Volume 9 Number 10 1981 Nucleic Acids Research

Characterization of a cloned histone gene cluster of the newt Notophthalmus viridescens

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Received 19 March 1981

ABSTRACT
We report the cloning and characterization of a histone gene cluster of the newt Notophthalmus viridescens. Fragments containing newt histone genes were identified in whole genome Southern blots; these fragments were cloned into a bacteriophage lambda cloning vector constructed for this purpose. The positions of most of the histone genes were determined by hybridizing subcloned sea urchin histone genes to digests of the cloned newt gene cluster. The position of each gene was verified, and its polarity determined by sequencing a portion of each. The order of the genes in the cloned segment is H1-H3-H2B-H2A-H4, with each of the genes but H2B being transcribed in the same direction. Subcloned segments of the histone gene repeat were used to determine the size of each newt oocyte histone mRNA.

INTRODUCTION

The primary structures of histones have been rigidly conserved in an evolutionary sense, presumably reflecting limitations inherent in fulfilling conserved functions. The genes which encode the histones have been examined in some organisms, and it is clear that several aspects of their organization have been conserved as well. The genes are linked and are repetitive in Drosophila, sea urchins, and Xenopus, although details of the pattern are different in each case. In Drosophila and sea urchins, genes for the five histones are clustered and tandemly reiterated several hundred times per haploid genome, although the order and polarity of the genes within the cluster differs between Drosophila and the several sea urchin species examined (1). In Xenopus, histone genes are clustered but are arranged differently from either Drosophila or sea urchin genes (2,3). Further, Xenopus histone genes are reiterated tens rather than hundreds of times per haploid genome (4). The relevance of these similarities and differences to the function of the genes is not clear. The fact that the five genes are clustered in higher animals may reflect the necessity for equal synthesis of the histone proteins. Even so, the mechanism by which clustered genes might be expressed coordinately remains unclear.

The lampbrush chromosomes of oocytes are decondensed and transcriptionally active. It has generally been assumed that the mRNAs accumulated in the egg are products of the lampbrush stage (5), although recent experiments cast doubt upon this interpretation (6). We wished to examine this problem in more detail, with special attention to the questions of how and at what stages mRNAs accumulate, the stability of these messages, and the significance of lampbrush chromosome transcription. We chose to study the structure and transcription of the histone genes of the newt Notophthalmus viridescens for two reasons. First, large quantities of histone mRNA are synthesized and stored in newt oocytes (7,8); we thus expected the transcripts to be prominent and amenable to study. Second, we expected the histone genes of the newt to be repeated, as they are in other animals, facilitating their isolation and characterization.

We report here on the first phase of this project. We describe the construction and selection of recombinant phage carrying newt histone genes, the mapping of the positions of the genes on this cloned segment, and the determination of the sizes of each of the oocyte histone mRNAs.

EXPERIMENTAL PROCEDURES

Animals, DNA and RNA. Newts (Notophthalmus viridescens viridescens) were obtained from Lee's Newt Farm, Oak Ridge, Tennessee, or were collected near Wells State Forest in Massachusetts.

DNA from testes, or from pooled livers and spleens, was prepared as described (9). Some DNA was the gift of Dr. Manuel Diaz.

Total ovary RNA was prepared as described (10), and was the gift of Dr. Milan Jamrich.

Enzyme reactions. Restriction enzymes were obtained from New England Biolabs, Bethesda Research Labs or Boehringer-Mannheim Biochemicals. Sma I digests were carried out in 30 mM Tris-HCl, pH 9.0, 3 mM $MgCl_2$, and 15 mM KCl. All others were in 6 mM Tris-HCl, pH 7.5, 6 mM $MgCl_2$, 60 mM NaCl, 6 mM β-mercaptoethanol and 10 μg/ml bovine serum albumin. Digests were at 37°C, except for Taq I and Bst EII digests, which were at 65°C.

Ligations were performed as described (11), using T4 polynucleotide ligase obtained from New England Biolabs.

Nick translations were performed as described (12). E. coli DNA polymerase I (3-5 units per reaction) was obtained from Boehringer-Mannheim Biochemicals.

