Scattering of repetitive DNA sequences in the albumin and vitellogenin gene loci of *Xenopus laevis*

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ABSTRACT

We have analyzed middle repetitive DNA in the albumin and vitellogenin gene families of *Xenopus laevis*. Mapping specific repetitive DNA sequences derived from introns of the A1 vitellogenin gene reveals that these sequences are scattered within and around the four vitellogenin genes (A1, A2, B1 and B2) and the two albumin genes (74 kd and 68 kd). Three repetitive DNA elements present in the A1 vitellogenin transcriptional unit are also located in introns of the 74 kd albumin gene. This apparently random distribution of middle repetitive DNA in the two gene families suggests that the analyzed sequences are not involved in gene regulation, but rather that they might represent unstable genetic elements. This hypothesis is further supported by the finding that size polymorphism in the A1 vitellogenin gene and in the 74 kd albumin gene is correlated with the presence or absence of repetitive DNA.

INTRODUCTION

The DNA of eukaryotes mostly consists of single copy DNA interspersed with middle repetitive DNA (reviewed in 3). Both types of DNAs are expressed and based on the observation that specific repetitive DNA transcripts are found in differentiated cells it has been postulated that repetitive DNA is involved in gene regulation (4). This hypothesis has been strengthened by the observation that specific gene transcripts in *Dictyostelium* contain dispersed repetitive DNA common for many developmentally regulated mRNAs (5, 6). Repetitive DNA was found in specific genes quite frequently in introns but also in exons (reviewed in 7). The functional significance of these repetitive sequences present in defined transcriptional units is not clear, but the data obtained were not collected systematically.

We have recently isolated two albumin (8) and four vitellogenin genes (9, 10, 11) of *Xenopus laevis*. These genes are expressed in a tissue and
stage specific manner: the albumin genes are constitutively transcribed in liver cells of the tadpole and the frog (unpublished data), whereas the vitellogenin genes can only be activated by estrogen in the liver of the frog but not in the tadpole prior to metamorphosis (reviewed in 12). The two albumin genes coding for the 74 kd and 68 kd albumin have a sequence divergence of about 8% in their mRNA coding sequence (13) while the intron sequences differ more extensively (14). The four vitellogenin genes form two groups, A and B (15), that differ in about 20% of their mRNA coding sequence. Each group is composed of two closely related genes A1, A2 and B1, B2 that differ in about 5% of their exon sequence. The introns of the four vitellogenin genes, however, are very different in sequence and in length. Whereas the A1 and B1 vitellogenin genes are linked (10) it is not known at the present time whether there is a linkage between the A2 and B2 genes.

Analysis of the A1 and A2 vitellogenin genes has revealed many repetitive DNA elements within and around these genes (12). Moreover we have identified and mapped six different repetitive DNA sequences within the transcribed introns of the A1 vitellogenin gene (16). To get more insight into possible roles of repetitive DNA sequences we first tested the two closely related A1 and A2 vitellogenin transcriptional units for common repetitive DNA sequences in their introns and second we systematically analyzed the distribution of the six repetitive DNA sequences of the A1 transcriptional unit in the 4 vitellogenin and in the two albumin gene loci. Together these specific genes, including their flanking regions, cover 210 kb of the Xenopus DNA. Such an analysis seems especially attractive in this system, because the available data suggest that both the albumin and vitellogenin gene family in Xenopus laevis arose by gene and genome duplications (10, 14, 17). In addition, the members of each gene family seem to be coordinately expressed (12, 18 and unpublished data). Therefore if repetitive DNA elements are involved in regulation of these genes they are expected to have conserved their sequence and position in the duplicated loci whereas repetitive DNA sequences, not essential for gene regulation, are likely to have diverged after the duplication events.
MATERIALS AND METHODS

Enrichment of repetitive DNA specific for a given genomic clone

A nitrocellulose filter containing 12 µg of λX1v 128 DNA was hybridized under standard conditions (16) in 5 ml at 37°C with 1.4 x 10^8 cpm Xenopus DNA labelled by nicktranslation with 32P. After washing the hybridized DNA was eluted with 90% formamide in 1 mM Tris-HCl, 1 mM EDTA (pH 8.0) at 47°C. The recovered DNA (1.2 x 10^6 cpm) was ethanol precipitated and used for hybridization.

