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Comparative analysis of Xenopus tropicalis and Xenopus laevis vitellogenin gene sequences

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ABSTRACT

Analysis of cDNA clones synthesized from vitellogenin mRNA of X. tropicalis revealed three different types of cDNA clones, i.e. A, A* and B. A and A* clones have a sequence divergence of about 6 % and are both related to X. laevis vitellogenin cDNAs of subgroup A1 as well as A2 with a sequence divergence of 6 - 9 %. B clones however, are related to X. laevis cDNA clones of subgroup B1 and B2 with a sequence divergence of about 7 %. While the A and B clones correspond to vitellogenin mRNAs of similar abundance, A* clone is complementary to a vitellogenin mRNA about 100 fold less abundant than A and B mRNAs although all three vitellogenin mRNAs are encoded by single copy genes. Furthermore, two forms of A* mRNA were found. One of the two is lacking an internal fragment of about 900 bp. Since this DNA fragment is highly repeated in the genome, we suggest that this A* clone was synthesized from a processing intermediate of the A* precursor vitellogenin mRNA.

INTRODUCTION

The estrogen regulated synthesis of vitellogenin, the egg yolk precursor protein, has been investigated in the liver of amphibians (1) and birds (2). In the frog Xenopus laevis vitellogenin is encoded in four genes (3). Analysis of the sequence relatedness between vitellogenin cDNAs has shown that the vitellogenin mRNAs fall into two main groups, A and B, which differ from each other in approximately 20 % of their nucleotides. Each main group contains two subgroups A1/A2 and B1/B2 which differ from each other by about 5 % sequence divergence.

Analysis of several Xenopus species led to the assumption that genome duplications had occurred during evolution: While X. laevis would have a tetraploid set of chromosomes, X. tropicalis which is a very ancient form of Xenopus would only contain a diploid set of chromosomes (4,5). Since genome duplication involves all genes, one would expect two vitellogenin genes in X. tropicalis in contrast to the four genes identified in X. laevis.

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To test whether this simple model of genome duplication is tenable, we have cloned sequences complementary to vitellogenin mRNA of X. tropicalis and determined the number of different sequences expressed and their relatedness with vitellogenin sequences of X. laevis.

MATERIAL AND METHODS

Preparation of cDNA clones

X. tropicalis frogs were obtained from Centre Suisse de Recherche Scientifique (Adiopodoumé, Ivory Coast). Vitellogenin mRNA synthesis was induced by injection of 500 µg estrogen 9 days and 150 µg 6, 3 and 1 day before RNA extraction. 10 µg 28S poly(A)+ RNA prepared from liver cells (6,7), was transcribed into cDNA as described (8). Synthesis of the second strand was done with E. coli DNA-polymerase I (Boehringer) (9). This enzyme proved to be much more efficient than reverse transcriptase. The reaction mixture was made 50 mM Tris (pH 8.0), 10 mM EDTA and 10 µg/ml tRNA (Miles), extracted with an equal volume of phenol and precipitated with ethanol. Double-stranded cDNA was digested with 15 units S1 nuclease (a gift of Dr. U. Schibler) for 30 min in a final volume of 100 µl, extracted with phenol/chloroform (1:1) and ethanol precipitated. The cDNA was separated on a 1% agarose gel (low gel temperature, Bio-Rad) containing 40 mM Tris, 5 mM sodium acetate, 1 mM EDTA, pH 7.8. The fraction of the gel containing cDNA fragments of 1 - 3.5 kb was adjusted to 0.5 M NaCl and incubated at 70°C for 10 min. After cooling to room temperature, the cDNA was extracted with an equal volume of phenol and precipitated with ethanol. After two reprecipitations, the cDNA was dissolved in 3 mM Tris (pH 8.0) and 100 ng were tailed with about 40 residues dC, annealed directly with Pst I digested pBR322 (tailed with poly dG) and used for transformation (8). Ampicillin sensitive clones were grown up for further analysis. All the work involving recombinant DNA was carried out according to the NIH-guidelines for recombinant DNA.

RNA gel electrophoresis

50 ng poly(A)+ RNA was glyoxylated for 3 min at 50° C and run on a 1% agarose gel (10). The RNA was transferred immediately after electrophoresis to a nitrocellulose filter (11) and hybridized with nick-translated cDNA fragments. The filters were washed (12) and exposed to a preflashed X-ray film (RP-2, Agfa-Gevaert).

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R-loop and heteroduplex analysis

R-loops were formed between vitellogenin mRNA and Bam HI digested plasmids as described (13). Heteroduplexes were formed between plasmids linearized with Bam HI and spread for electron microscopy (3).

Filter hybridization and hybrid melting experiments

Chromosomal DNA digested with a restriction enzyme, was run on a 1.2 % agarose gel and transferred to a nitrocellulose filter (14). Hybridization and washing of the filters was as described (12). For hybrid melting experiments, 150 ng plasmid DNA was fixed on nitrocellulose filters (15) and hybridized with nick-translated fragments of cDNA clones over night. Filters were washed and the hybridized DNA then eluted at increasing temperatures in 30 mM NaCl, 8 mM Tris (pH 8.0), 1 mM EDTA. The radioactivity released was measured by Cerenkov counting.

RESULTS

Identification of vitellogenin in X. tropicalis

The induction of vitellogenin by estrogen has been intensively studied only in the X. laevis frog (16), whereas nothing has been known as of yet about other Xenopus species. Therefore, the plasma of a X. tropicalis was analyzed 1, 3 and 6 days after a single injection of 200 μg estrogen. As can be seen in Fig. 1, a new protein accumulates in the plasma of estrogen-treated frogs (slots 3 - 5) which is absent in untreated plasma of males (slot 2). The protein has a molecular weight of about 190,000 dalton which is very similar to X. laevis vitellogenin (slot 1). Since this estrogen-induced protein can be precipitated with an antibody directed against X. laevis vitellogenin (slot 6), we conclude that it represents the vitellogenin of X. tropicalis. The observation of a single band of vitellogenin in X. tropicalis and of at least two different bands in X. laevis may indicate that vitellogenin is less complex in X. tropicalis than in X. laevis.

