Volume 10 Number 23 1982 Nucleic Acids Research

Characterization of histone genes isolated from *Xenopus laevis* and *Xenopus tropicalis* genomic libraries

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Received 12 October 1982; Accepted 3 November 1982

ABSTRACT

Using a cDNA clone for the histone H3 we have isolated, from two genomic libraries of Xenopus laevis and Xenopus tropicalis, clones containing four different histone gene clusters. The structural organization of X. laevis histone genes has been determined by restriction mapping, Southern blot hybridization and translation of the mRNAs which hybridize to the various restriction fragments. The arrangement of the histone genes in X. tropicalis has been determined by Southern analysis using X. laevis genomic fragments, containing individual genes, as probes. Histone genes are clustered in the genome of X. laevis and X. tropicalis and, compared to invertebrates, show a higher organization heterogeneity as demonstrated by structural analysis of the four genomic clones. In fact, the order of the genes within individual clusters is not conserved.

INTRODUCTION

Histone genes are rehiterated several fold in the genome of most systems studied. 10 copies are present in the chicken genome (1), 10-20 copies in mouse (2), 20-50 copies in X. laevis (3), 110 copies in Drosophila melanogaster (4) and several hundred copies in sea urchin (5). Only two copies of the H2A and H2B genes are present in yeast (6).

The most extensive studies on the organization of histone genes have been carried out on sea urchin and Drosophila (for a review, see 7). In these systems the five histone genes, separated by spacer DNA, are present in repeating units tandemly organized. The order of the genes in each cluster is constant within species. In sea urchin the order is H4-H2B-H3-H2A-H1 (8) while in Drosophila is H3-H4-H2A-H2B-H1 (4). In sea urchin all five genes are transcribed off the same strand (8) while in Drosophila two proteins (H4-H2B) are coded on one strand and three (H3-H2A-H1) on

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the other (4). In the sea urchin Psammechinus miliaris, in addition to the major repeating unit, several other clusters are present; they have different restriction patterns and contain genes for variant proteins (9).

Recently, preliminary characterizations of recombinant DNA molecules containing histone genes have been reported for several vertebrates (for a review, see 10). Moorman et al. (11) have described the presence of four histone genes on a X. laevis genomic fragment in the order H3-H4-H2A-H2B; the sequences coding for H3, H4 and H2B are present on one strand and those coding for H2A on the other (quoted in 10). Two other X. laevis genomic clones, analyzed by Zernik et al. (12), present different gene arrangements and contain two different H1 variants. The organization of histone genes in the newt Notophthalmus viridescens is completely different from that of Xenopus laevis. The majority of histone genes are arranged in homogeneous 9 Kb clusters. The gene order is H1-H3-H2B-H2A-H4. These clusters are not tandemly repeated, but are separated by 50 Kb of DNA made up of a 225 bp repeated sequence (13). The analysis of several chicken histone genomic clones indicates the absence of a constant repeating unit (14). Similar organization heterogeneity emerges from the analysis of mouse (15, 16) and human (17, 18) genomic clones.

In the present work we have isolated and characterized different clusters of histone genes: two from X. laevis and two from X. tropicalis. The characterization includes the construction of restriction maps and the definition of the gene order.

MATERIALS AND METHODS

Preparation of nucleic acids.

DNA and RNA of X. laevis and DNA of recombinant plasmids were extracted and purified as previously described (19). Phage growth was essentially as described by Blattner et al. (20) and DNA purification was as already described (21).

Isolation of histone RNA by hybridization selection.

Hybridization selection experiments were performed with 20 μg of λ CH4 recombinant DNA or 5 μg of plasmids DNA or 1-3 μg of purified ge-

nomic fragments as described by Bozzoni et al. (19).

In vitro translation and analysis of products.

RNA samples were tested in a non preincubated wheat germ lysate (22) or in a rabbit reticulocyte lysate system (NEN translation kit). The wheat germ protein synthesis assay was essentially as described by Alton and Lodish (23). ^{3}H-lysine (100 Ci/mM) was used as radioactive precursor. Products of the cell-free translations were extracted with 66% acetic acid and analyzed by acrylamide gel-electrophoresis according to Panyim and Chalkley (24) or on two-dimensional acrylamide gel-electrophoresis as described (25). Purified X. laevis histone markers were added to each sample before electrophoresis. Gels were processed for fluorography according to Laskey and Mills (26) and exposed to Kodak-X-Omat films at -70°C.

Peptide mapping.