For 5' end-labeling of DNA fragments for sequencing, DNA (1-3 µg) was digested with the first restriction enzyme, as described above, in a reaction volume of 10 µl. The digestion was stopped by heating at 65°C for 5 minutes. The reaction was made to 50 mM imidizole-HCl, pH 6.6, 10 mM $MgCl_2$, 5mM dithiotheitol, 0.1 mM spermidine, 60 µM ADP and 1 µM γ-^{32}P ATP (2000-3000 Ci/mmole, New England Nuclear), in reaction volume of 25 µl. 10 units of polynucleotide kinase (P-L Biochemicals) were added, and the reaction held at 37°C for 45 minutes. The reaction was terminated by heating at 65°C for 5 minutes. Seven volumes of restriction enzyme buffer and the second restriction enzyme were added. After a suitable digestion, the DNA was phenol extracted, ethanol precipitated and electrophoresed on a polyacrylamide gel. Gel slices containing the desired fragments were chopped to a fine paste with a fresh razor blade, 3 volumes of 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, were added, and the mixture held at 42°C overnight. Acrylamide crumbs were removed by filtration through 0.45 µm pore size nitrocellulose and the labeled DNA recovered by ethanol precipitation.

Sequencing was carried out using the nick-translation method of Seif et al (13). Backward and forward reactions were carried out exactly as described. Reactions were terminated by addition of an equal volume of 10 mM EDTA, pH 8.0 and dried; DNA was resuspended in the loading mixture described, and run in 8% polyacrylamide gels prepared as described (14). Most preparations of Boehringer-Mannheim DNA polymerase I contained enough endogenous nuclease to make the reactions work well. To other batches we added 1 µl 10^{-7} g/ml DNase I per 8 µl polymerase I.

Electrophoresis. 0.3% to 2.0% agarose gels were run as described (15). 3.5% or 5.0% acrylamide gels were prepared and run as described (16), except that the running buffer was the same used for agarose gels.

Elution of DNA from gels onto nitrocellulose filters and the hybridization or radioactive probes to these filters was as previously described (17).

RNA and DNA were denatured with glyoxal and dimethylsulfoxide, electrophoresed as described (18); transfer of the RNA to diazotized paper and hybridization with radioactive probes was as described (19).

For preparative electrophoresis, agarose gels were poured using low gelling temperature agarose (Marine Colloids) and allowed to harden at 4°C for 1 hour. Gels were run, stained, and photographed, and appropriate bands were excised from the gel. The volume of the gel slice was estimated, 1/10 volume of 3 M sodium acetate added, and the tube held at 65°C until the agarose melted. The DNA was extracted twice with phenol, once with phenol:chloroform

(1:1), and three times with ether, and ethanol precipitated twice in the presence of E. coli tRNA carrier.

Recombinant DNA. All plasmids were propagated in E. coli strain HB101. pC01, which contains a full repeat of the Strongylocentrotus purpuratus histone cluster in pBR313 (20) was obtained from E. Weinberg. pTs323, pTs317 and pTs304, subclones of the Psammechinus miliaris histone repeat h22 (21), containing the genes and surrounding spacer sequences for H2A, H2B and H3, respectively, in pCR1, were obtained from M. Birnstiel.

pNv6-2 is a recombinant plasmid which contains a newt H4 gene and some surrounding spacer sequences. This clone was originally identified by hybridization with pC01, and its identity confirmed by sequencing. Details of the construction and characterization of this clone will be presented elsewhere.

Newt DNA fragments originally isolated as recombinant phage were often recloned into pBR322 for detailed analysis and sequencing. Gel purified fragments were ligated as described above, and used to transform bacteria as described (22). Recombinant plasmids were selected by sensitivity of the host bacterium to tetracycline. Plasmid DNA was prepared as described (23).

Lambda phage were grown on E. coli strains DP50 or DP50supF, obtained from F. Blattner. Charon 10 (24) was obtained from F. Blattner. λ NM570 BV2 (25) was obtained from K. Murray. Phage were grown and harvested as described (26) and DNA was prepared by phenol extraction of purified phage.

