nucleotides from the 3' ends. The bubble observed in heteroduplexes between plasmids of the 2 groups is probably due to a difference in the mRNA sequences and not to a preferential melting of an AT-rich region derived from the poly(A) tail attached to the 3' end of the mRNA because heteroduplexes formed between 2 clones of the same group (pcXa 11 and pcXa 12) showed only the loop due to the difference in the length of the cDNA inserts but no bubble (fig. 4b) when spread under more stringent conditions (56% formamide, 2.8 M urea).

We also spread heteroduplexes, formed between plasmids of the 2 groups (pcXa 1 and pcXa 11) under the more stringent conditions used for the heteroduplexes shown in fig. 4b. About 50% of these heteroduplexes contained 3 bubbles of unpaired DNA separated by short stretches of paired DNA (fig. 4c) whereas in the other 50%, only the vector DNA remained hybridised (not shown). The high proportion of heteroduplexes similar to those in fig. 4c suggests that the differences between the 2 albumin mRNAs are not evenly distributed over the entire sequence.

To quantitate the difference in sequence between the 2 albumin mRNAs, the melting temperature(Tm) of heteroduplexes between pcXa 1 and other clones representative of the 2 groups was determined. The large Pst 1 fragment from clone pcXa 1 (see fig. 2) was nick-translated and hybridised to plasmid DNA immobilised on nitrocellulose filters and then the hybridised DNA was eluted at increasing temperature. Fig. 5 shows that the Tm of the homoduplex between pcXa 1 and the large Pst 1 fragment of pcXa 1 was 76° C and that heteroduplexes formed with plasmids of the same group (pcXa 2 and 3) also melted at this temperature. In contrast, heteroduplexes with plasmids of the other group (pcXa 11, 12 and 13) all melted with a Tm of 68° C. This 8° C difference in the Tm corresponds to a sequence difference between the 2 albumin mRNAs of about 8% (18, 19).

Abundance of the Albumin mRNAs

The abundance of albumin mRNA in the poly(A)+ RNA was measured by hybridizing the Hpa II fragment of clone pcXa1 to a large excess of liver poly(A)+ RNA under non-stringent hybridization conditions in which both albumin mRNAs hybridize to

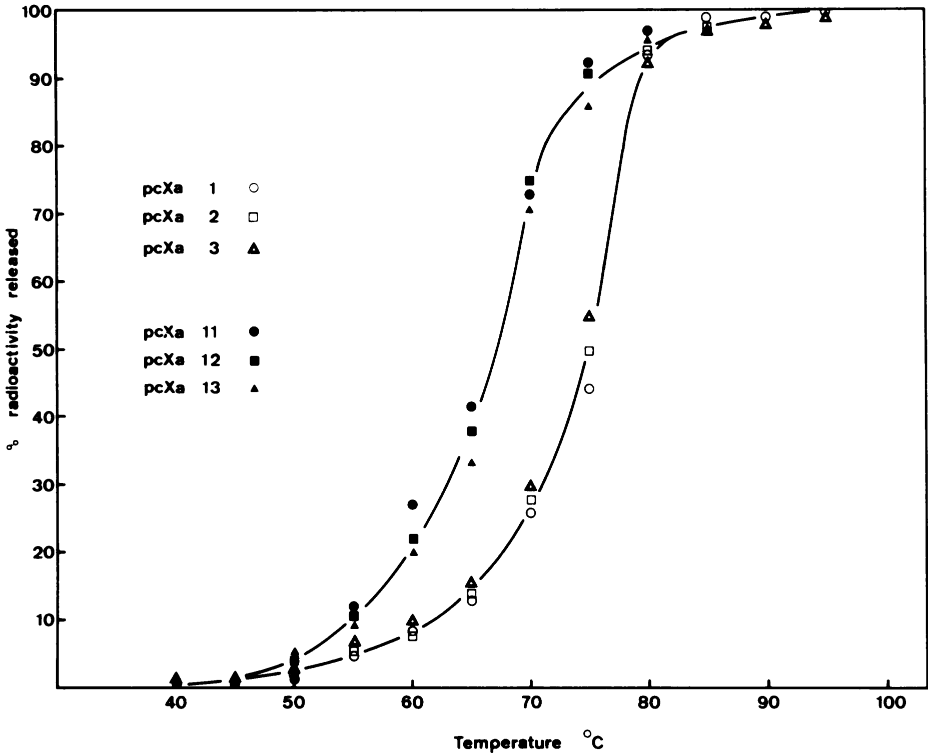

Fig 5. Melting curves of heteroduplexes between pcXa 1 and other plasmids containing albumin cDNA.