Hybridizations

The intron subclones 6b, 8b, 13, 18, 23 and 33a (16) were labelled by nicktranslation and the specific radioactivity was adjusted to 10^7 cpm/µg. Prehybridization and hybridization with 2 x 10^6 cpm for 16 hr were as described (19) and washing was also performed under conditions described (20).

Heteroduplex analysis

0.3 µg DNA of each genomic clone were denatured in 200 µl 70% formamide, 0.3 M NaCl, 5 mM EDTA and 10 mM Tris-HCl (pH 8.0) for 5 min at 65°C and renatured for 90 min at 35°C. The mixture was diluted 10-fold into 20% formamide, 1 M urea, 30 mM Tris-HCl (pH 8.5), 1 mM EDTA, 40 µg/ml of cytochrome c and spread onto a hypophase of distilled water. Electron microscopy was done as described (15).

RESULTS

The closely related A1 and A2 vitellogenin genes have no common repetitive DNA sequences in their transcribed regions

Analysis of intron subclones of the A1 vitellogenin gene enabled us to isolate and characterize middle repetitive DNA sequences of the introns 6, 8, 13, 18, 23 and 33 (16). Since these six repetitive DNA sequences were selected as A1 vitellogenin gene regions not present in the A2 gene, any repetitive DNA common to the A1 and A2 vitellogenin genes would have been missed. To search specifically for repetitive DNA common for the A1 and A2 vitellogenin gene, DNA of the genomic clone λX1v 128 covering the entire A2 vitellogenin gene (9 and Fig.4) was immobilized on a nitrocellulose filter and hybridized with total Xenopus DNA labelled by nicktranslation. Under these conditions mainly repetitive DNA present in the genomic clone...
Figure 1: Selection of repetitive DNA present in the transcriptional unit of the A2 vitellogenin gene.

The genomic clones of the A1 vitellogenin gene λXlv 109, λXlv 107, λXlv 104 and the A2 vitellogenin genomic clone λXlv 128 (see Fig. 4) as well as the A1 vitellogenin cDNA clone pXlv 23 (15) and the subclone from intron 6 of the A1 vitellogenin gene (clone 6b in ref. 16) were restricted with Eco RI and separated electrophoretically (panel a). The DNA was blotted and hybridized with total Xenopus laevis DNA labelled by nicktranslation as described (16). The autoradiogram obtained is shown in panel b. An identical blot was hybridized with Xenopus DNA selected by hybridization to the genomic A2 vitellogenin clone λXlv 128 (see Materials and Methods). The corresponding autoradiogram is shown in panel c. The intensity of hybridization to the 5.5 kb fragment in lane 104 of panel c is overestimated through the photographic processing of the original autoradiogram. Moreover, it hybridizes quite well to the selected Xenopus DNA, since it contains many exon sequences of the A1 vitellogenin gene that crosshybridize with the A2 vitellogenin exon sequences (17). In fact, the isolated 5.5 kb fragment of the A1 gene hybridizes exclusively to fragments of A2 genomic clones containing homologous exons (data not shown).

hybridizes (16 and Fig. 1b). The bound DNA enriched for repetitive DNA present in the A2 vitellogenin gene was melted off and probed for sequences also contained in the A1 vitellogenin gene locus. As can be seen from the autoradiogram in Fig. 1c the eluted DNA reacted as expected strongly with
Figure 2: Mapping of the repetitive DNA common to the A1 and A2 vitellogenin gene loci.

A subclone containing the 0.95 kb Eco RI fragment of the A2 vitellogenin gene was hybridized to blots containing the A1 genomic clones λXⅣv 109, λXⅣv 107, λXⅣv 106, λXⅣv 104, λXⅣv 101 and the A2 genomic clones λXⅣv 127, λXⅣv 125, λXⅣv 128 and λXⅣv 129 (ref. 9). The clones were restricted with Eco RI (), HindIII (), Bam HI () or Sal I (). The hybridizing fragments were scored and a summary of these data is given for the A1 and A2 vitellogenin gene loci. The region containing the repetitive sequence is indicated by the dotted box. The 1.6 kb Eco RI fragment within the A1 vitellogenin gene also reacts with the 0.95 kb probe, because crosshybridization is due to the close sequence relationship of the exon sequences present in the 0.95 kb and 1.6 kb fragment (see 17).