ES6, a lambda phage derivative with replaceable central Bam HI fragments, was constructed by in vitro recombination of NM570 BV2 and Ch10. Phage DNAs were cut with Bam HI, and the left arm of BV2, the right arm of Ch10, and the C and D fragments of wildtype λ (C_I857 Sam7) were purified by preparative electrophoresis. These fragments were ligated together as described above, packaged into phage capsids using packaging extracts prepared as described in an unpublished procedure of F. Blattner, and used to infect DP50. One of the recombinant phage recovered was named ES6. Restriction enzyme mapping confirmed that this phage contained the fragments expected. A combination of the left and right arms of this phage should be just too small to form viable phage (27), allowing physical selection for insertion of foreign DNA.

The cohesive termini generated by Bam HI and Bgl II have the same nucleotide sequence, and can thus be easily ligated together. Bam HI arms of ES6 and 8-12 kb Bgl II-digested newt DNA were purified by preparative electrophoresis, ligated together, packaged into phage capsids, and used to infect bacteria. At least 80% of the phage prepared in this manner contained

newt DNA inserts.

About 10,000 phage from the library constructed in this fashion were screened as described (28), using nick-translated pNv6-2 as the hybridization probe. After two rounds of screening, well separated positive plaques were picked and DNA prepared.

All recombinant DNA experiments were carried out in accordance with the guidelines established by the NIH.

RESULTS

Preliminary characterization and cloning of newt histone genes. We used a subcloned newt H4 gene to investigate the organization of the histone genes in the newt genome. Figure 1 shows the results obtained when pNv6-2 (see Experimental Procedures) was hybridized to newt DNA which had been digested with various restriction enzymes, fractionated by gel electrophoresis, and transferred to nitrocellulose as described (29). The probe hybridized to DNA at the limit mobility of the gel after Bam HI, Eco RI or Hind III digestion, and to bands of 9.0 and 4.3 kb in the Bgl II lane. This result and others of this type indicated that one or more major families of the newt histone genes were cut at regular intervals by Bgl II, but were not cut by Bam HI,

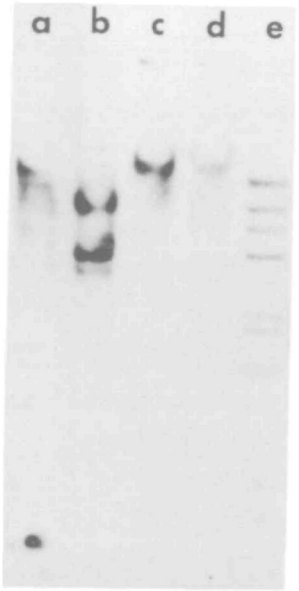

Figure 1. Hybridization of a newt H4 gene to digests of newt DNA. pNv6-2, a clone containing a single newt H4 gene and a small amount of surrounding spacer sequences, was hybridized to digests of newt DNA, which had been fractionated in a 1% agarose gel. Digests shown are (a) Bam HI. (b) Bgl II, (c) Eco RI, (d) Hind III, (e) ^{32}P-labeled λ DNA cut with Hind III. Sizes for bands in the λ Hind III digest are 23.8, 9.6, 6.8, 4.4, 2.4, 2.1 and 0.56 kb.

Eco RI, or Hind III.

We prepared a library of 8-12 kb Bgl II fragments of newt DNA in λES6 and screened about 10,000 of the phage with pNv6-2, as described in Experimental Procedures. Preliminary restriction enzyme maps of four positive clones revealed that three had the same basic restriction enzyme pattern. Slight differences in the positions of some of the enzyme sites, and differences in the orientation of the newt DNA in the phage indicated that these three clones were independent isolates of a major family of related sequences. We report here on the results of our analysis of one of these clones, ES6 Nv51.

Positions of histone genes in Nv51. A restriction enzyme map of ES6 Nv51 was constructed by appropriate single and double digests of ES6 Nv51, and of segments of Nv51 subcloned into pBR322. Positions for some enzyme sites are shown in Figures 2 through 5. The positions of most of the histone genes in Nv51 were determined by hybridizing subcloned sea urchin histone genes to restriction enzyme digests of ES6 Nv51 DNA. The location of each gene was verified, and the direction of transcription was determined by sequencing parts of each.

