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Are there major developmentally regulated H4 gene classes in *Xenopus?*

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ABSTRACT

Primer extension analysis has been used to study the principal H4 mRNAs present at different developmental stages and in several adult tissues of Xenopus borealis and X. laevis. In X. borealis a single sequence class predominates in oocytes, tadpoles and cultured fibroblasts. There is also a polymorphic minor type which shows no developmental regulation. The primer extension bands obtained from adult liver and kidney RNA appear to be the same as ovary and therefore these tissues almost certainly contain the same major H4 mRNA species. This is confirmed by S1 mapping of the 3' end of the mRNA. Thus for H4 genes in X. borealis there is no evidence of the kind of switches in histone gene expression seen in sea urchins or certain protostomes. The situation in X. laevis is complicated by considerably higher gene variability both within and between individuals. Nevertheless, in this species, as in X. borealis, there seems to be no major developmental switch in the regulation of H4 gene expression, a conclusion that also holds for an H1C and an H3 gene.

INTRODUCTION

The first histone genes to be analysed in detail were those of sea urchins, which have since been taken as a paradigm for histone genes in general. They show two remarkable major switches in development; the subject of this paper is whether such switches are a phenomenon that extends to amphibians.

The genome of sea urchins contains 200-500 quintets of histone genes each quintet encoding the five histone classes. These "early" quintets are nearly identical and are expressed between the time that the oocyte undergoes its reduction divisions and the blastula stage (1-3). Through oogenesis an uncharacterised class of histone genes produces the "cleavage stage" (cs) histones (4), and from blastula to larval stages histones are produced from "late" genes, which are small in number (5-12 of each class), different from early genes in sequence, and they are not present in quintets (5). There is no evidence that developmental switches of this kind occur in phyla other than the Echinodermata, although there is evidence of switches in histone subclass in the mollusc Ilyanassa. It has been found that Drosophila has a major class

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of histone gene quintets superficially similar to the early sea urchin genes (7,8), however changes in their developmental expression have not been reported, so it is not clear if they are a functional analogue of the sea urchin early genes.

In vertebrates it is not known if there are early and late genes analagous to those of sea urchins. In some vertebrates, such as chickens and mammals (2,9-17), there are only 10-20 genes encoding each histone class, they are not arranged in quintets and the genes are diverse in sequence. Some amphibians on the other hand contain highly reiterated histone genes, many of which are contained in a highly conserved quintet. This is clearly shown in the newt Notophthalmus viridescens (18,19) and the frog Xenopus borealis (20). X. laevis approaches this situation, in that several major cluster orders may be recognised (20,21). These animals also contain minor groups of genes, few of which have been analysed in detail. They thus resemble sea urchins in having both a major histone gene quintet, and in having minor genes, arranged in more diverse ways. These analogies in structure naturally suggest that there may be developmental regulation in the expression of amphibian histone genes comparable to that seen in urchins. This possibility fits the similarities of the early development of the two groups, which are both characterised by rapid cell divisions that are accommodated by histone synthesis uncoupled from cell division (22-25). We have tested this possibility for the H4 genes in particular, and find that it does not apply; similar gene sequences provide the major H4 mRNAs throughout development.

METHODS

RNA Extraction

(i) Extraction from ovaries, early embryos, tadpoles and testes. Tissues were homogenized in a buffer containing 10 mM Tris pH 7.4, 1.5 mM $MgCl_2$, 10 mM NaCl, 1% SDS and proteinase K at 1 mg/ml (29), and incubated at room temperature for 1 hour. This homogenate was extracted with phenol/chloroform, then chloroform, and ethanol precipitated. In the case of ovaries, eggs, and early embryos, which contain only small amounts of DNA, the tissues were reprecipitated and used for analysis. RNA from other tissues was dissolved in 10 mM Tris pH 7.4, 10 mM NaCl, 3 mM $MgCl_2$ and incubated with DNase I (Worthington) at 50 µg/ml on ice for 30 min. The solution was extracted with phenol/chloroform, chloroform, and then ethanol precipitated.