In addition to the 3.5 kb Eco RI fragment several other Eco RI fragments
of the A1 gene also hybridized but weakly with the DNA selected with the A2 gene sequences. Since the enriched DNA hybridized also with the A1 specific cDNA clone pXIvc 23 representing single copy DNA we conclude that the weaker bands correspond to crosshybridizations of single copy sequences common to the two closely related A vitellogenin genes. As a control for the specificity of the enrichment a subclone containing a repetitive DNA specific for the A1 vitellogenin gene (the repetitive DNA in intron 6) was also included in the blot. This DNA hybridized with total Xenopus DNA (Fig. 1b) but not to the DNA enriched by hybridization to the A2 genomic clone (Fig. 1c).

In conclusion, these experiments demonstrate that none of the transcribed repetitive sequence is sheared by the two closely related A1 and A2 transcriptional units. However, transcribed repetitive sequences in one of the two genes can be found in the non-transcribed flanking regions of its close relative (see also below).

Repetitive DNA sequences of the A1 vitellogenin transcriptional unit are present in other gene loci.

As mentioned above the 6 different repetitive DNA sequences of the A1 vitellogenin gene that are derived from introns 6, 8, 13, 18, 23 and 33 are not found in the transcribed region of the closely related A2 gene. Further it was of interest to see whether these 6 repetitive sequences present within transcribed regions of the A1 gene are also found in flanking regions of the A1 and A2 genes, in the vitellogenin B1 and B2 loci as well as in the two albumin loci and whether their positions reflect some regular distribution. Therefore, genomic clones covering all albumin and vitellogenin genes, extending some 210 kb of Xenopus DNA, were restricted with Eco RI, separated electrophoretically and blotted onto filters. The blots were probed with the subclones containing the various repetitive DNA sequences of the A1 vitellogenin gene. Fig. 3 illustrates such an experiment where the A1 specific intron 23 subclone, containing the repetitive DNA derived from intron 23, was hybridized to digests of genomic clones. As expected the intron 23 subclone hybridized to the restriction fragments of the A1 vitellogenin gene regions containing intron 23, i.e. fragments of the genomic clones λXIv 106 and λXIv 104 (Fig. 3). In addition clear hybridization was also observed with Eco RI fragments of the A2 (clones λXIv 127 and λXIv 125) and
Figure 3: Hybridization of the intron 23 subclone, derived from the A1 vitellogenin gene, to genomic clones of the albumin and vitellogenin genes.

The DNA of the genomic clones listed in Figure 4 as well as Charon 4A DNA (Ch) were restricted with Eco RI and separated on an agarose gel (upper panel). The transferred DNA was hybridized with the subclone of intron 23 of the A1 vitellogenin gene. The autoradiogram as obtained after stringent washing conditions is given in the lower panel. The hybridizing fragments are marked with an arrow in the upper panel.

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of the B1 (clone λX1v 209) vitellogenin genes as well as with regions of the 74 kd (clones λX74a 101 and λX74a 111) and 68 kd (clone λX68a 209) albumin gene loci (see Fig. 3). Mapping of the hybridizing fragments in the known restriction map of the genes revealed that the hybridization locates in the 5'-flanking region of the A2 gene as well as in the 3'-flanking sequence of the B2 vitellogenin and 68 kd albumin gene (Fig. 4). In contrast to these locations outside of the transcriptional units, the hybridizing Eco RI
fragment of the 74 kd albumin gene is clearly within the gene (Fig. 4). The repeated element locates in an intron of the 74 kd albumin gene, since mature albumin mRNA does not react with the intron 23 subclone (data not shown).

Using the same approach the 6 gene loci were analyzed for the presence of the other 5 repetitive sequences identified within the A1 vitellogenin transcriptional unit (autoradiograms not shown). The mapping of these repetitive sequences is included in Fig. 4. All these repetitive DNA elements were found at up to three different locations. Only the repetitive DNA sequence of intron 13 could not be observed at any other gene locus. The distribution of these repetitive sequences does not reveal any systematic arrangement. Furthermore, the repetitive DNA elements isolated as sequences present within the A1 vitellogenin transcriptional unit are predominantly located in flanking regions of the analyzed genes. In addition to subclone 23 (see above) only the subclone derived from intron 18 of the A1 vitellogenin gene was found within other transcriptional units: this sequence is present within the B1 vitellogenin as well as the 74 kd albumin transcriptional units. In the B1 vitellogenin gene this sequence might reside at a homologous position, i.e. in intron 18, since the hybridizing Eco RI fragment corresponds to the region containing introns 16 to 21 (compare the Eco RI restriction fragments in ref. 10 with the exon-intron map given in ref. 11).