H2A:

We located the H2A gene by hybridizing pTs323, a subclone of the Psammechinus miliaris h22 histone repeat to restriction enzyme digests of Nv51. The sea urchin subclone hybridized to a 1400 bp fragment produced by Bam HI and Hind III digestion, and to 1900 and 500 bp Sma I fragments (data not shown). Figure 2a shows the sequencing reactions carried out to position the H2A gene more accurately, and Figure 2b shows the nucleotide sequence and amino acid assignments from a region of the H2A gene. Of the three reading frames on each of the two strands, only one allows codon assignments consistent with known sequences of H2A proteins. The 26 unambiguous codons in this sequence exactly match a region of the cow H2A protein (30).

H2B:

Psammechinus miliaris h22 subclone pTs317 hybridized to a 365 bp Hind III fragment, and to a 1400 bp Bam HI/Hind III fragment of Nv51 (data not shown). Sequencing reactions performed to locate the H2B gene are shown in Figure 2a, and nucleotide sequences obtained in this region are shown in Figure 2c. 29 of the 32 unambiguously assignable codons match a region of the trout H2B protein sequence (31).

H3:

P. miliaris subclone pTs304 hybridized to a 2300 bp Bam HI/Hind III

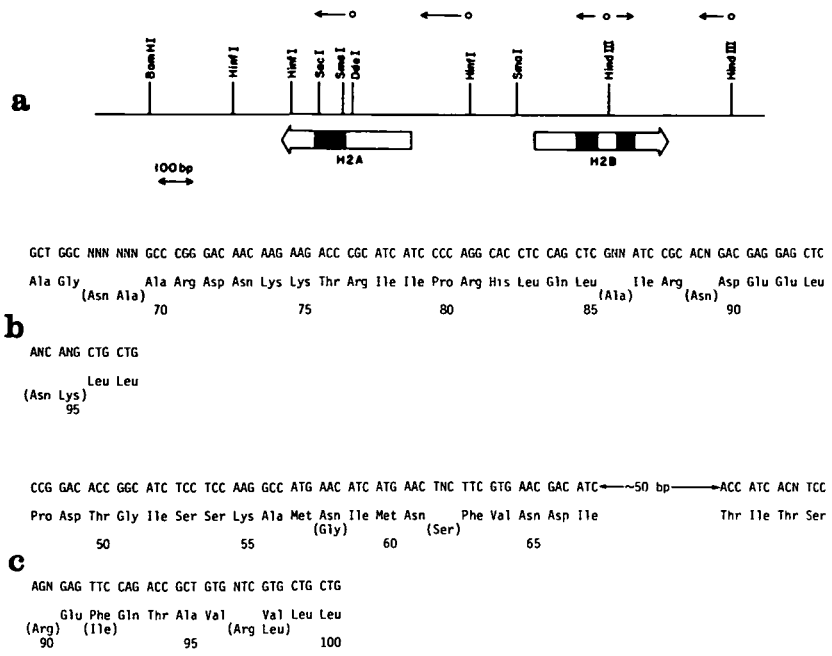

Figure 2. Detailed restriction enzyme map, sequencing reactions and nucleotide sequences from the H2A and H2B gene region of Nv51. (a) Sequencing reactions performed in this region are shown by the thin arrows. The open circles indicate the restriction enzyme site labeled for each reaction, and the length of the arrow indicates the length of the sequence obtained. The thick arrows show protein coding regions for each gene, based on the assumption that newt histone proteins are about the same sizes as other vertebrate histones and that the genes do not contain intervening sequences. The solid area of each gene is the portion sequenced. The Dde I site shown is the only one that has been mapped in this region. (b) Nucleotide sequence from the H2A gene, and below, amino acids assigned to one reading frame of the sequence. N = unknown base. The reading frame shown is the only one which matches the amino acid sequence of a reference histone. For simplicity, most of the reference histone sequence has been omitted from the figure. Amino acids from the reference sequence are shown in parentheses below the predicted newt protein sequence only where the newt sequence is ambiguous or does not match the reference. Numbers refer to amino acid positions in the reference histone. The reference here is cow H2A (30). (c) Nucleotide sequence from two regions of the H2B gene, as in 2b. The reference histone is trout H2B (31).

fragment and to 2050 and 3800 bp Sac I fragments of Nv51 (data not shown). Figure 3a shows sequencing reactions performed to locate the H3 gene. Nucleotide sequences and codon assignments are shown in Figure 3b. The 26 unambiguous codons exactly match a region of the cow H3 protein (32).