Analysis of peptides of in vitro synthesized H3 protein was as previously described (19).

DNA sequence analysis.

Sequencing of 5'-end labeled DNA was performed by the method of Maxam and Gilbert (27).

Construction and screening of the libraries.

The construction of the X. laevis library, from DNA of a single individual, has been reported by Bozzoni et al. (21). The X. tropicalis genomic library was constructed, using DNA extracted from erythrocytes of a single X. tropicalis female, in λ Charon 4 as the X. laevis library. In both cases the cloned DNA was obtained by partial EcoRI digestion. Screening was carried out according to the in situ hybridization technique of Benton and Davis (28), as already described (21).

Analysis of restriction enzyme sites.

The restriction maps were determined by analysis of fragments produced by single or double digestions with various restriction enzymes or following the method of partial digestions described by Smith and Birnstiel (29).

Electrophoresis, blotting, hybridization.

DNA was transferred from the gels onto nitrocellulose filters according

to the technique of Southern (30). Northern transfer was carried out as described by Pierandrei-Amaldi et al. (31). Prehybridization was performed in 2xSSC (1xSSC=0.15M NaCl, 0.015M Na-citrate), 1x Denhardt (32) for 4 hr at 65°C and hybridization was in 2xSSC, 0.1% SDS at 65°C for 16 hr. The filters were washed in 2xSSC at 65°C and then in 0.1xSSC at 55°C.

DNA labeling.

The DNAs of the recombinant phages and plasmids were digested with restriction enzymes, run in agarose gels and stained. The fragments of interest were isolated by electroelution (33). The entire inserts of the recombinant phages and plasmids, or fragments of these, were used to prepare radioactive probes by nick-translation (34). Radioactive cDNA complementary to mRNA was synthesized according to Efstradiatis et al. (35).

RESULTS

Isolation and characterization of a Xenopus laevis cDNA clone containing sequences for the histone H3.

It was previously shown that in X. laevis oocytes a specific fraction of the histone mRNAs contains poly-A tails (36). For this reason we have been able to isolate, from a cDNA bank of X. laevis oocyte poly(A)$^+$ RNA (19), a clone (pXom32) containing sequences specific for the histone H3. It was identified by its ability to select a mRNA whose in vitro translation product comigrates with a X. laevis H3 non radioactive marker in a two-dimensional gel-electrophoresis specific for basic proteins. The identification has been confirmed by comparison of the peptide map obtained by S. aureus V8 digestion of the in vitro translation product with that of the corresponding histone protein marker.

In Figure 1 it is shown the nucleotide sequence of the cDNA insert of plasmid pXom32. It appears that 45 adenine residues occur in the central region of the inserted fragment; moreover the two regions separated by the poly-dA show two open reading frames. Thus two cDNA fragments have been inserted in the same plasmid, probably due to the blunt end ligation performed in order to attach the EcoRI linkers to the double stranded cDNA molecules. From Figure 1 it is also evident that the sequence from the nu-

1 AATTCGCGGGGAGCGTCCTTAATTTTTTTGAATTTTTTTTTACAAATTGTACATAAGTGTGATGTCCTTTTATTTTATAAAGGGTTTGTT

91 AACTGTAGAGTAGTTGTTAGTAAGCATTTTATATGACATTCCCTTAATCCTCAGGTTTTTCAGAAATTTGTATTTGC<u>AGC</u>TAGTCTACTT (AluI)

181 TGTGACCTCTCATTTAAACCATAGTGCATGCCCACATCATTCTTTACATCATTTAGTATCTGTTTAACTCCATTGTAAATAAACTTTCCA

271 CTACCAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAATTCAAAGAGGCCAACCAGATAAGCCTCGCTTGCTTCCTGA

361 AGAGCACCAATAGCTGCACTCTGGAACTTCAAGACTGACTTGAGGTTCCAGAGTGC<u>AGCT</u>ATTGGTGCTCTTCAGGAAGCAAGCGAGGCT (AluI, AluI)
77 AsnPheLysThrAspLeuArgPheGlnSerAlaAlaIleGlyAlaLeuGlnGluAlaSerGluAla

451 TATCTGGTTGGCCTCTTTGAAGATACCAACCTGTGTGCCATTCATGCCAAGAGAGTAACCATTATGCCCAAGGACATCCAGTTAGCCAGAAG
TyrLeuValGlyLeuPheGluAspThrAsnLeuCysAlaIleHisAlaLysArgValThrIleMetProLysAspIleGlnLeuAlaArg 128