(ii) Extraction from liver and kidney. The method used was modified from Cox (30) and Chirgwin et al. (31). Tissue was homogenised on ice in 7 M guanidine-

HCl, 10 mM Tris pH 7.4, 1 mM EDTA, 0.2% SDS, 100 mM beta-mercaptoethanol, and left on ice 10-15 minutes. The DNA released was sonicated, then 1/20th volume of 1 M acetic acid, 1/20th volume of 2 M potassium acetate pH 5.0 and 0.5 volume of ethanol were added to precipitate the RNA. The pellet was dissolved in 10 ml 5.4 M guanidine-HCl, 22.5 mM EDTA pH 7.6, 10 mM dithiothreitol (DTT), vortexed, warmed to 65°C for 3 min then re-precipitated several times with 0.5 ml 2 M potassium acetate pH 5.0 and 5.0 ml ethanol. The pellet was dissolved in 25 mM EDTA and extracted with phenol/chloroform until the interface was clean, then the aqueous phase was precipitated with 2 volumes of 4.5 M potassium acetate, pH 6.0.

(iii) Extraction from tissue culture cells. Cells were washed with cold phosphate buffered saline (PBS), scraped off the bottle and spun down in cold PBS, in the presence of vanadyl ribonucleoside complex (10 mM). The pellet of cells was resuspended in lysis buffer (0.14 M NaCl, 1.5 mM $MgCl_2$, 10 mM Tris pH 8.6, 0.5% NP40) vortexed for 10 seconds, left on ice for 5 minutes and then the nuclei spun down in an Eppendorf microfuge. An equal volume of buffer (0.2 M Tris pH 7.5, 25 mM EDTA, 0.3 M NaCl, 2% SDS, and 1 mg/ml proteinase K was added to the supernatant and it was incubated at 37°C for 30 minutes. The supernatant was extracted with phenol/chloroform, then chloroform, and finally ethanol precipitated.

Primer extension on H4 mRNA.

The probe was prepared from the X. laevis H4 cDNA clone pcX1H4W2 (26). The eukaryotic insert was purified by digestion with Bam Hl. It was cut with Dde I and end-labelled with gamma ^{32}P-dATP using T4 polynucleotide kinase. It was cut again with Mbo II and fractionated on a 12% acrylamide, 7 M urea sequencing gel (27). A 31 nucleotide fragment of the non-coding strand, corresponding to residues 19 to 50 of the coding region, was isolated.

Typically this probe was hybridized to 4-10 µg RNA in 10 µl 0.4 M NaCl, 10 mM Pipes, pH 6.4, for 4-18h at temperatures varying from 50°C to 75°C. The mix was added to 80 µl of primer extension solution (50 mM Tris pH 8.2, 10 mM DTT, 25 µg/ml Actinomycin D, 6 mM $MgCl_2$, 2 mM ATP, GTP, CTP, TTP) containing 10 units of reverse transcriptase and incubated at 42°C for 1 h. The product was extracted with phenol/chloroform, ethanol precipitated, dissolved in formamide sample buffer, heated at 100°C for 3 minutes and analysed on a 12% acrylamide, 7 M urea sequencing gel (27). The gel was then exposed to X-ray film at -70°C with an intensifying screen.

In some experiments the hybridisation was performed as above, but with the addition of 1% SDS to inhibit degradation by RNase. The mixture was then

ethanol precipitated and redissolved in 10 µl 0.4 M NaCl, 10 mM Pipes pH 6.4 before addition to the primer extension mixture. For sequencing reactions the input RNA concentration was raised to 100 µg and the volumes increased to accommodate this. In the 70°C hybridisation chemical degradation of the RNA was appreciable. To prevent this, hybridisation in 50% formamide, 0.4 M NaCl, 10 mM Pipes pH 6.4 at 45°C was sometimes substituted. The same extension bands were observed as with 70°C hybridization.

Sequencing of Primer Extension Products

The bands from the above gel were located by autoradiography, eluted overnight in 0.5 M NH_4 acetate, 10 mM Mg acetate, 1 mM EDTA, 0.1% SDS, pH 7.5 at 37° and ethanol precipitated. They were sequenced as described by Maxam and Gilbert (27).

S1 Nuclease Analysis of H4 mRNA

The probe was made from an X. borealis cDNA clone, pcXbH4W1 (26). The insert of this clone in pAT 153 contains a single Bam H1 site at the 3' end of its coding strand, the other Bam H1 site having been lost during cloning. The clone was opened with Tth 111I at a unique site 160 residues from the end of the 3' poly(A) tract. It was labelled by addition of an alpha-^{32}P-dGMP residue to the non-coding strand using T4 polymerase and then cut with Bam H1. The product was strand-separated on a sequencing gel (8% acrylamide, 7 M urea) and the single-stranded probe recovered as described above.