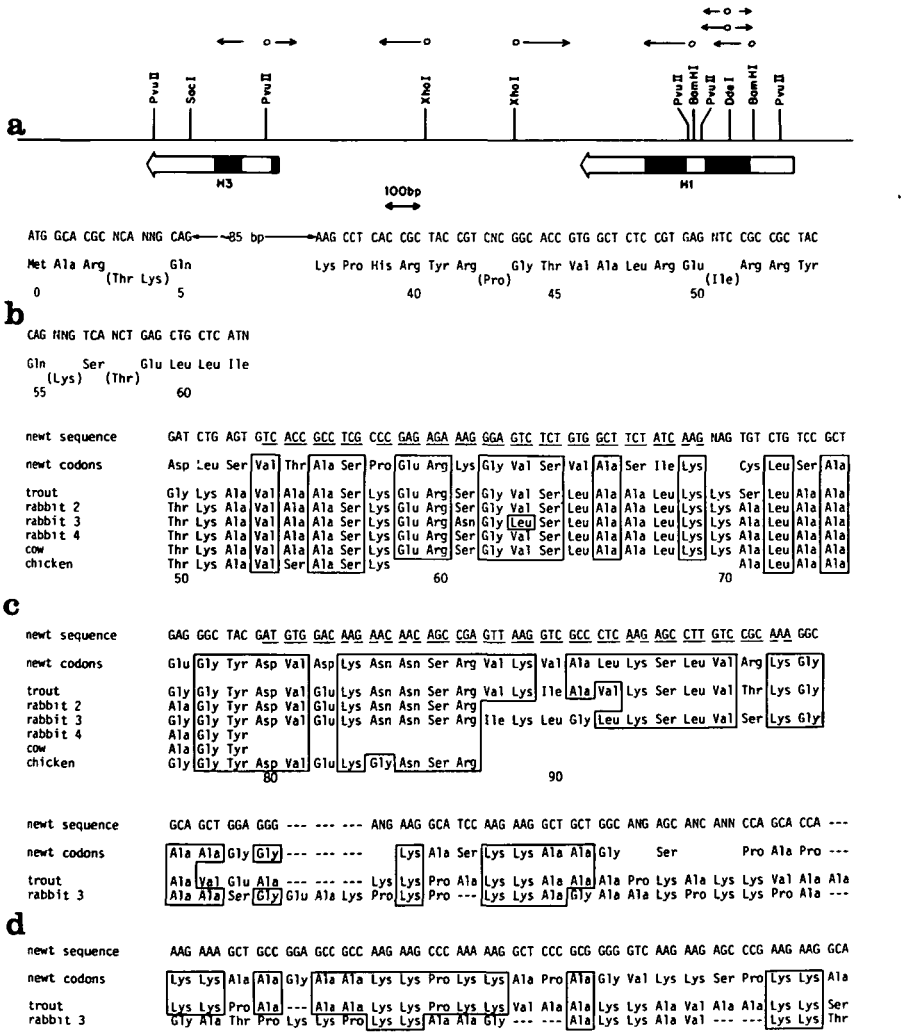

Figure 3. Detailed restriction enzyme map and nucleotide sequences from the H3 and H1 regions of Nv51. (a) As in 2a, Dde I sites have been mapped only between the Bam HI sites. (b) Nucleotide sequence from two regions of the H3 gene, as in 2b. The reference histone is cow H3 (32). (c) Nucleotide sequence from the more N-terminal part of the H1 gene. Underlined nucleotides have been determined two or more times. Codons assigned to the newt sequence are compared to the sequences of several vertebrate H1 proteins. Regions of homology between newt and the reference histones have been boxed. Reference sequences were taken from Von Holt et al. (33). Numbers are the alignment positions of Von Holt et al. (d) Nucleotide sequence from the more C-terminal part of the H1 gene, as in 3c. Gaps, indicated by dashed lines, have been introduced into the alignment to increase the homology.