Position and size of DNA sequences common to the A1 vitellogenin and 74 kd albumin genes

To locate and measure the size of the repetitive DNA elements common for the A1 vitellogenin and the 74 kd albumin genes we made heteroduplexes

Figure 4: Scattering of the repetitive DNA in the albumin and vitellogenin gene loci.

The genomic clones of the vitellogenin and albumin genes are given with their Eco RI sites. The transcriptional units of the albumin genes are indicated as deduced from R-loop structures between genomic clones and albumin mRNA (14). For the vitellogenin genes the indicated sizes of the transcriptional units are based on R-loop structures as well as on in vitro transcription experiments (10, 11, 17). Restriction fragments hybridizing with the intron subclones are marked with the symbols given in the Figure. The size of specific restriction fragments is given in kb. The three 3' side Eco RI fragments in clone λX74a 102 represent a cloning artefact, since they are not linked to the 74 kd albumin gene locus (Ryffel, unpublished).
Figure 5: Heteroduplex between the Al vitellogenin and the 74 kd albumin gene.

A heteroduplex between clone \( \lambda Xlv 106 \) and \( \lambda X74a 111 \) is shown and the structure is interpreted. The sizes of the single-stranded DNA loops are given in kb. The double-stranded paired regions a, b, c and d measure 0.40, 0.19, 0.19 and 0.11 kb, respectively. The right (R.A.) and left (L.A.) arm of the Charon DNA are indicated and the vitellogenin and albumin DNA is drawn by a dotted and broken line, respectively.

between the corresponding genomic clones and spread them for electron microscopy. In Fig. 5 a heteroduplex between the Al vitellogenin genomic clone \( \lambda Xlv 106 \) and the 74 kd albumin clone \( \lambda X74a 111 \) (see Fig. 4 for their locations) is given. Clearly there are four positions (a, b, c and d) where pairing of 100 to 400 nucleotides between the albumin and vitellogenin gene is observed. From the picture it is evident that all four paired regions have the same orientation relative to the Charon arms. In a control experiment hybridizing the same Al vitellogenin genomic clone \( \lambda Xlv 106 \) with a 74 kd albumin clone containing the Xenopus insert in opposite orientation no paired regions could be observed. The heteroduplex shown in Fig. 5 could unambiguously be interpreted since the orientation of the inserts in the Charon DNA as well as the location of crosshybridizing sequences was established (see Fig. 4). Knowing that the 6.0 kb Eco RI fragment of the albumin clone \( \lambda X74a 111 \) contains sequences present in the 3.2 kb and 4.1 kb Eco RI fragments of the Al vitellogenin clone \( \lambda Xlv 106 \), the single-stranded DNA could be identified as vitellogenin and albumin gene sequences as given
in the drawing of Fig. 5. Furthermore, size measurements on 21 heteroduplexes allowed to deduce that the paired regions a and b contain hybridized repetitive DNA of the Al vitellogenin gene derived from intron 18 and 23, respectively. The paired region c involves an additional sequence derived from intron 23 of the Al vitellogenin gene that can hybridize to the 74 kd albumin transcriptional unit. Precise mapping in the albumin gene reveals that this sequence resides in the 5.2 kb Eco RI fragment that is at the 3'-side of the 6 kb Eco RI fragment in the 74 kd albumin clone λX74a 111 (see Fig. 4). The finding of this additional sequence c common for the Al vitellogenin and the 74 kd albumin gene could be verified by showing that the 5.5 kb Eco RI fragment of the Al vitellogenin clone λXIV 104 hybridizes also to the 5.2 and 5.4 kb Eco RI fragments of the 74 kd albumin clones (see Fig. 4, actual data not shown).

Comparing the location of these paired regions on the albumin clone with the exon-intron map of the 74 kd albumin gene (14) suggests that the regions a, b and c hybridizing to the Al vitellogenin gene are located in the 74 kd albumin gene in intron 3, 5 and 8, respectively. The paired region d involves a region common to the Al vitellogenin gene (possibly intron 31) hybridizing to the 3'-flanking region of the 74 kd albumin gene.