Figure 1. Nucleotide sequence of cloned cDNA fragment of pXom32 and the deduced aminoacid sequence of the H3 protein (residues 77 to 128 of standard numbering).

cleotide 388 to 322 is inverted with respect to the sequence from 406 to 472. This could be due to artifactual events which might have occurred during cDNA synthesis before cloning, as it has been described (37, 38). We were able, from published data (11) and from analysis of the aminoacid composition, to attribute histone coding capacity to the region downstream to nucleotide 386. This region corresponds to the carboxy -terminal portion of the histone H3. As mentioned the region upstream to the poly-dA has also an open reading frame but the encoded peptide is so far unknown. The presence of this second coding sequence has not interferred with the hybrid released translation experiment either because the corresponding mRNA is not abundant in the oocyte or because the coded protein has acidic properties preventing its migration into the gel utilized for protein analysis (25).

In order to obtain a pure probe for screening the genomic libraries we subcloned the histone coding portion of pXom32 by isolation of the AluI-EcoRI 123 bp fragment (nucleotides 419-542) and its insertion into pBR322 via EcoRI linkers (pXom32-50).

Isolation and characterization of genomic DNA clones containing histone genes.

The *X. laevis* (21) and the *X. tropicalis* genomic libraries were screened for phages containing the H3 genes using ^{32}P-labeled pXom32-50 as probe

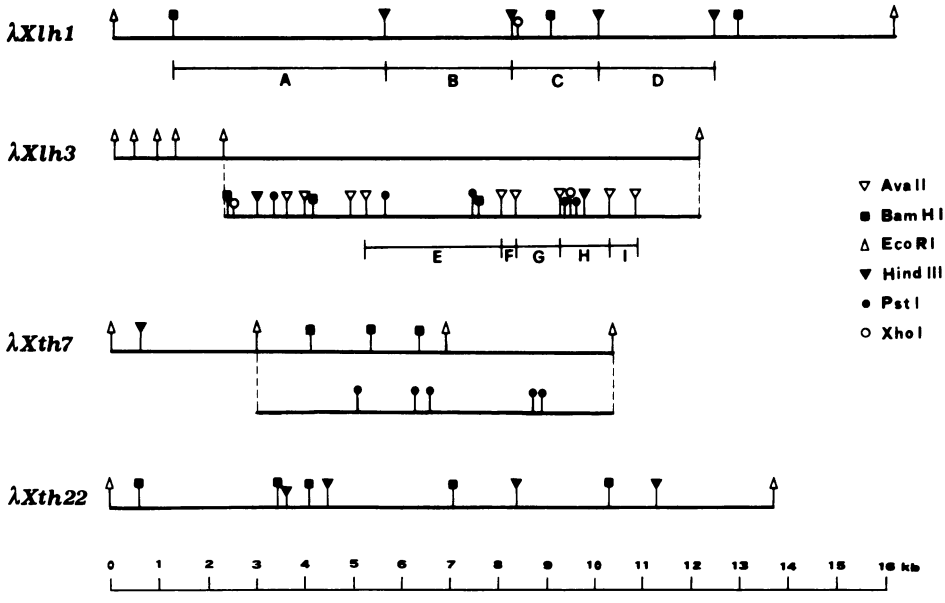

Figure 2. Restriction maps of λ Xlh1, λ Xlh3, λ Xth7 and λ Xth22 inserts. Letters A, B, C, D (λ Xlh1) and E, F, G, H, I (λ Xlh3) indicate the fragments utilized for the hybridization and for the hybrid released translation experiments.

in the in situ plaque hybridization procedure (28).

The screening of 4 x 10^4 X. laevis recombinant phages yielded four positive plaques. These (λ Xlh1, λ Xlh3, λ Xlh4, λ Xlh20) were singularly purified and amplified for further analysis. λ Xlh3, λ Xlh4, λ Xlh20 have identical restriction patterns while λ Xlh1 is completely different. λ XlH1 and λ Xlh3 have been chosen for the study presented in this paper. λ Xlh1 contains a single EcoRI genomic fragment of 16 Kb. λ Xlh3 contains a partial EcoRI fragment 12 Kb of total lenght. The insert, upon digestion with EcoRI, gives five fragments: four quite small (400-1000 bp), which don't hybridize to ^{32}P-labeled cDNA obtained from X. laevis oocyte poly(A)$^+$ RNA and one of 9.8 Kb which hybridizes to the H3 probe. The restriction maps of the 16 Kb EcoRI insert of λ Xlh1 and of the 9.8 Kb EcoRI fragment of λ Xlh3 have been determined and are shown in Figure 2a and b.