H1:

As we show in the next section, both subclones pNv51-3 and 51-6 hybridize to a 760 nucleotide oocyte RNA, which, because of its size, must be the mRNA for histone H1. This RNA is the only hybridizing species which would be large enough to encode a protein the size of H1, which ranges from 210-220 amino acids in the vertebrates examined (33). Figure 3a shows sequencing reactions performed to locate the H1 gene, and Figure 3c shows a nucleotide sequence obtained from one gene region. The codons assigned to one of the six reading frames of this sequence are compared with the primary sequences of several reference H1 proteins. A region of the trout H1 protein, with 30 matches out of 46 codons, best corresponds to the assigned protein sequence. Of the 16 mismatches, 11 can be accounted for by single base substitutions. The newt gene can also be matched to mammalian H1 proteins with only slightly less homology, ranging from 54-62%. Of the five histones, H1 is the least conserved evolutionarily, although the sequence shown comes from a region of the protein which is relatively well conserved.

Another nucleotide sequence from the H1 gene is shown in Figure 3d. If the H1 gene is positioned so as to place the sequence shown in 3c near the middle of the protein coding region, the sequence in 3d falls within the carboxy terminal half of the H1 protein, a region which consists almost entirely of alanine, proline and lysine residues, and is not evolutionarily well conserved. Of the 120 bases in this sequence, only 7 are thymine, while adenine, guanine and cytosine are each about equally represented. Alanine, lysine and proline can be specified by codons lacking in thymine, and one reading frame in this sequence, shown in Figure 3d, would produce a peptide consisting almost entirely of these amino acids. This amino acid sequence cannot be matched to reference H1's with as much confidence as can the sequence in 3c, although some homology, notably with the trout H1 protein, is apparent.

H4:

pNv6-2, an independently isolated subcloned newt H4 gene, hybridized within the 2800 bp fragment limited by the leftmost Bam HI site and an Eco RI site within the λ vector, and to an 860 bp Taq I fragment within this region (data not shown). Figure 4a shows sequencing reactions performed to localize the H4 gene, and Figure 4b gives the sequence obtained in this region. The 49 codons assigned in this sequence exactly match a region of the cow H4 protein (34).

Figure 5 summarizes the positions of each of the histone genes in Nv51. We have sequenced at least 20% of each coding region and have found that each

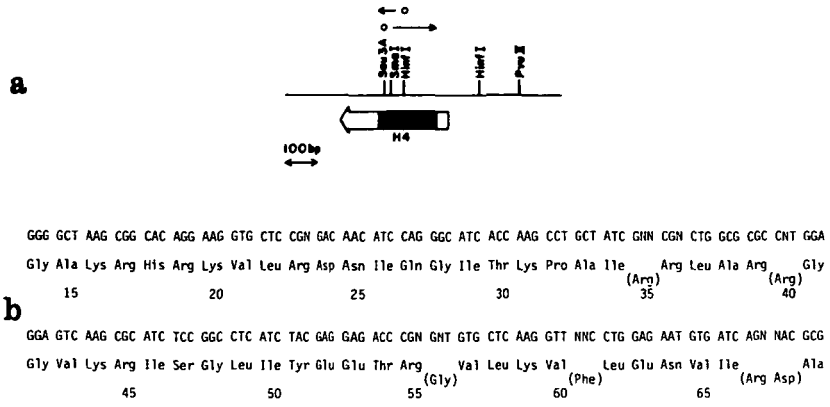

Figure 4. Detailed restriction enzyme map and sequencing reactions from the H4 gene region of Nv51. (a) As in 2a. (b) As in 2b. The reference is cow H4 (34).

predicts a protein which can be matched with the corresponding protein sequence of a reference histone. The sequencing data are not intended to be final or complete, since most nucleotides have been determined only once. They do however, allow assignment of an unambiguous position and polarity for