The large Pst 1 fragment of pcXa 1 was nick-translated and hybridised to the other plasmid DNAs immobilised on nitrocellulose. The hybridised DNA was then eluted at increasing temperatures and the eluted DNA measured by Cherenkov counting.

the albumin cDNA of either group. Fig. 6a shows that a maximum of 45% of the cDNA hybridized to the RNA. This is close to the theoretical maximum as only one strand of the cDNA can hybridize to the RNA and as 10% of the Hpa II fragment is derived from the pBR 322. The cDNA hybridized to the RNA with a $Rot_{\frac{1}{2}}$ of 1.5×10^{-2} Ms. From standard values for the $Rot_{\frac{1}{2}}$ of globin cDNA hybridizing to pure globin mRNA at the same temperature and salt concentration (20) and taking the length of the albumin mRNA to be 2,300 nucleotide (see text relating to fig. 6), pure Xenopus albumin mRNA would hybridize to its cDNA with a $Rot_{\frac{1}{2}}$ of 1.3×10^{-3} Ms. From the $Rot_{\frac{1}{2}}$ value obtained experimentally, therefore, albumin mRNA represents about 9% of the total poly(A)+ RNA in male

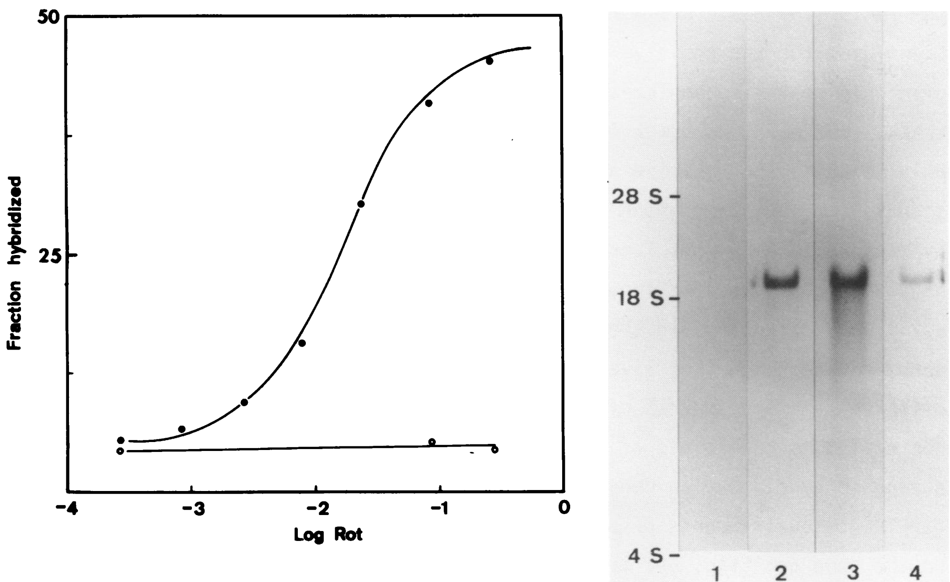

Fig. 6. Albumin mRNA levels in male Xenopus liver.
A. Measurement of total albumin mRNA

1,000 cpm of the Hpa II fragment of clone pcXa 1 nick-translated to a specific activity of 10^8 cpm/µg DNA was hybridized to 7.5 ng of poly(A)+ RNA from male Xenopus liver (•) or 7.5 ng of calf thymus DNA (o) in 5 µl of 0.36 M NaCl, 10 mM Tris pH 7.4 and 1 mM EDTA. After various lengths of time, the amount of DNA hybridized was measured using S1 nuclease digestion. Hybridization in the presence of calf thymus DNA shows that there was insignificant reannealing of the cloned cDNA.

B. Measurement of the relative amounts of the 2 albumin mRNAs

1 µg of poly(A)+ RNA from male Xenopus liver (tracks 2 and 4) and positively selected 68K albumin mRNA (tracks 1 and 3) was glyoxylated, electrophoresed on a 1.2% agarose gel and then transferred to nitrocellulose (21). The filters were then hybridised to 2 x 10^6 cpm of nick-translated pcXa 1 (tracks 1 and 2) or pcXa 12 (tracks 3 and 4) under stringent hybridization conditions for 15 h. After washing for 2 x 1 h in 50% formamide, 0.2% SDS, 4 x SSC and 30 min in 0.2% SDS, 2 x SSC all at 37° C, the filters were exposed to X-ray film. The markers were 28 and 18S Xenopus laevis ribosomal RNA and yeast tRNA.