Three types of vitellogenin cDNA clones from X. tropicalis

The complexity of the vitellogenin gene products of X. tropicalis was investigated by cloning cDNA complementary to vitellogenin mRNA. The cDNA clones were characterized by restriction mapping as well as R-loop and heteroduplex analysis.

To compare vitellogenin cDNA sequences of X. tropicalis with cDNA sequences of X. laevis, which have been studied previously, clones located in the 3' re-

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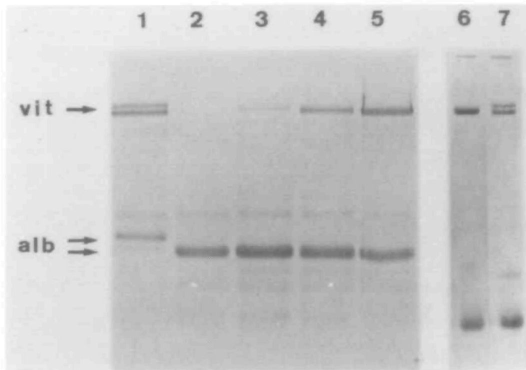

Fig. 1. Identification of vitellogenin in X. tropicalis.
0.2 µl plasma of a male X. tropicalis not treated with estrogen (slot 2) and stimulated with a single injection of 200 µg estrogen for 1 day (slot 3), 3 days (slot 4) or 6 days (slot 5) was run on a SDS polyacrylamide gel (17) and stained with Coomassie brilliant blue. For comparison, 0.1 µl plasma of a female X. laevis stimulated with estrogen for 7 days was separated (slot 1). Slot 6 and 7 are immunoprecipitates of slot 5 and 1, respectively. Vitellogenin (vit) and albumin (alb) are indicated with arrows.

gion of the mRNA were selected, where most of the X. laevis cDNAs clones originated from (3). Therefore, plasmid DNA of 79 independent clones was separated by agarose gel electrophoresis, transferred to nitrocellulose filters and hybridized with a short cDNA of high specific radioactivity synthesized from 28S poly(A)+ RNA of estrogen induced X. tropicalis. This radioactive cDNA was mainly complementary to the 3' region of the mRNA template, since reverse transcription starts within the poly(A) of the mRNA. 12 individual clones hybridizing strongly with this cDNA probe and having a cDNA insert of about 2 kb were selected.

For restriction analysis the Pst I fragments from clone pXtvc 1 (Fig. 2) were isolated and the fragments designated as the 3' fragment (a) and the internal fragments (b) and (c). Similarly, the fragments from pXtvc 11 and 14 were designated as the 3' fragment (f) and the internal fragments (g) and (e) (see Fig. 2). Comparing the hybridization of these isolated fragments to X. tropicalis and X. laevis cDNA restriction fragments enabled the construction of the restriction map of the cDNA clones shown in Fig. 2. Furthermore, these hybridizations yielded a clear distinction between X. tropicalis cDNA clones either reacting with group A or group B vitellogenin cDNAs of X. laevis. The two

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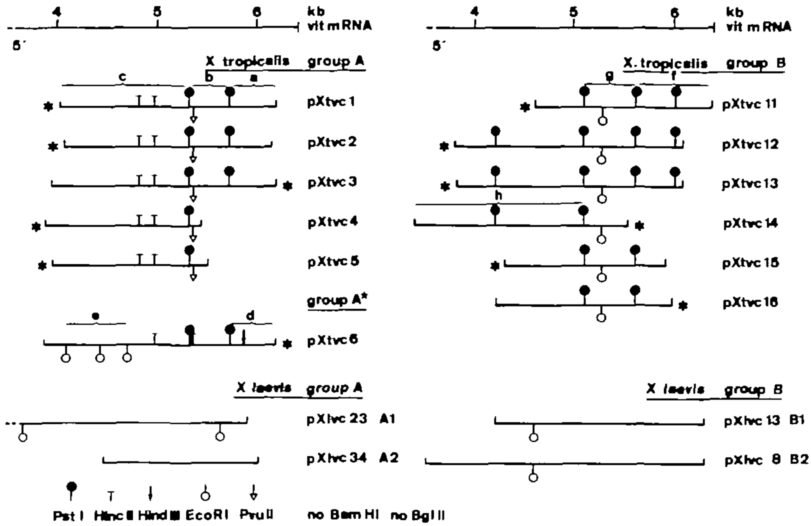

Fig. 2. Restriction map of vitellogenin cDNA clones.
The vitellogenin cDNA clones are aligned to the vitellogenin mRNA drawn on the top. Specific restriction fragments are designated a - h. The asterisks indicate the end of the cloned cDNA closest to the single Eco R I site in pBR322. X. laevis cDNA clones used in our analysis are drawn with their Eco R I sites at the bottom (3). For the B group, only the Pst I and the Eco R I sites were mapped.

groups were designated as A and B. While the B clones were all identical in their restriction sites, two different types of cDNA clones were found in A. They were designated as A (pXtvc 1 - 5) and A* (pXtvc 6).

A more precise mapping of the cloned cDNA on the vitellogenin mRNA was obtained by electron microscopic measurements of R-loop molecules formed between individual clones and the mRNA. For all isolated clones R-loop structures were found and the average length of the mRNA was 6,300 ± 500 nucleotides (S.D., N = 250 molecules), the size expected for vitellogenin mRNA (13). The length of the RNA/DNA hybrid in R-loops with the B clones pXtvc 11 and 16 agreed with the length of the insert obtained from the restriction analysis and the position of the cDNA on the mRNA (designated as B mRNA) also agreed with the mapping based on the restriction data indicated in Fig. 2.