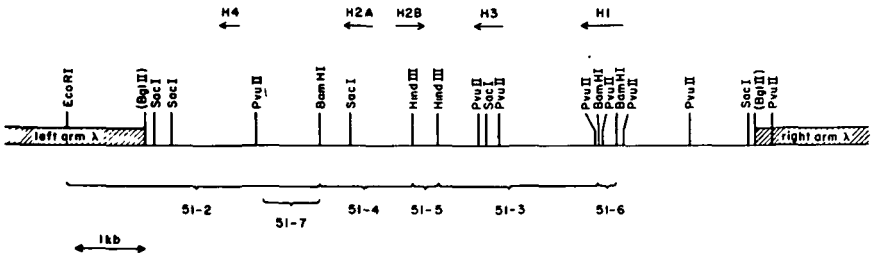

Figure 5. Map of ES6 Nv51. The Bgl II sites in parentheses indicate the newt:λ DNA boundary. Because a newt Bgl II fragment was cloned into a Bam HI site, these sites were destroyed when the clone was constructed. The position of the left boundary of the clone is the position of a Sau 3A site (Sau 3A recognizes the four nucleotide sequence common to both Bam HI and Bgl II recognition sites); the position of the right boundary was deduced from the distances between sites in the newt and λ DNA. Fragments recloned into pBR322 are shown below the restriction enzyme map. The endpoint of each subcloned segment is a Bam HI, Eco RI or Hind III site, except for pNv51-7 which has as its left end a Taq I site which is not shown on the map; this Taq I end was ligated into the Cla I site of pBR322. All other sites were ligated into the corresponding sites of pBR322. Arrows above the restriction map indicates the positions and polarities of protein coding regions of the histone genes.

each gene. Figures 2, 3, 4, and 5 show the position and length of the protein coding region for each of the histones. The length of each coding region was calculated from the size of the reference histone, since newt histones are about the same sizes as other vertebrate histones (8, 35), assuming the genes do not contain intervening sequences.

Sizes of oocyte histone mRNAs. Total oocyte RNA was denatured by glyoxal and DMSO treatment, fractionated by gel electrophoresis and transferred to diazotized paper as described in Experimental Procedures. In Figure 6, lane a shows the ethidium bromide staining pattern of this RNA and lane b shows the hybridization of Nv51 to the transferred RNA. Nv51 hybridizes to four RNA bands of sizes 760, 540, 500 and 440 nucleotides. These sizes were determined by comparison with a similarly denatured ^{32}P labeled Taq I digest of pBR322 run in an adjacent lane.

Hybridization of five subcloned fragments of Nv51 to oocyte RNA is shown in lanes c through g. Subclone pNv51-2 (see Figure 5), from the leftmost end of Nv51, hybridizes only to the smallest RNA. Since our sequencing placed only

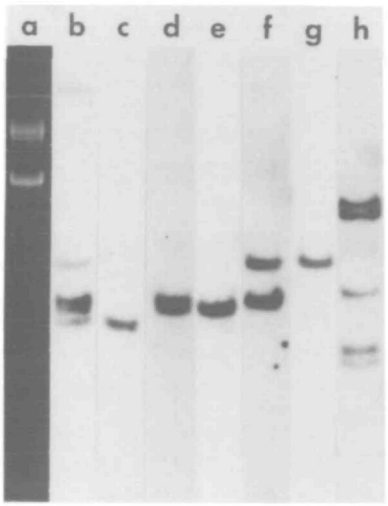

Figure 6. Sizes of oocyte histone mRNAs. The ethidium bromide staining pattern of denatured oocyte RNA electrophoresed in a 2% agarose gel is shown in lane (a). Lanes (b)-(g) show the hybridization of various histone probes to this RNA. The probes and coding regions which they include (see Figure 5) were (b) ES6 Nv51 (all five histone genes), (c) pNv51-2 (H4 gene), (d) pNv51-4 (H2A and H2B), (e) pNv51-5 (H2B), (f) pNv51-3 (H3 and H1), and (g) pNv51-6 (H1). Lane (h) is a ^{32}P-labeled Taq I digest of pBR322 DNA, run in an adjacent lane. Sizes of the bands are 1440, 1310, 620, 370 and 310 bases.

the H4 gene within this region, we conclude that H4 mRNA in newt oocytes is 440 nucleotides long. pNv51-4 hybridizes to the 540 and 500 nucleotide RNAs (lane d), while pNv51-5 hybridizes only to the smaller of these (lane e). Our sequencing data placed the H2A gene wholly within the pNv51-4 subclone and the H2B gene in both pNv51-4 and in 51-5. Therefore, we conclude that oocyte H2A and H2B mRNAs contain 540 and 500 nucleotides respectively. pNv51-3 hybridizes to bands of 760 and 540 nucleotides, while pNv51-6 hybridizes only to the larger of these (lane g). We conclude that the oocyte H1 mRNA contains 760 nucleotides, and H3 mRNA 540 nucleotides.