The probe was hybridised to 6-12 µg total RNA in 10 µl 0.4 M NaCl, 10 mM Pipes pH 6.4 for 3 h, before addition to 180 µl 0.28 M NaCl, 50 mM Na acetate pH 6.4, 4.5 mM $ZnSO_4$ containing 20 µg/ml sonicated, denatured salmon sperm DNA. The mixture was incubated with 100 units S1 nuclease for 30 minutes at 25°C. The product was extracted with phenol/chloroform, ethanol precipitated and then analysed on an 8% acrylamide sequencing gel as described above.

RESULTS

Rationale and Conditions of the Primer Extension Reaction

A probe which hybridises to an mRNA encoding a highly conserved protein, should permit all varieties of this mRNA to be detected by primer extension. If the primer is labelled at its 5' end and the extension products can be fractionated, they can be sequenced by the procedure of Maxam and Gilbert (27). This approach reveals the range of mRNA molecules actually present in a given RNA preparation, and has been used in exactly this way to study the actin genes expressed at different stages of the life cycle of Dictyostelium (32). It is particularly appropriate to the study of a histone gene like that

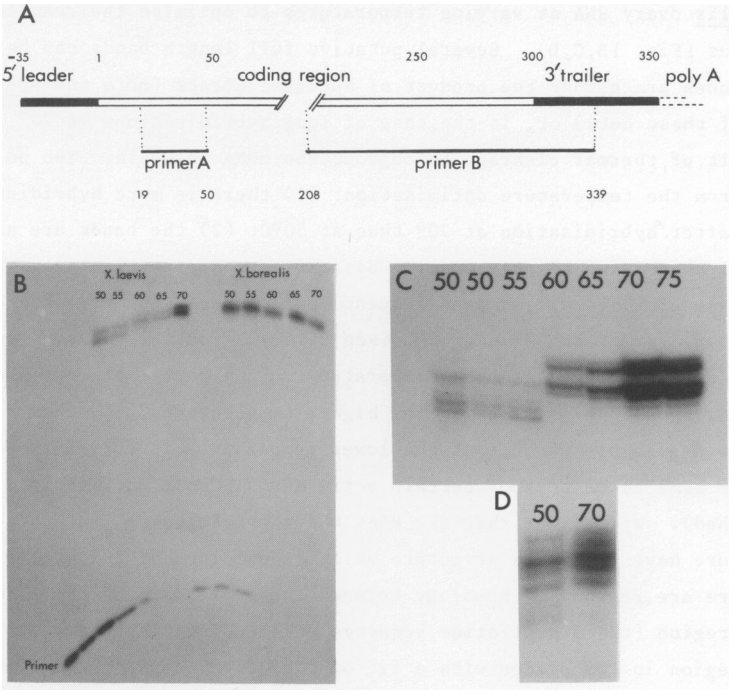


Figure 1. Primer extension analysis of H4 mRNAs. (A) Diagram of an H4 mRNA based on the structure of clone pcXlH4W2. However this clone lacks a 5' leader (26). Primer A was the one used in all studies, except that shown in Figure 10. (B) 5µg of total ovary mRNA was annealed to primer A at the range of temperatures shown, extended with reverse transcriptase and analysed on 12% sequencing-type polyacrylamide gels (27). Electrophoresis was from top to bottom. The temperature of the hybridization is shown above each track. Enlargements of the extension product region of a similar gel is shown in (C) for X. laevis and (D) for X. borealis. In the left hand 50° track of (C) the hybridization mixture was heated at 90° for 3 min before overnight hybridization at 50°.

specifying H4, since the H4 protein molecule shows absolute conservation of amino acid sequence within the phylum Chordata, and there is even efficient cross-hybridisation of nucleic acid probes between different phyla. Although one can never be certain that some unsuspected molecules are indetectable with the H4 primer extension probe, we know that it will detect a number of H4 mRNAs in mouse cells, and thus is likely to detect most, if not all, H4 mRNA sequences in Xenopus. However, our results do identify a set of circumstances inwhich hypothetical mRNAs could be missed (see below).