However, analysis of R-loops with the A clone pXtvc 1 revealed two different types of structures. In Fig. 3A a representative of the more frequent type is shown. In this case, the length of the RNA/DNA hybrid agrees with the size of

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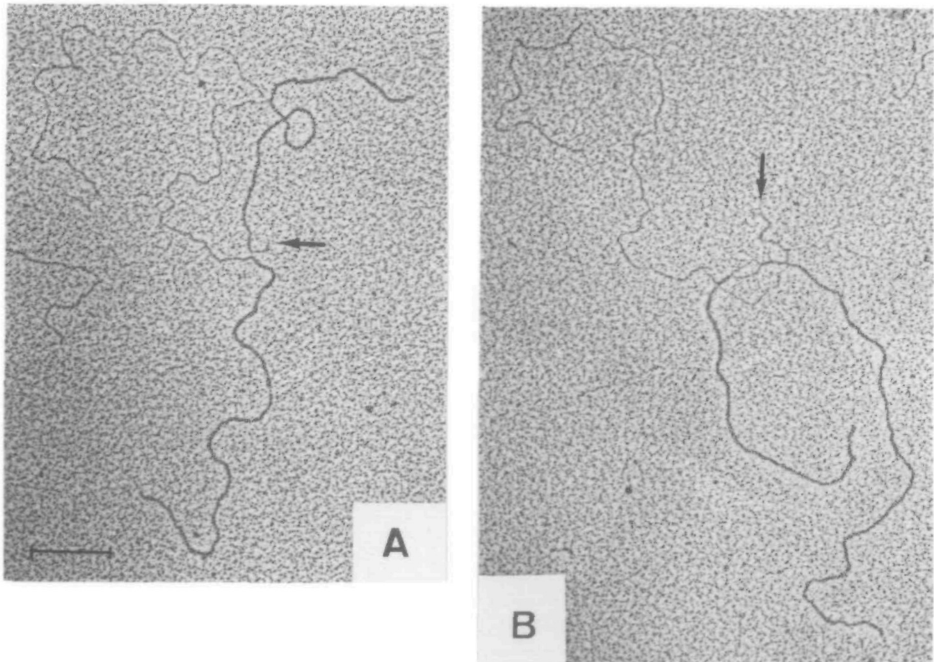

Fig. 3. R-loops between vitellogenin mRNA and the A clone pXtvc 1.
The 3' end of the mRNA is indicated with an arrow. See Fig. 5A and 5B for schematic drawings of the two types of R-loops shown in A and B, respectively. The bar represents 0.5 kb.

the inserted cDNA determined by restriction analysis. Since the cDNA maps close to the end of the mRNA and crosshybridizes with X. laevis clones pXlvc 23 and 34, known to originate from the 3' portion of the vitellogenin mRNA A1 and A2, respectively (3), we conclude that this clone is complementary to the 3' portion of X. tropicalis vitellogenin mRNA (designated as A mRNA). In the second case, only a short RNA/DNA hybrid of 380 bp was found in the R-loop formed between pXtvc 1 and the mRNA (Fig. 3B). In this unusual case, which was only rarely observed, the RNA/DNA hybrid maps 770 nucleotides from the 3' end of the mRNA and contains a characteristic substitution loop in its middle part. A schematic representation of the two types of R-loops is given in Fig. 5A and 5B, where all R-loop molecules are aligned at the 3' end of the vitellogenin mRNA (indicated with an arrow).

Using the A* clone pXtvc 6, three different R-loop structures were observed

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within the same preparation. In only three out of 170 molecules, R-loop structures of the usual type were found with a long RNA/DNA hybrid of 1920 bp located 250 nucleotides from the 3' end of the mRNA (Fig. 4A and schematically in Fig. 5C) as expected from the restriction data. The two other R-loop structu-

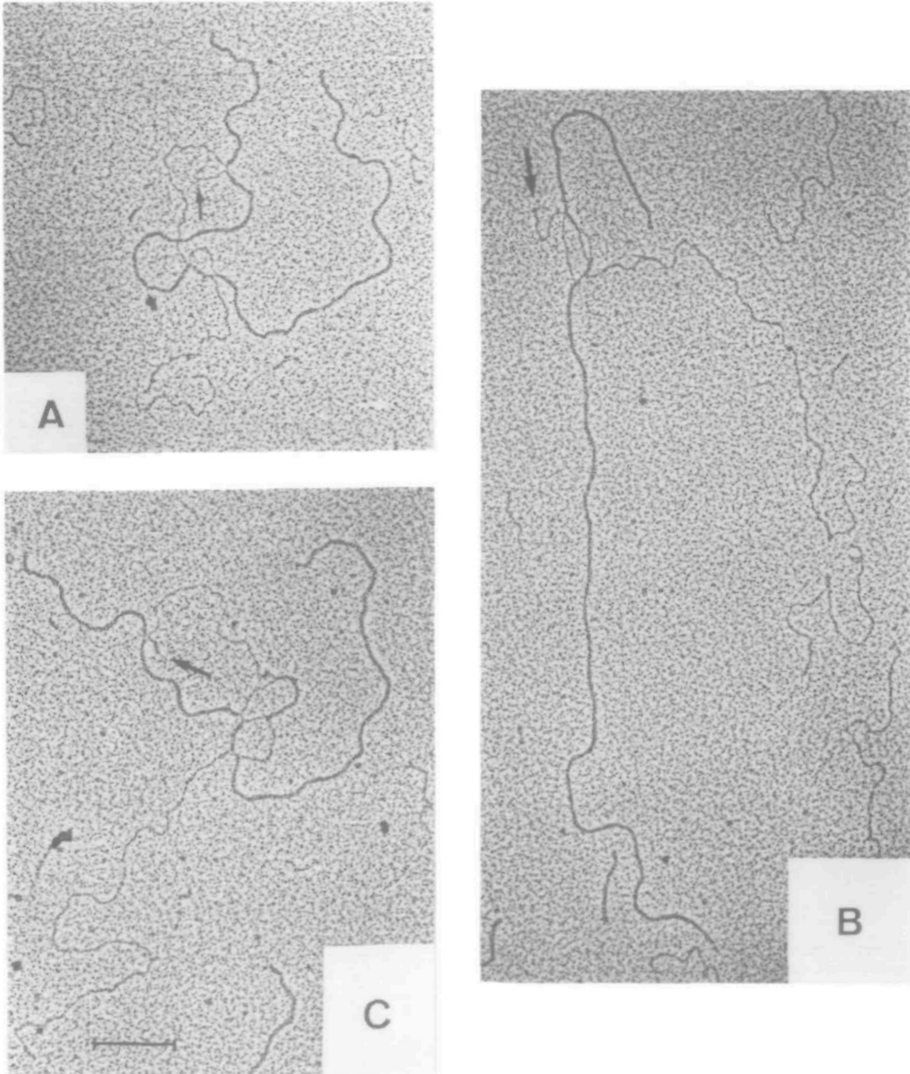

Fig. 4. R-loops between vitellogenin mRNA and the A* clone pXtvc 6.
The 3' end of the mRNA is indicated with an arrow. Schematic drawings of the three types of R-loop structures A - C are shown in Fig. 5C - 5E, respectively. The bar represents 0.5 kb.