DISCUSSION

We describe here the construction and characterization of a cloned DNA segment containing genes for the five histones of the newt Notophthalmus viridescens. As we show in another report, the cloned segment Nv51 is a representative of the major family of newt histone genes, which are largely homogeneous and reiterated 600 to 800 times per haploid genome (17). The structure of Nv51 thus reflects the arrangement of a large family of gene sequences. We located each gene on the cloned segment by hybridization; the position of each gene was verified, and its polarity determined by sequencing a portion of each. The sequences provide a convenient and straightforward way to map the histone genes. Since most of the sequences presented here have been determined only once, and are incomplete, we view them as preliminary.

The order of the newt histone genes on the repeating unit is H1-H3-H2B-H2A-H4, with all but the H2B gene transcribed in the same direction. This arrangement differs from the single pattern found in sea urchins, from that of Drosophila melanogaster, and from that of two Xenopus laevis clusters (1,2,3). Of these four groups of animals, no two possess histone gene clusters with the genes in the same order or polarity. Clearly the selective pressures which have kept histone genes clustered in diverse groups of animals have not maintained a common arrangement of the genes within the cluster. Compare the diversity of histone gene order with the evolutionary conservation of the genes for the large rRNAs, which are arranged in the order 18s-5.8s-28s in all eukaryotes examined. This arrangement is presumably related to a common, highly conserved mechanism for transcribing the genes and processing the transcripts into mature ribosomal RNAs. Models have been proposed to account for the transcription of the histone genes based on their arrangement; for instance, that sea urchin genes, which are all on the same strand, are

transcribed as a multicistronic precursor (36). This model has some experimental support (37,38), but given the variability in histone gene organization, no model based on the arrangement in one organism can be universally valid. It seems more likely that histone genes are usually transcribed individually, and that their relative order is unimportant for expression.

The newt histone gene cluster Nv51 and its subclones were used to investigate the sizes of oocyte histone mRNAs. Each of the histone mRNAs is 100-150 nucleotides longer than necessary to encode its protein suggesting, not unexpectedly, that each mRNA contains some untranslated sequences. The early histone mRNAs of two sea urchins, S. purpuratus and P. miliaris contain about 100-120 extra nucleotides; these nucleotides contribute about equally to the mRNA regions preceding and following the coding region (39,40). Most histone mRNAs lack 3' poly(A) tracts, however some of the oocyte histone mRNAs of both Xenopus and Notophthalmus do possess poly(A) tails (7,8). Thus some of the extra nucleotides in newt oocyte mRNA probably comprise 3' poly(A) tracts.

The results of Ruderman and Pardue are inconsistent in one respect with those we present here. They found oocyte histone mRNA in both poly(A)$^+$ and poly(A)$^-$ fractions, and suggested that polyadenylated histone mRNA contained 60 to 120 adenylic acid residues. The mRNAs should thus be heterogeneous in size, comprising either two separate size classes representing adenylated and nonadenylated messages, or comprising a single broad size class if adenylic acid tracts were heterogeneous in length. Our studies show instead that each mRNA species has a rather narrow size distribution. The width of each band would have obscured size differences of 20 nucleotides or less, but the heterogeneity implied by the results of Ruderman and Pardue should have been detectable. Resolution of this discrepancy will require further investigation. With cloned probes now available, it should be possible to isolate the histone mRNAs and determine the lengths of their poly(A) tracts by conventional means.

ACKNOWLEDGEMENTS. We thank Drs. Manuel Diaz and Milan Jamrich for gifts of materials, and Dr. Peter Rae for helpful comments on the manuscript. This work was supported by grant GM 12427 from the NIH. E.C.S. was supported by NIH training grant GM 07223. This work is part of a thesis submitted by E.C.S. in partial fulfillment of the Ph.D. degree.

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