Four X. tropicalis recombinant phages (λ Xth7, λ Xth9, λ Xth19, λ Xth22)con-

taining sequences for histone H3, were isolated out of 7 x 10^4 plaques. After plaque purification, the restriction patterns of these clones were compared. After digestion with EcoRI they did not show any common band. λ Xth7 and λ Xth22 were used for further analysis. The insert of λ Xth7, upon digestion with EcoRI, gives three fragments (3.9, 3.6 and 3.0 Kb), while λ Xth22 contains a single EcoRI fragment of 13.6 Kb. Their restriction maps are shown in Figure 2c and d.

Clustering of histone genes in Xenopus laevis.

To determine whether the genes encoding the five histones are clustered in the genome, as in invertebrates, the X. laevis genomic clones, which had been selected with an H3 probe, were assayed for the presence of other histone coding sequences by hybrid selection of mRNAs and gel-electrophoresis analysis of their in vitro translation products. 20 μg of recombinant DNA from each clone were bound to nitrocellulose filters and hybridized in 50 % formamide to 20 μg of X. laevis oocyte poly(A)$^+$RNA. The selectively bound mRNAs were eluted from filters, translated in a wheat germ cell-free system and the products were analyzed by gel-electrophoresis (24).

The electrophoretic pattern of the radioactive products coded by mRNAs hybridized to λ Xlh1 and λ Xlh3 DNAs are shown in Figure 3. DNA from clone λ Xlh1 selects mRNAs coding for the five histones (Fig. 3a), while H1 is missing among the products of mRNAs hybridized to λ Xlh3 DNA (Fig. 3b).

Arrangement of the histone genes in the X. laevis genomic clones.

Using as probe ^{32}P-labeled cDNA obtained from X. laevis oocyte poly(A)$^+$ RNA we were able to localize, in the genomic fragments, the regions carrying coding sequences expressed in the oocyte (not shown). The fragments giving positive signals, A, B, C, D of clone λ Xlh1 (see Fig. 2a) and E, F, G, H, I of clone λ Xlh3 (see Fig. 2b), were used in the hybrid released translation technique to locate individual histone genes along the genomic fragments.

Figure 4 shows the two-dimensional electrophoretic patterns of the products coded by the mRNAs specifically hybridized to each of the fragments E, F, G, H, I of λ Xlh3. These products have been identified by comigration

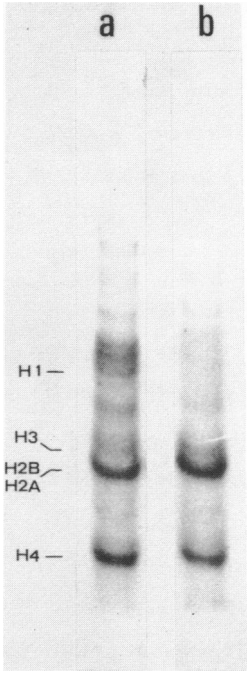

Figure 3. Electrophoretic analysis of radioactive products coded by mRNAs selected by hybridization to the X. laevis genomic clones λ Xlh1 (lane a) and λ Xlh3 (lane b).

with X. laevis histone markers. As previously shown, λ Xlh3 DNA selects mRNAs coding the histones H2A, H2B, H3 and H4 (Fig. 4a). Fragment E selects only mRNA encoding the H3 protein (Fig. 4b), both F and G select H4 mRNA (Fig. 4c and d), H selects mRNAs encoding H2A and H2B (Fig. 4e) while I selects only H2B mRNA (Fig. 4f). The localization on the restriction map of the H3 coding sequences was better determined by hybridization of the H3 probe (pXom32-50) to Southern blot of PstI digested λ Xlh3 (not shown).

The same analysis performed on λ Xlh1 shows that the fragments A, B, C, D select mRNAs encoding H1 (A), H4 (B), H3 and H2A (C) and H2B (D) proteins. To know the relative position of H3 and H2A we utilized the H3 cDNA clone as probe in a blot hybridization experiment on HindIII-BamHI double digested of λ Xlh1 (not shown).

The results of these experiments establish that the order of the X. laevis histone genes in λ Xlh1 is H1-H4-H3-H2A-H2B (Fig. 5a) and in λ Xlh3 is H3-H4-H2A-H2B (Fig. 5b).