Xenopus liver.

To measure the relative amounts of the 2 albumin mRNAs in male liver, glyoxylated poly(A)+RNA was electrophoresed and transferred to nitrocellulose (21). It was then hybridised with nick-translated cDNA complementary to either

the 74K or the 68K albumin mRNA under the same stringent hybridization conditions used for the positive selection experiments under which the cDNAs hybridise only to their complementary RNA (fig. 6b). Both cDNA clones (pcXa 1, track 2 and pcXa 12, track 4) hybridised to an RNA of 2,300 nucleotides in poly(A)+ RNA. The relative amounts of the two albumin mRNAs were measured by scanning the autoradiograph shown in fig. 6b to measure the amount of cDNA hybridised to each RNA. This showed that the 74K albumin mRNA is about twice as abundant as the 68K albumin mRNA in poly(A)+ male liver RNA. As there is a 2-3 fold difference in the relative amounts of the 74K and 68K albumins in serum and secreted from liver (see fig. 3, track 1), this suggests that the difference in the amounts of the two proteins synthesized by the liver is controlled by the levels of their respective mRNAs. To show that under these stringent hybridization conditions the cDNA only hybridises to its complementary RNA, equal amounts of purified 68K albumin mRNA were also electrophoresed on the same gel (fig. 6b, tracks 1 and 3). Nick-translated pcXa 12 (track 1) but not pcXa 1 (track 3) hybridised to the purified 68K albumin mRNA which confirms that these hybridization conditions can be used to distinguish the 2 albumin mRNAs in total poly(A)+ RNA.

DISSCUSSION

In this study we have shown that there are two closely related but distinct mRNAs coding for serum albumin in Xenopus laevis and that these two distinct albumin mRNAs can be isolated by positive selection and translated in Xenopus oocytes which then secrete the 2 albumins characteristic of Xenopus serum. This proves that the 2 proteins of 74,000 and 68,000 daltons which were previously thought to represent 2 albumins in Xenopus serum are in fact the translational products of 2 distinct mRNAs. This is different from serum albumin in all mammals studied so far, for example rat (22) and chicken (23), which only have one albumin.

We have characterised the differences between the 2 albumin mRNAs in 3 ways. Initially they were characterised by restriction enzyme and heteroduplex analysis of the cDNA clones and then by measuring the melting temperatures of heteroduplexes between various pairs of cDNA clones. These studies all suggest that the differences between the 2 mRNAs are distributed over the whole length of the cloned sequence and that the 2 mRNAs are mismatched by about 8%. This indicates that the 2 mRNAs are encoded by 2 closely related but distinct genes

rather than constructed by differential splicing of a single gene product as appears to be the case with the α-amylase gene (24). In fact we have recently obtained direct evidence for the presence of 2 albumin genes in the Xenopus laevis genome by analysis of Southern transfers of genomic DNA and clones isolated from a gene bank (May et al., unpublished).

The 74K albumin is about twice as abundant as the 68K albumin in X. laevis serum; probably due to different rates of protein synthesis as the liver secretes more of the 74K than the 68K albumin (see track 1, fig. 3). This is correlated with the relative levels of the 2 mRNAs in liver poly(A)+ RNA as measured by their hybridization to the cloned cDNA probes under conditions where the cDNA hybridises only to its complementary mRNA.

The different levels of mRNA coding for the 2 albumins could be due to different rates of transcription, processing or to different stabilities of the mRNAs and comparison of the 2 albumin genes should provide insight into the factors which regulate their differential expression.

Bisbee et al. (4) first suggested that the number of albumins detected by electrophoretic techniques in polyploid series of Xenopus species increases with the number of chromosomes. Thus X. tropicalis (20 chromosomes) has one detectable albumin, X. laevis (36 chromosomes) has 2 albumins and X. Ruwenzoriensis (108 chromosomes) has three albumins. Our demonstration at the nucleic acid level that there are 2 closely related albumin mRNAs and genes in X. laevis proves as suggested from the study at the protein level, that there are 2 albumin genes in X. laevis and strengthens the view that the genome of X. laevis is tetraploid due to a duplication during its recent evolution. This is also supported by the analysis of the X. laevis vitellogenin (16), globin (25) and some ribosomal protein sequences (26) where pairs of closely related genes have also been identified.

The genome duplication which gave rise to Xenopus laevis is thought to have occurred as recently as 30 million years ago (4) and it should now be possible to analyse the evolutionary processes by which the 2 genes have diverged since the duplication event using the cloned probes for the 2 closely related albumin genes and mRNAs described in this study.

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