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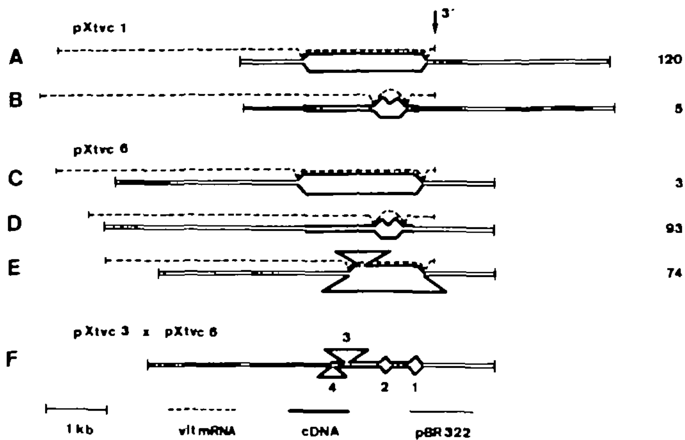

Fig. 5. Schematic drawing of R-loops and heteroduplexes formed with A and A* clones.

Individual R-loops formed between vitellogenin mRNA and pXtvc 1 (A and B) or pXtvc 6 (C, D and E) were measured and the average lengths are drawn. The length of individual structures is drawn horizontally. The R-loop structures (A - E) were aligned at the 3' end of the vitellogenin mRNA (indicated with an arrow). The number of molecules analyzed of each type is given. Heteroduplexes formed between the A clone pXtvc 1 and the A* clone pXtvc 6 (Fig. 6) were measured and the average lengths of 21 molecules are drawn in F. The heteroduplex molecule is aligned at the short end of the plasmid DNA in E.

res which were observed more frequently and had about the same abundance within the preparation, were of quite unexpected structure. In one case, the R-loop structure contained a short RNA/DNA hybrid of 460 $\pm$ 35 bp (S.D.) located 595 $\pm$ 65 bp (S.D.) from the 3' end of the mRNA (Fig. 4B and schematically in Fig. 5D). Within the RNA/DNA hybrids a characteristic substitution loop was observed in all these molecules. In the other case, the R-loop structure as illustrated in Fig. 4C and schematically in Fig. 5E contained an RNA/DNA hybrid of 1220 $\pm$ 120 bp (S.D.) which was located more closely to the 3' end of the mRNA (235 $\pm$ 60 bp, S.D.). The RNA/DNA hybrid is interrupted by the looping out of a single stranded DNA sequence of 890 $\pm$ 90 bp (S.D.). (In many cases, this additional DNA sequence reannealed with the complementary DNA strand as in Fig. 4C, whereas in other structures the short RNA/DNA hybrid 5' of this DNA loop was absent. Hence, an R-loop of the usual type but with a shorter RNA/DNA hybrid was observed.) Comparing this unusual R-loop structure formed with the A* clone pXtvc 6 (Fig. 4C) with the restriction data given in Fig. 2 suggests that the three Eco R I restriction sites found in A* but not in A, map within this

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additional DNA. Direct evidence that the DNA sequence between these Eco R I sites of A* is missing in A clones was obtained, when these two Eco R I restriction fragments were isolated and hybridized back to clones pXtvc 1 – 6. Hybridization occurred exclusively with the A* clone pXtvc 6 but not with the other A clones.

Since all three different cDNA types A, A* and B, formed at least in some cases R-loop structures of the usual type with the mRNA, we conclude that they represent three different vitellogenin mRNAs present in the mRNA population. From the observation that all B clones were identical in their restriction sites and formed a single type of R-loop structures, we assume that only one vitellogenin mRNA of type B is present in X. tropicalis. On the other hand we postulate two different but related vitellogenin mRNAs in X. tropicalis, designated as A and A*. In this case, apart from the usual R-loops, structures with short RNA/DNA hybrids containing a typical substitution loop were observed. These structures can best be explained by the formation of R-loops containing a mRNA only partially complementary to the cDNA. Since the hybrid as well as the substitution loop in A and A* clones map exactly at the same position as referred to the mRNA (Fig. 5B and 5D), we suggest that in these R-loop molecules the related vitellogenin mRNA A* and A is present, respectively. In addition, two forms of A* mRNA must be present in the preparation – one containing all the sequences cloned in pXtvc 6 (Fig. 4A) – the other lacking a 900 bp sequence in the region of the three Eco R I restriction sites.