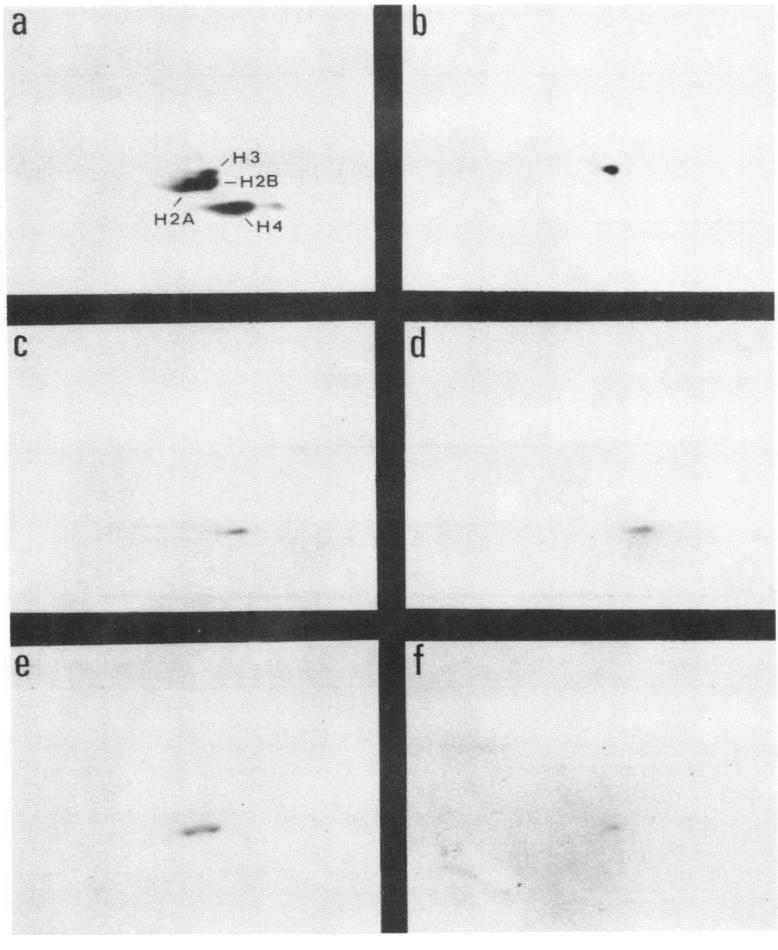

Figure 4. Two-dimensional analysis of radioactive products coded by: a) mRNA hybridized to recombinant clone λ Xlh3, b-f) mRNA hybridized to the fragments E, F, G, H, I respectively.

The fragments A, B, C, D of λ Xlh1 have been used also in hybridization experiments on Northern blots of oocyte poly(A)$^+$ RNA. As shown in Fig. 6 each fragment hybridizes to a single band which fits to the expected molecular weight of a histone mRNA. In fact fragment A, which contains the H1 sequence, hybridizes to the largest mRNA, while fragment B, containing the H4 sequence, to the smaller. A broader band results from the