The primer extension probe (Fig. 1A) was hybridised to Xenopus laevis and

X. borealis ovary RNA at varying temperatures to optimise the reaction conditions (Fig. 1B,C,D). Several putative full length bands can be seen. Shorter ones are either the product of nuclease attack (note the specific nature of these cuts) or, in the case of long hybridisations at 70°, they are the result of thermal cleavage throughout the mRNA molecule. Two points emerge from the temperature optimisation: (1) there is more hybridisation product after hybridisation at 70° than at 50°C; (2) the bands are not identical at the two temperatures. These effects are particularly pronounced in the case of X. laevis, indeed sequencing shows that the two sets of bands are quite different at 50° and 70°C (see below). For this reason most analyses were performed at both temperatures. The fact that more product of some mRNAs should be obtained at the higher temperatures, and that some mRNAs even give negligible product at the lower temperature is curious. We have found the same to be true of certain actin mRNAs (Cross and Woodland, unpublished). We believe that the RNAs which hybridise only at the higher temperature have secondary structure which is unfolded at this temperature; in fact there are regions of homology between the 5' leader and the primer-binding region (the 9 nucleotide sequence CCTTTGCGC can hybridize to the primer region in two places with a fit of 8 of the 9 bases). Similar effects have been previously noted by Casey and Davidson (53), who invoked a smilar interpretation. Unfortunately this observation means that certain H4 mRNAs might not be detected by primer extension; i.e. they need to hybridise at the high temperature, but have diverged from the primer sequence.

H4 mRNAs at Different Stages of Development and in Different Cell Types

Primer extension bands differing in length by only one nucleotide may be resolved by gel electrophoresis and the bands obtained after hybridisation at 50° and 70° are different, particularly in X. laevis. This high degree of discrimination means that electrophoresis of the primer extension products from different RNA preparations should provide a good indication of whether they contain different H4 mRNAs.

Fig. 2 shows the extension products obtained using RNAs obtained from different stages of development. It can be seen that in both X. laevis and X. borealis there is no qualitative change between the oocyte and larval stages, and both are similar to the testis. Fig. 3 shows that the same is true when a number of adult organs are compared. Thus there is no gross change in the kinds of H4 mRNA expressed at different stages of development, or in the differentiated cell types we have examined.

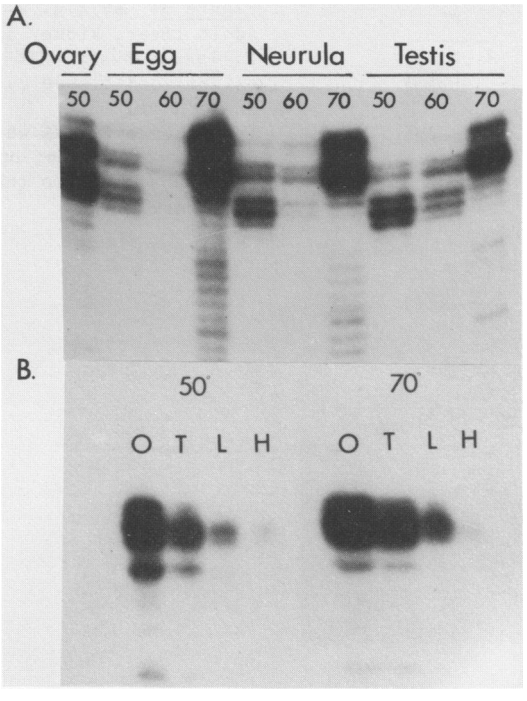


Figure 2. Primer extensions on H4 mRNAs from a variety of RNA preparations. Only the extension product regions of the gel are shown. (A) X. laevis. Total RNA preparations were used and hybridized at the temperatures indicated. (B) X. borealis. O, ovary; T, tadpole; L, lung primary culture; H, heart primary culture.

Sequences of H4 mRNA Leaders in X. borealis Oocytes

In the first instance the H4 mRNAs of one ovary were analysed. Bands of the type shown in Fig. 1D were cut out and sequenced (Fig 4). All but the shortest band at 50° have the same sequence, except of course that they vary slightly in length. The only possible ambiguity was in a residue near the 5' terminus which seemed to be A or G in this animal. The shortest band after 50° hybridisation was too faint for complete sequencing, but it is clear that it is completely unrelated to the other bands in the leader and that it differs at three nucleotide positions in the coding sequence. X. borealis contains a major, conserved family of histone gene quintets and a smaller number of other histone genes (20). It is tempting to suggest that the main group of sequences is derived from the major set of quintets, and the divergent one from a minor set. The coding region of the minor mRNA is