Since the albumin and vitellogenin clones used for heteroduplex formation contain the gene in the same orientation relative to the Charon DNA and since the paired regions a, b and c are not inverted to one another, we conclude that the primary transcripts of the Al vitellogenin and the 74 kd albumin gene cannot pair because the same strand of the three common elements (a, b and c) is transcribed.

Polymorphic genes are characterized by additional repetitive DNA

Analysis of the Al vitellogenin gene has revealed that a size polymorphism involving a deletion of about 400 nucleotides in intron 11 is correlated with the absence of a repetitive DNA element (12). As Figure 4 illustrates the long variant of the Al vitellogenin gene is characterized by a 1.5 kb Eco RI fragment as found in the clones λXIV 109 and λXIV 106 whereas the short variant present in clone λXIV 107 contains a 1.1 kb Eco RI fragment at this position.

Size polymorphism has also been observed in the 74 kd albumin gene (8)
Figure 6: Repetitive DNA involved in the size polymorphism of the 74 kd albumin gene.

The genomic clones \( \lambda X74a \) 101 and \( \lambda X74a \) 102 representing the long gene variant as well as the clones \( \lambda X74a \) 111 and \( \lambda X74a \) 112 containing the short gene variant of the 74 kd albumin gene were restricted with Eco RI and separated on an agarose gel (left panel). The transferred digests were hybridized to total Xenopus DNA and the autoradiogram obtained after stringent washing is given in the right panel. The restriction fragments containing the size polymorphism are indicated by arrows.

and from electron microscopy of R-loops this heterogeneity was mapped to intron 9 (ref. 14). In this case, the long variant has a 5.4 kb Eco RI fragment as found in the genomic clones \( \lambda X74a \) 101 and \( \lambda X74a \) 102 whereas the short variant in clones \( \lambda X74a \) 111 and \( \lambda X74a \) 112 is defined by a corresponding fragment of 5.2 kb (see Fig. 4). To analyze whether this size polymorphism is also correlated with an additional repetitive DNA element, we restricted genomic clones representing the two polymorphic forms with Eco RI and blotted the DNA after electrophoresis onto nitrocellulose filter (Fig. 6a). The blots were probed for repetitive DNA by hybridization with total Xenopus DNA as described previously (16). After stringent washing only two bands remained hybridized (Fig. 6b). Clearly the 5.4 kb Eco RI fragment specific for the long variant of the 74 kd albumin gene is strongly labelled whereas the corresponding 5.2 kb fragment of the short variant cannot be seen on the autoradiogram. Based on this experiment we conclude that the long variant of the 74 kd albumin gene contains a repetitive DNA not found in the short variant.

Knowing that in two genes of Xenopus size polymorphism is correlated with additional repetitive DNA, we were interested to analyze whether the repetitive DNA element involved is the same. Therefore, we hybridized the 1.5 kb Eco RI fragment of clone \( \lambda X1v \) 107 (see Fig. 4) that contains the repetitive DNA characteristic of the long Al vitellogenin gene variant, to
the 74 kd albumin genomic clones digested with Eco RI (data not shown). The lack of crosshybridization between the polymorphic region of the A1 vitellogenin gene and the polymorphic part of the 74 kd albumin gene demonstrates that different repetitive DNA sequences are involved.