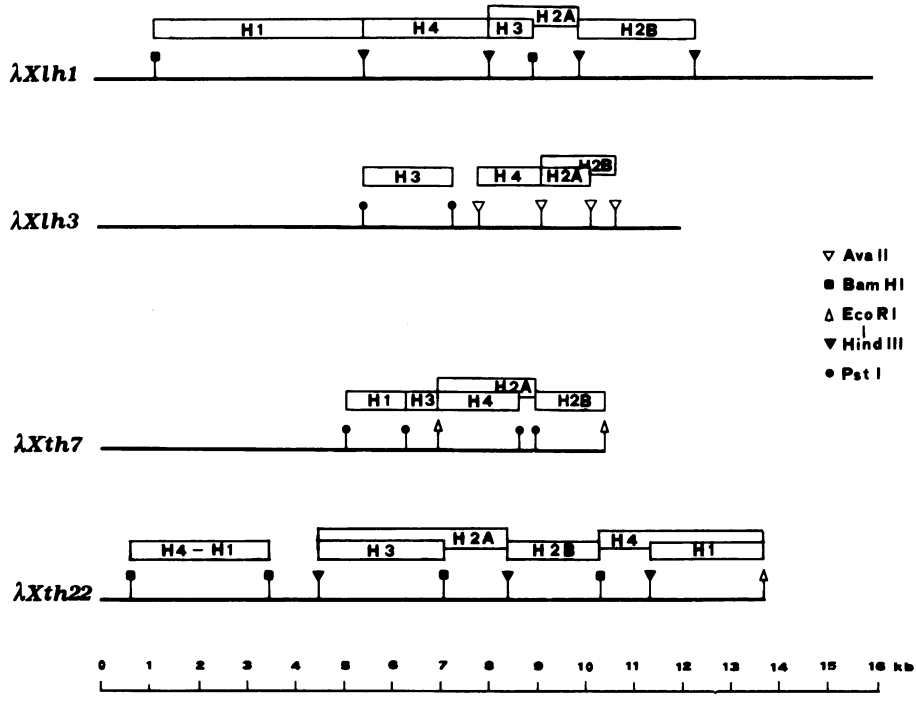

Figure 5. Histone genes arrangements in λ Xlh1, λ Xlh3, λ Xth7 and λ Xth22. Only relevant restriction sites are indicated (compare with restriction maps of Fig. 2).

hybridization with fragment C, which in fact contains sequences for both H3 and H2A.

Similar experiments have been carried out with λ Xlh3 clone (not shown). In this case no hybridization has been detected in the region of H1 mRNA in agreement with the hybrid released translation experiments (see above).

Arrangement of the histone genes in the X. tropicalis genomic clones.

In order to determine the arrangement of the histone genes in the Xenopus tropicalis clones we carried out heterologous hybridization experiments. We have used the BamHI-HindIII fragments: A (H1), B (H4), C (H3-H2A) and D (H2B) of λ Xlh1 X. laevis clone. The H3 cDNA clone has been used to distinguish H3 from H2A sequences. These probes have been utilized on Southern blots of λ Xth7 and λ Xth22 digested with several enzymes. As

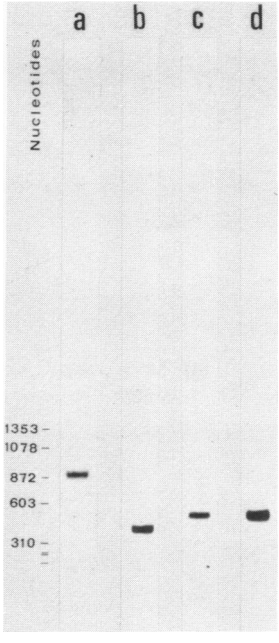

Figure 6. Northern blot analysis of RNA poly(A)$^+$ hybridized to nick-translated fragment A(a), B(b), C(c) and D(d) of λ Xlh1. Molecular weight standards were derived from denatured end-labeled HaeIII digestion fragments of ΦX RF DNA.

an example Figure 7A shows the EcoRI-PstI double digestion of λXth7 DNA (lane 1) and its hybridization with the various probes (lane 2-6). It appears that each one of them gives a specific band of hybridization. Knowing the localization of the EcoRI and PstI sites on the restriction map we have been able to order the different coding regions (Fig. 5c). Similarly, Figure 7B shows, as an example, λ Xth22 DNA digested with BamHI and HindIII. The analysis of this clone demonstrates the presence of two coding sequences for H1 and H4 and one for H2A, H2B and H3 (Fig. 5d).

From these experiments we concluded that both X. tropicalis clones contain sequences coding for the five histones indicating that also in this case they are clustered. The gene order is H1-H3-H4-H2A-H2B (λ Xth7) and H4, H1-H3-H2A-H2B-H4-H1 (λ Xth22).

Organization of the histone genes in the total X. laevis and X. tropicalis genomic DNA.

It seemed interesting to verify the representativity of our cloned fragments with respect to the total genomic histone gene complements. Since Southern blot analysis for histone sequences has shown, in X. laevis, an