The sequence relatedness between the three types of cDNA clones was further tested by heteroduplex analysis in the electron microscope. Hybrid molecules were formed between A clone pXtvc 1 and B clone pXtvc 11 known to have the same orientation within the vector DNA. The analysis revealed that all the molecules were paired within the vector DNA but open within the cloned cDNA sequence. When heteroduplexes were formed between A clone pXtvc 3 and A* clone pXtvc 6, a reproducible pattern of paired and unpaired cDNA sequences (numbered 1 – 4) was obtained (Fig. 6 and schematically in Fig. 5E). Although the 3' end of both cDNA clones maps at the same position on the mRNA (Fig. 2), a single-stranded DNA sequence (loop 1) was observed in this region of the heteroduplex molecules. Since homoduplexes were always paired under the spreading conditions used, we conclude that this loop must be due to base pair substitutions between A and A* clones. For the same reason, loop 2 found in these heteroduplex molecules must also be due to base pair substitutions between A and A* clones. The

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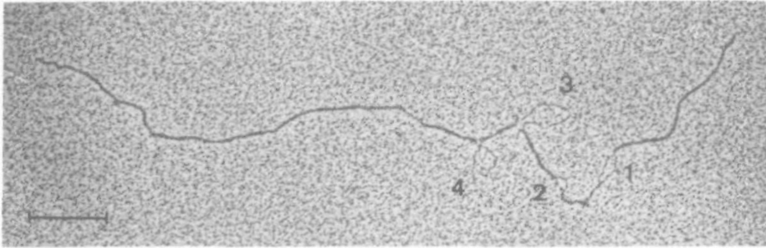

Fig. 6. Heteroduplex molecules between A clone pXtvc 3 and A* clone pXtvc 6. A schematic drawing of the molecules is given in Fig. 5E. The bar represents 0.5 kb.

single-stranded DNA in loop 3 has a size of 770 $\pm$ 150 nucleotides and maps 1230 bp from the beginning of the cDNA corresponding to the region of the three Eco R I restriction sites in A* clone pXtvc 6. Since the DNA between these Eco R I sites is absent in A clones, we conclude that this loop contains A* specific DNA. Since both cDNA clones pXtvc 3 and 6 have a similar length, but pXtvc 6 contains sequences not present in pXtvc 3, we expect additional DNA looping out at the 5' end of the paired cDNA region. Loop 4 shown in Fig. 6 represents this deletion loop which is generated by the A clone pXtvc 3.

The fact that the substitution loop 2 maps at the same position as the substitution loop found in R-loops (compare Fig. 5B and 5D with 5F) strongly supports the hypothesis that in these structures the A mRNA forms an R-loop with the A* clone and vice versa.

Sequence relatedness of X. tropicalis and X. laevis vitellogenin cDNA clones

To quantitate the difference in sequence between the three vitellogenin cDNAs of X. tropicalis and the vitellogenin cDNAs of X. laevis, the melting temperature (Tm) of homoduplexes and heteroduplexes was determined. The 3' fragment (a) (see Fig. 2) was nick-translated and hybridized to filters containing X. tropicalis plasmid cDNA (pXtvc 1, 3, 6 and 11) or X. laevis cDNA of group A1 (pXlvc 23) and group A2 (pXlvc 34). The bound radiocactivity was eluted at increasing temperature. Fig. 7A shows that the Tm of homoduplexes between this 3' fragment (a) and the A clone pXtvc 1 was 68.5° C and that heteroduplexes with another A clone (pXtvc 3) also melted off at this temperature (solid line in Fig. 7). In contrast, heteroduplexes with A* clone pXtvc 6 and with X. laevis clones of group A1 and A2 all melted off with a Tm of 62.5° C. This 6° C difference in the Tm corresponds to a sequence divergence of about

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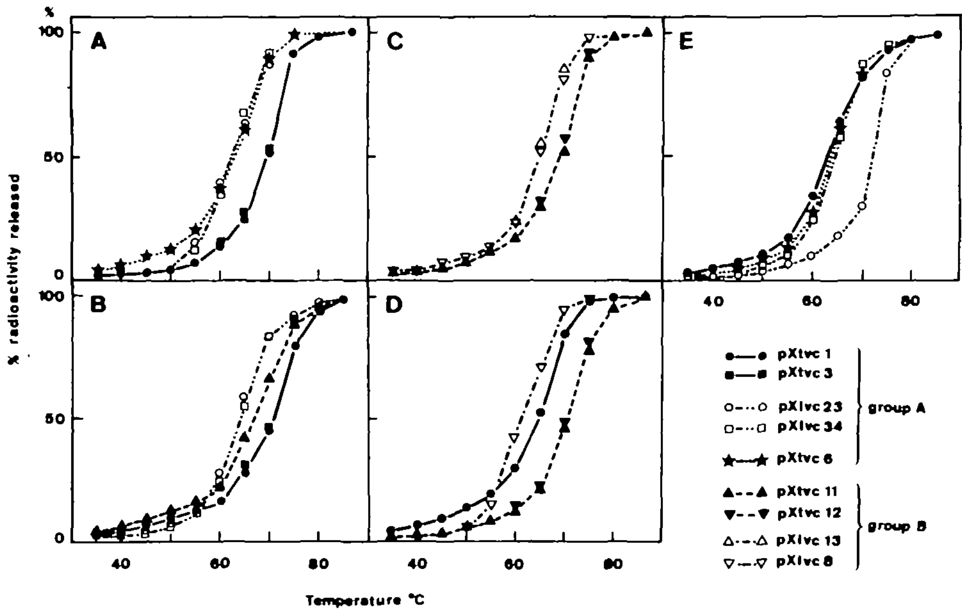

Fig. 7. Sequence relatedness between the vitellogenin cDNAs.
The 3' fragment (a) (panel A), the internal fragment (c) (panel B) of the A clone pXtvc 1, the 3' fragment (f) (panel C) of the B clone pXtvc 11 or the internal fragment (h) (panel D) of the B clone pXtvc 14 were hybridized to filter bound cDNA clones listed in the figure and melted at increasing temperature. Panel E represents a control experiment using the Eco RI fragment of X. laevis A1 clone pXlvc 23. For the location of the cDNA clones see Fig. 2.

6 % between the A clone fragment (a) and the A* clone of X. tropicalis or group A1 and A2 clones of X. laevis.

In an analogous experiment when the internal fragment (c) of A clone pXtvc 1 (see Fig. 2) was hybridized, a Tm of 70° C was found for A clones pXtvc 1 and 3 and the X. laevis cDNA clones melted off with a Tm of 64° C, corresponding again to a sequence divergence of about 6 % in this region (Fig. 7B). In contrast to the 3' fragment (a), the internal fragment (c) also hybridized with B clone pXtvc 11. The Tm was 66° C, indicating a sequence divergence of about 4 % between A and B clones in this internal region.