**DISCUSSION**

The chromosome number and DNA content in different Xenopus species suggests that Xenopus laevis evolved by genome duplication. The albumin gene pair coding for the 74 kd and 68 kd albumin would represent the duplicated genes in Xenopus laevis. In the case of the vitellogenin genes, it seems likely that the A1 and B1 vitellogenin genes that are linked (10) represent one cluster whereas the A2 and the B2 vitellogenin genes where linkage has not been found yet would represent the second cluster that arose during genome duplication. Analysis of the exon-intron structures within the albumin as well as the vitellogenin gene family revealed identical patterns: the exon sequences within the 74 kd - 68 kd albumin, the A1 - A2 or the B1 - B2 vitellogenin gene pairs are quite similar (5-8 % sequence divergence), whereas most intron sequences differ in each pair extensively in sequence and in length (11,14, 17). These differences in the corresponding introns can best be explained by insertion or deletion events in which repetitive DNA sequences are probably involved. Our analysis on the distribution of repetitive DNA in these duplicated genes support this hypothesis. The location of 6 specific repetitive DNA elements in the albumin and vitellogenin gene families reveals that only one repetitive element is found probably at a homologous position (intron 18) in two members (A1 and B1) of this gene family. The other repetitive elements analyzed show a scattered distribution within the members of the two gene families. Moreover a specific repetitive DNA can be located within, but also outside, of a transcriptional unit. Our analysis was mainly limited to the six middle repetitive DNA elements initially characterized in the A1 vitellogenin transcriptional unit. From several experiments we know that there are additional repetitive DNA elements in the A1, A2, B1 and B2 vitellogenin genes (12; S. Gerber-Huber and J.-L. Schubiger, unpublished results) as well as in the albumin genes (Ryffel et
al., unpublished results). Therefore, we cannot exclude that we missed repetitive sequences common to all members of the analyzed gene families.

The scattering of the analyzed repetitive DNA elements in the duplicated albumin and vitellogenin gene loci illustrates that DNA structure changes throughout evolution extensively. Obviously, deletions and insertions of repeated sequences seem to play an important role. In the changing genome, the size and order of exons specifying structural genes remain conserved. Rearrangement in exons as a result of insertions or deletions most likely destroy the function of genes and are therefore removed by selection. However, we may find it intriguing that we have not observed one or the other duplicated albumin or vitellogenin gene that were inactivated by such rearrangements since one functional copy of the duplicated gene would survive and might be sufficient for the needs of the animal. At the level of the population, however, only a slight relative advantage of animals with a complete functional set of genes might lead to the disappearance of the mutated forms.

Further evidence for dynamic changes involving repetitive DNA sequences comes from the size variants in the A1 vitellogenin and the 74 kd albumin gene that both are characterized by an additional repetitive DNA sequence. In addition, previous experiments have shown that the repetitive DNA of intron 13, 18 and 23 of the A1 vitellogenin gene are not present in the related species Xenopus tropicalis (16) whereas the other repetitive elements as well as vitellogenin exon sequences can readily be observed (21). From hybridizations of small nuclear RNA of Xenopus (22) to the albumin and vitellogenin genomic clones, we also know that the scattered repetitive DNA sequences do not correspond to such sequences, since no crossreaction was observed (unpublished data in collaboration with R. Zeller, I. Mattaj and E. De Robertis). In addition we could also show that the repetitive DNA sequences described in this paper do not hybridize with the repetitive DNA containing a possible replication origin (23). Together, these observations support the hypothesis that the analyzed sequences have no obvious regulatory function and therefore suggest that they are not involved in the coordinate control of the members of the two gene families.

We believe that this finding is of general significance: middle
repetitive DNA elements as measured by standard hybridization techniques have been found in flanking regions and also within transcriptional units of many structural genes in higher eukaryotes (reviewed in 24). In none of these cases any clear indication has been observed to suggest some regulatory function. A notable exception are developmentally regulated genes in Dictyostelium, a lower eukaryote (5, 6). In contrast, many recent experiments demonstrate that very short repeats of about 10 to 20 nucleotides are involved in coordinate regulation of specific genes (reviewed in 21). These short repeats however do not correspond to the classical dispersed middle repetitive DNA.

Our previous analysis (16) has shown that transcripts of all 6 repetitive DNA present in the estrogen inducible A1 vitellogenin transcriptional unit are also contained in transcripts made in the absence of estrogen. The present data substantiate this finding by showing that the repetitive DNA of intron 18 and 23 of the A1 vitellogenin gene are also found in introns of the 74 kd albumin gene. Since albumin gene transcription is constitutive in liver cells (17), it is expected, that transcripts complementary to the repetitive DNA of intron 18 and 23 are present in unstimulated liver cells of males. We assume that the repetitive DNA sequences analyzed are also present in other transcriptional units, because probing of nuclear RNA with specific repetitive DNA reveals many different transcripts (data not shown). We conclude, therefore, that tissue-specific repetitive DNA transcripts can just reflect differential gene activity and do not necessarily represent sequences responsible for differential gene regulation. Based on all these data, the analyzed repetitive DNA in the albumin and vitellogenin gene loci show characteristics of selfish DNA (as defined by ref. 25 and 26) rather than characteristics of regulatory elements.

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