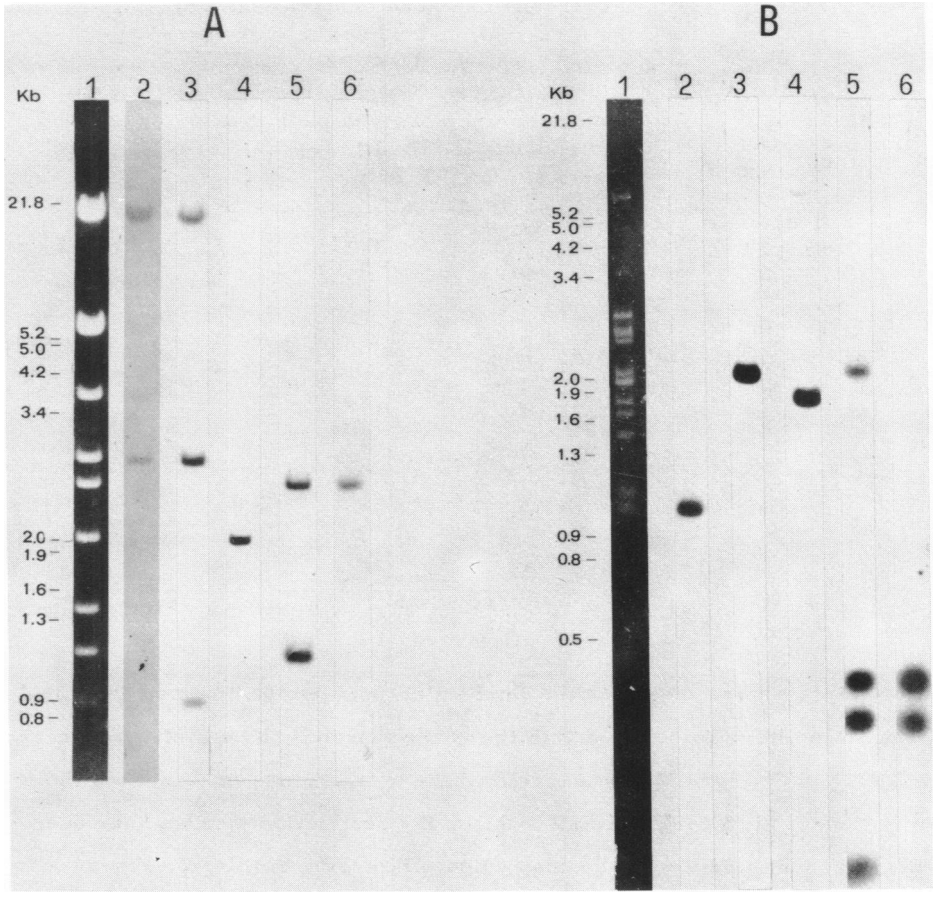

Figure 7. Southern blot analysis of DNA from λ Xth7 double digested with EcoRI and PstI (A) and λ Xth22 double digested with BamHI and HindIII (B). The digested DNAs (lane 1) were hybridized to the radioactive fragments A (lane 2), B (lane 3), C (lane 4), D (lane 5) and to pXom32-50 (lane 6). Molecular weight standards were derived from EcoRI-HindIII digested λ+DNA.

high level of population polymorphism (39) we have compared the histone hybridization pattern of the cloned fragments with that of total genomic DNA of the same two individuals of X. laevis and X. tropicalis utilized for the construction of the genomic libraries. In Figure 8a it is shown the hybridization pattern of histone genes in the total genomic DNA of the X. laevis. Figures 8b and c show that our cloned fragments correspond to the 16 Kb EcoRI unit (λ Xlh1) and to the 9.8 Kb EcoRI unit (λ Xlh3). Neither of them is the

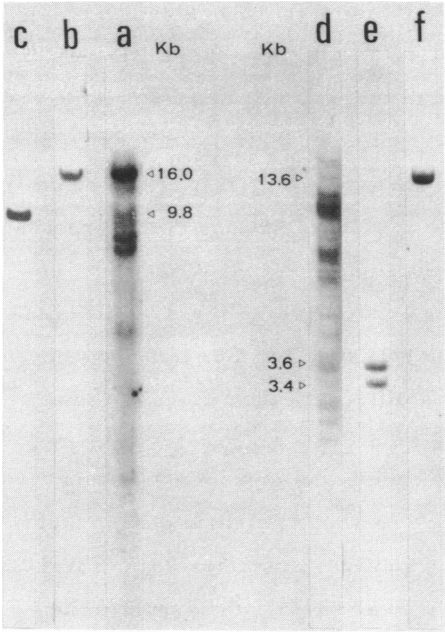

Figure 8. Southern blot analysis of EcoRI digests of: X. laevis genomic DNA (a), X. tropicalis genomic DNA (d), λ Xlh1 DNA (b) λ Xlh3 DNA (c) λ Xth7 DNA (e) and λ Xth22 DNA(f). A mixture of nick-translated fragments A, B, C, D has been used as probe. Molecular weight standards were derived from EcoRI digested λ+DNA.

"major repeating unit" described in all the individuals analyzed (39), which is represented by two 8.9 Kb and 5.1 Kb EcoRI fragments.