When the 3' fragment (f) of the B clone pXtvc 11 and the internal fragment (g) of clone pXtvc 14 (Fig. 2) were hybridized with A and B clones, a Tm of 69 - 70° C was obtained for B clones over the whole sequence (Fig. 7C and 7D). A clone pXtvc 1 only hybridized in the region of the internal fragment (g) and the Tm was 64.5° C corresponding to a sequence divergence of about 5%. X. lae-

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vis cDNA clones of group B1 (pXlvc 13) and B2 (pXlvc 8) melted off at 63°C which corresponds to a sequence divergence of about 7 % between X. tropicalis B clones and X. laevis group B clones.

In a control experiment where the internal Eco R I fragment of X. laevis clone pXlvc 23 of group A1 (see Fig. 2) was hybridized with X. tropicalis and X. laevis cDNA clones immobilized on nitrocellulose filters, the Tm of homoduplexes was 72° C. Heteroduplexes between group A1 and A2 of X. laevis melted off at 64° C corresponding to a sequence divergence of about 8 % (Fig. 7E). Hybrids formed with X. tropicalis A clones pXtvc 1, 3 and 6 revealed a Tm of 63° C, indicating a sequence divergence of about 9 %.

From these melting curves we conclude that X. tropicalis A and A* clones have an equal sequence divergence of about 7 % to X. laevis clones of either subgroup A1 and A2. Similarly, B clones of X. tropicalis are related to subgroup B1 and B2 with a similar sequence divergence of about 7 %. Furthermore, A and B clones of X. tropicalis contain related sequences within the internal fragments (c) or (e), respectively. In addition, A and A* cDNAs of X. tropicalis have a sequence divergence of about 6 % over the whole sequence present in pXtvc 1 (data only shown for 3' fragment (a) of pXtvc 1).

To ascertain that no other related sequences than A and A* are present in our cDNA collection, the Tm of 17 different cDNA clones all belonging to the group A was determined with the internal fragment (b) of pXtvc 1. All clones apart from pXtvc 5 and 6 behaved identically as the A clones pXtvc 1 and 3 with a Tm of 69° C. pXtvc 5 which melted off at 65.6° C was shown to be an A clone by restriction mapping (Fig. 2) and heteroduplex analysis in the electron microscope (data not shown). Since this clone only contains a short sequence complementary to the radioactive probe, it leads most probably to less stable hybrids and therefore to a reduced Tm although belonging to the A clone sequences. As expected, the A* clone pXtvc 6 melted off at a lower temperature with a Tm of 63° C being the only representative of the A* type. Similarly, the Tm of 25 clones crossreacting with the internal fragment (g) of the clone pXtvc 11 was determined. Since they all melted off at 69 – 70° C as homoduplexes, we conclude that they all contain sequences identical to the B clones.

Abundance of the vitellogenin mRNAs

To estimate the abundance of the three vitellogenin mRNAs, 28S poly(A)+ liver RNA of estrogen-treated animals was run in parallel with poly(A)+ RNA of untreated males on an agarose gel, transferred to a nitrocellulose filter and

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hybridized with internal fragments (b) of the A clone (pXtvc 1), (g) of the B clone (pXtvc 11) and the two Eco R I fragments (e) specific for the A* clone pXtvc 6. The results demonstrated that the RNA of estrogen-treated animals hybridized with all three probes (slots 1 in Fig. 8A - 8C), whereas poly(A)+ RNA of untreated males failed to hybridize (slots 2 in Fig. 8A - 8C). The size of the hybridizing RNA was identical to the size of X. laevis vitellogenin mRNA. Since the experimental protocol for all three hybridizations was identical and the size of the radioactive probe about the same, the abundance of each type of mRNA could be estimated if the time of exposure of the X-ray film was taken into consideration. Therefore, A* mRNA screened with the two internal Eco R I fragments (e) (exposure for 8 days) was at least 100 fold less abundant than A and B mRNAs (exposure for 2 h) which both have about the same abundance. On the other hand we know from the analysis in the electron microscope that the two internal Eco R I fragments (e) of pXtvc 6 are part of the cDNA sequence looping out in a high proportion of the RNA/DNA hybrids in the R-loop structures analyzed. Therefore no estimation of the abundance can be made for this other form of the A* mRNA.

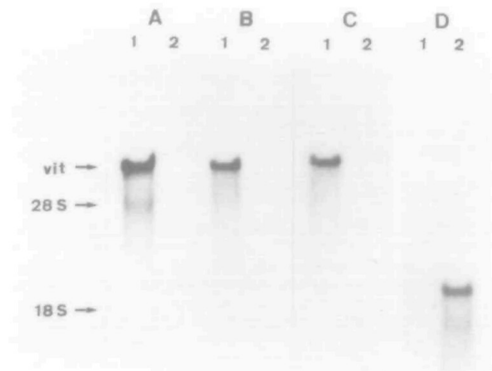

Fig. 8. Relative amount of the three vitellogenin mRNAs.
28S poly(A)+ RNA of estrogen-treated animals (slots 1) and total poly(A)+ RNA of unstimulated males (slots 2) were separated electrophoretically, transferred to a nitrocellulose filter and hybridized with the isolated fragment (b) of the A clone pXtvc 1 (filter A), the fragment (g) of the B clone pXtvc 11 (filter B), the fragment (e) of the A* clone pXtvc 6 (filter C) and the X. laevis albumin cDNA insert (filter D) isolated from pcXa 11 and pcXa 13 (8). The X-ray film was exposed for 2 h (filter A and B), 8 days (filter C) and 5 days (filter D). The mobility of vitellogenin mRNA (vit) and ribosomal RNA (28S and 18S) as revealed after staining, is indicated.

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To show that the RNA extracted from males was not degraded, one of the filters was washed and rehybridized with a cDNA probe containing X. laevis 68k albumin sequences (8). As expected for intact RNA, a single band showed up in poly(A)+ RNA of X. tropicalis males (slot 2 in Fig. 8D) which had about the same mobility in the gel as X. laevis albumin mRNA.

Number of vitellogenin gene copies in genomic DNA

We have shown that in X. tropicalis three vitellogenin mRNAs, i.e. A, A* and B are expressed. To determine the number of the vitellogenin gene copies in the genome, chromosomal DNA was digested with Bgl II or Eco R I, run on an agarose gel and transferred to a nitrocellulose filter. Increasing amounts of Eco R I digested DNA of A clone pXtvc 1 corresponding to half, one, two and five gene copy equivalents were run as standards on the same gel. In Fig. 9A an autoradiograph of a filter hybridized with the internal fragment (b) of the A clone pXtvc 1 is shown. Since the intensity of the bands correlates with the amount of complementary DNA bound to the filter, the number of gene copy equivalents can be estimated by comparison of the intensities of the bands from chromosomal DNA with the standard cDNAs. In such an experiment the intensity of the band obtained from Eco R I digested chromosomal DNA (slot 7) as well as the sum of the intensities of the three bands obtained from Bgl II digested chromosomal DNA (slot 6) is similar to the intensity of the plasmid DNA which corresponds to two gene equivalents (slot 4). Since we know that A and A* cDNA clones crosshybridize under the conditions used, we conclude that A and A* sequences are encoded each by single copy genes. Analysis of a single copy gene equivalent of the B clone pXtvc 12 reveals that no crosshybridization of A and B sequences can be detected in this experiment (slot 1 in Fig. 9A).

In an analogous experiment, B clone pXtvc 12 was linearized with Bam H I and run in standard amounts on the same gel with Bgl II digested chromosmal DNA. Hybridization with the internal fragment (g) of pXtvc 11 reveals that B sequences are encoded by a single copy gene (slots 3 and 6 in Fig. 9B) and that no crosshybridization between B and A sequences takes place (slot 1 in Fig. 9B). On the other hand, X. laevis vitellogenin sequences of the B group having a sequence divergence of about 7 % to X. tropicalis sequences, partially crosshybridized under the conditions used (slot 7 in Fig. 9B), therefore we conclude that the B clones found represent the only B type sequence present in chromosomal DNA of X. tropicalis.

From the restriction analysis of A and A* clones with different restric-

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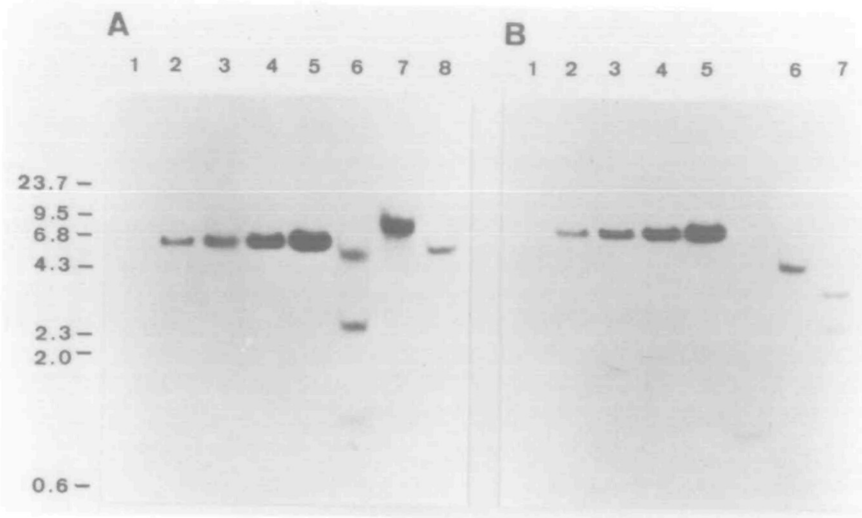

Fig. 9. Number of vitellogenin gene sequences in X. tropicalis.

Filter A: 10 µg X. tropicalis DNA digested with Bgl II (slot 6) or Eco R I (slot 7) and 10 µg X. laevis DNA digested with Eco R I (slot 8) were separated electrophoretically. The A clone pXtvc 1 linearized with Eco R I was used as standard for half (20 pg in slot 2), one (40 pg in slot 3), two (80 pg in slot 4) and five (200 pg in slot 5) gene copy equivalents. In slot 1, 20 pg of the B clone pXtvc 12 was run. The transferred DNA was hybridized with the internal fragment (b) of the A clone pXtvc 1 and washed under conditions that allowed related sequences to be stable (12).

Filter B: 10 µg X. tropicalis DNA digested with Bgl II (slot 6) and 10 µg X. laevis DNA digested with Eco R I (slot 7) were separated. 20, 40, 80 and 200 pg of the B clone pXtvc 12 (slots 2 – 5) digested with Bam H I were run as standards. In slot 1, 20 pg of the A clone pXtvc 1 was separated. The filter was hybridized with the internal fragment (g) of the B clone pXtvc 11 and washed as filter A.

tion enzymes (Fig. 2), differences in the restriction pattern of genomic DNA were expected. In Fig. 10 an experiment is shown, where X. tropicalis DNA was digested with Bgl II and hybridized with the 3' fragment (a) of the A clone pXtvc 1 and the 3' fragment (d) of the A* clone pXtvc 6 which both cover the same region of the two related vitellogenin mRNAs. While the pattern of fragments hybridizing under non-stringent conditions (12) was very similar (slots 2 and 3 for the fragments (a) and (d), respectively), the pattern of fragments forming stable hybrids under more stringent conditions (12) differs considerably (slots 1 and 4 in Fig. 10A).

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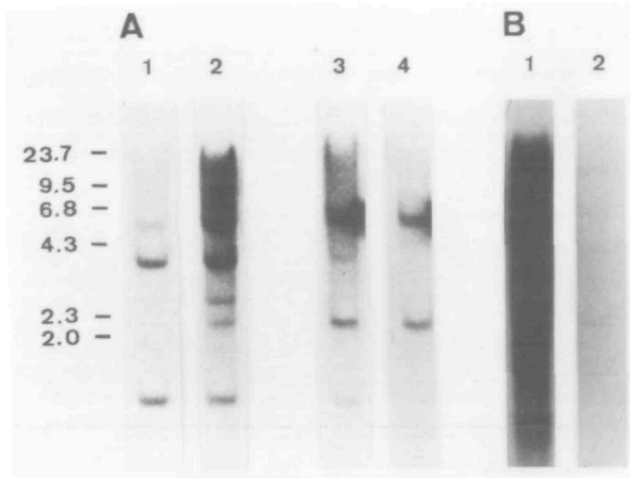

Fig. 10. A and A* vitellogenin mRNAs are encoded by separated genes and repetitive DNA is present in the cloned A* sequence.