From Figure 8d it is evident that histone genes are highly heterogeneous also in the X. tropicalis genome. The fragments cloned in λ Xth7 (Fig. 8e) and λ Xth22 (Fig. 8 f) represent low copy number units. Two hybridization bands are present in λ Xth7, due to an EcoRI site internal to the cluster, as expected from the restriction map.

DISCUSSION

From a cDNA bank of X. laevis oocyte poly(A)$^+$ RNA (19), we have isolated a clone containing sequences specific for histone H3. Determination of the nucleotide sequence of the cloned cDNA has shown, in fact, the presence of a region coding for 52 aminoacids of the carboxy-terminal portion of H3. This aminoacid sequence can be compared with the one derived by Moorman et al. (11) from a X. laevis H3 gene. The two sequences differ for 4 out of 52 aminoacids. These differences are mainly localized in a small region (aa. 87-90) where -Ala-Ala-Ile-Gly- are present in our sequence while

-Ser-Ala-Val-Met- have been found by Moorman et al. (11). It is interesting to note that this last sequence is identical to all other animal H3 sequences known (40). This suggests that our clone corresponds to a H3 histone variant expressed in the oocyte. Another interesting observation comes from the comparison of our H3 coding nucleotide sequence with that of Moorman et al. (11). Out of 156 nucleotides, the two sequences present 30 differences, most of them being sinonymous substitutions (6 are responsible of the mentioned aminoacid changes). The interesting point is that of these 30 differences, 24 are G or C in Moorman et al. gene (11) and A or T in our sequence, resulting in a different G C content and in a different codon usage of these two H3 variant genes within the same species.

Using as probe the H3 cDNA clone, we have isolated from a X. laevis and a X. tropicalis genomic libraries clones containing histone genes. Since in the Xenopus population a certain level of polymorphism has been described for several genes by restriction analysis (19, 39, 41), the genomic libraries were constructed with DNA extracted from single individuals. Two genomic clones from X. laevis and two from X. tropicalis have been characterized. This analysis includes the search for the presence of other histone sequences, the construction of restriction maps and the definition of the histone gene order.

A polymorphism has been observed by Southern blot analysis of DNA from several individuals which give different hybridization patterns with histone probes (39). Two bands are present, though, in all individuals analyzed by EcoRI digestion and derive from several copies of a gene unit constant in the population and called "major repeating unit". Neither the genomic fragments isolated by Moorman et al. (11) and Zernik et al. (12) nor our are representative of this major unit.

In the two clones analyzed (λ Xlh1 and λ Xlh3) we showed the presence of the 4 core histone genes and in one of them (λ Xlh1) the presence also of H1. We cannot exclude the possibility that a different H1 gene, not expressed in the oocyte, is present in clone λ Xlh3. In fact we have used only oocyte RNA in all the hybrid released translation and Northern blot hybridization experiments.

We found also that the order of the histone genes is different for the two genomic fragments. In fact the histone genes of λ Xlh3 are arranged in the order H3-H4-H2A-H2B while in λ Xlh1 they are H1-H4-H3-H2A-H2B, which is the same order found in the major unit by van Dongen et al. (39).

As for X. tropicalis we have also observed an heterogeneity of the histone gene units and a clustering of the five genes. Moreover, as previously reported for X. laevis (12), we found that the X. tropicalis histone gene clusters may be tandemly repeated. In fact we have shown that λ Xth22, containing a 13.6 Kb genomic fragment, has sequences specific for H1 and H4 repeated at the two extremities of the fragment. This suggests the occurrence of two adjacent repeats but the possibility of an anomalous cluster cannot be excluded. Even considering the case of adjacent repeats it must be noticed that the two regions containing H1 and H4 have different restriction maps. The arrangement of the histone genes is different for the two cloned genomic fragments of X. tropicalis: H1-H3-H4-H2A-H2B (λ Xth7) and H4, H1-H3-H2A-H2B-H4-H1 (λ Xth22).

Together with recent data (for a review see 10) our results indicate an extremely variable histone gene arrangement in Vertebrates.

ACKNOWLEDGMENTS

We thank Mr. A. Di Francesco and Mrs. N. Campioni for technical assistance and the partecipants to the EMBO course "Molecular cloning and gene structure" held in Naples (October 1981) for collaboration in the construction of the Xenopus tropicalis genomic library. We are grateful to Mrs. A. Sebastiano for typing the manuscript.

This work was partially supported by the Foundation "Istituto Pasteur-Fondazione Cenci Bolognetti".

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