10 µg X. tropicalis DNA digested with Bgl II (slots 1 – 4 in A) was separated electrophoretically, transferred to a nitrocellulose filter and hybridized with the 3' fragment (a) of the A clone pXtvc 1 (slot 1 and 2) or the 3' fragment (d) of the A* clone pXtvc 6 (slot 3 and 4). The filters washed under non-stringent conditions (12) were exposed for 1 day (slot 2 and 3) and reexposed for 3 days after stringent washing (slot 1 and 4).
In panel B, 10 µg X. tropicalis DNA digested with Bgl II (slot 1) and 10 µg X. laevis DNA digested with Eco R I (slot 2) were hybridized with the two Eco R I fragments (e) of the A* clone pXtvc 6. The filters were washed under non-stringent conditions (12) and exposed for 2 days.

A similar experiment was carried out with the two internal Eco R I fragments (e) of the A* clone pXtvc 6 which were present only in a few of the A* mRNA molecules. Hybridization of these fragments with genomic DNA led to the surprising result that this sequence is present in many copies within the genome of X. tropicalis (slot 1 in Fig. 10B) but absent in X. laevis DNA even under non-stringent washing conditions (slot 2 in Fig. 10B).

From these results we conclude that vitellogenin genes A, A* and B are encoded by single copy genes and that A* clone pXtvc 6 contains a repetitive DNA sequence, dispersed throughout the X. tropicalis genome.

DISCUSSION

In X. tropicalis, three different vitellogenin mRNAs i.e. A, A* and B are expressed after estrogen-treatment. While the abundance of the A and B mRNAs

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as well as the number of the corresponding cDNA clones found in our cDNA collection are about the same, the A* mRNA has a much lower frequency and a single A* clone (pXtvc 6) was found. Analysis of R-loop structures in the electron microscope has revealed that the A and the A* mRNA contain closely related sequences leading to R-loop formation between the A mRNA and the A* clone and vice versa. Since R-loops between the A mRNA and the A* clone were observed much more frequently (93 out of 170) than R-loops between the A* mRNA and the A clone (5 out of 125), we conclude that the A mRNA has a higher abundance in the vitellogenin mRNA population than the A* mRNA. Furthermore, in A* clone pXtvc 6 a 900 bp sequence was found which is not crossreacting with any sequence in the A clones. R-loop analysis between the A* clone and the vitellogenin mRNA has shown that this additional DNA was present only in three out of 170 molecules (Fig. 4A and 5C). Hybridization of chromosomal DNA with the Eco R I fragments (e) of the A* clone pXtvc 6 which are part of this additional DNA has shown that it is highly repeated in the genome (Fig. 10B). Both, the absence of this DNA fragment in a high proportion of all A* mRNA molecules and the fact that it contains repetitive DNA make it very likely that it represents an intron. In fact, introns containing repetitive DNA have been found in X. laevis vitellogenin gene A1 and A2 (16,18). From our analysis it cannot be decided conclusively whether pXtvc 6 has been cloned from an A* mRNA present in low abundance in the cytoplasm of liver cells or from a vitellogenin precursor RNA representing a nuclear contamination. However, it seems very unlikely that a mRNA containing such a repetitive DNA element codes for a protein (19).

Comparison of A and B clones of X. tropicalis has revealed a conserved region in a distance of 1 - 2 kb from the 3' end of the vitellogenin mRNA (Fig. 7). Closely related sequences have even been found between the X. laevis A2 vitellogenin gene and a chicken vitellogenin gen mapping in the same region of the mRNA (20). In is not known whether this conserved region has any important function on the mRNA or the vitellogenin proteins.

Analysis of chromosomal DNA has revealed that all three vitellogenin mRNAs are encoded by single copy genes (Fig. 9 and 10). Since all three vitellogenin mRNAs are induced by estrogen but have a different abundance in the cytoplasm of stimulated liver cells (Fig. 8), we conclude that the A* vitellogenin gene is transcribed at a lower rate than the A and B genes and/or the expression of the three vitellogenin mRNAs is differentially controlled at

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the level of processing or stability of the transcripts. A similar phenomenon has been reported for the genes of the ovalbumin gene family in ckicks, where both, the X and Y mRNAs are present at a lower abundance than the ovalbumin mRNA although all three genes are controlled by the same steroid hormone (21). However, our results are in contrast to the four vitellogenin mRNAs in X. laevis which were shown to be present at about the same abundance in estrogen-treated liver cells (22).

Based on the chromosome number and the DNA content per nucleus as well as immunological distances between albumins of various Xenopus species, it has been proposed that X. laevis has evolved from an ancestral form by a genome duplication about 30 million years ago (4,5). Recent studies in X. laevis have revealed pairs of related mRNAs for vitellogenin (3), albumin (8) and larval and adult α and β globins (23). The similar degree of divergence of 5-8% within each pair strongly supports the hypothesis that they all arouse simultaneously by a genome duplication and have evolved with an equal rate of divergence , the only exception being the larval globin genes differing by about 14% (23). On the other hand, X. tropicalis having only 20 chromosomes instead of 36 in X. laevis would be the only Xenopus species that did not undergo such a genome duplication. Therefore, one would expect single genes in X. tropicalis instead of pairs of related genes in X. laevis. For the vitellogenin genes this assumption is not valid since we have found an A and an A* vitellogenin gene in X. tropicalis. This unexpected finding indicates that the vitellogenin gene A has duplicated during evolution in contrast to the B gene which is present as a single copy gene. Since all A genes as well as all B genes of X. tropicalis and X. laevis have a similar sequence divergence of about 7%, we suggest that they all evolved independently for about the same duration. Therefore, we assume that the genome duplication occured in the same period when X. tropicalis and X. laevis diverged.

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