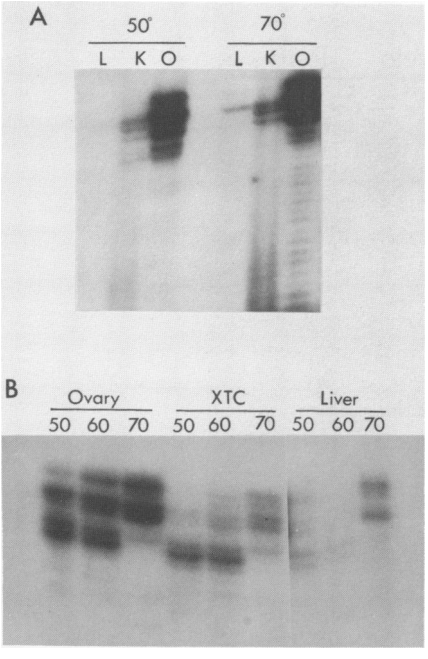
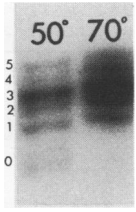


Figure 3. As Fig. 2. (A) X. borealis adult liver, kidney and ovary RNA. (B) X. laevis ovary, XTC-2 cell (an established tissue culture line) and adult liver RNA. In (A) the 50° signal from the liver RNA was too weak to show on the photograph; on the original a pattern similar to that of the liver was seen.

related to some of the 50° hybridisation bands in X. laevis (see below). Presumably it is also diverged in the region of the primer used, since it fails to give products at 70°.

H4 mRNA leaders from the ovaries of two other frogs have also been sequenced. One gave a single base difference from the animal described above (Ov2 in Fig. 5). Quantitative arguments demand that many histone genes are expressed in oogenesis (23). It therefore seems that in the animals studied many of the H4 genes must have identical leader sequences, but that different animals have slightly different sequences in the leaders of these genes.

The fact that the abundant class of H4 mRNAs has 4 different lengths of reverse transcriptase product might mean that there is heterogeneity in the cap sites of their genes. Typically mRNAs produce a double band in primer extension and we know that this is true of particular H4 genes injected into ocoytes (unpublished). Alternatively there might be slightly ragged initiation on a single set of genes. These possibilities can be distinguished by cloning a number of H4 genes from a single animal. We have now done this (Turner and Woodland, unpublished) and find no differences in the cap region of 4 different H4 genes. We therefore favour the view that there is ragged initiation.



NTCACATPuGTCGAGAGCTAGCTTCGAGATTTAAGTC ATG TCT GGA AGA GGC AAG G Band 5
NCNCATPuGTCGAGAGCTAGCTTCGAGATTTAAGTC ATG TCT GGA AGA GGC AAG G Band 4
NNNATPuGTCGAGAGCTAGCTTCGAGATTTAAGTC ATG TCT GGA AGA GGC AAG G Band 3 } (50° and 70°)
NCATPuGTCGAGAGCTAGCTTCGAGATTTAAGTC ATG TCT GGA AGA GGC AAG G Band 2
NTPuGTCGAGAGCTAGCTTCGAGATTTAAGTC ATG TCT GGA AGA GGC AAG G Band 1
.....CGCTCNCTTGCTATC ATG TCT GGA CGC GGC AAA G Band 0 (50°)

Figure 4. Sequences of X. borealis H4 leaders. The bands are identified above. Bands 1-5 gave identical sequences after hybridization at 50° and 70°. Band 0 was seen only at 50°, and could only be sequenced in part. The residues in which it differs from bands 1-5 are underlined.

(A) Major Species

Ov1	NTCACATAGTCGAGAGCTAGCTTCGAGATTTAAGTC ATG TCT GGA AGA GGC AAG G	Type 1
Ov2	NNNNNNTAGTCGAGAGCTAGCTTTGAGATTTAAGTC ATG TCT GGA AGA GGC AAG G	Type 2
Ov3	NNNNNATAGTCGAGAGCTAGCTTCGAGATTTAAGTC ATG TCT GGA AGA GGC AAG G	Type 1
Lung3	NNNNNNNAGTCGAGAGCTAGCTTCGAGATTTAAGTC ATG TCT GGA AGA GGC AAG G	Type 1
Tadpole	NCTGACATAGTCGAGAGCTAGCTTTGAGATTTAAGTC ATG TCT GGA AGA GGC AAG G	Type.2
	met ser gly arg gly lys	

(B) Small Minor Species

Ov1	...CGCTCTGCTTGCTATC ATG TCT GGT CGC GGC AAA G	Type A
Ov2	...CGCTTTAGGTC ATG TCT GGT CGC GGC AAA G	Type B
Ov3	...GCATCGCTTCTGCTTGATATC ATG TCT GGA CGC GGC AAA G	Types A + B
Lung3	...CGCTTCTGCTTGATATC ATG TCT GGA CGC GG	Types A + B
Tadpole	...CGCTTGCTATC ATG TCT GGT CGC GGC AAA G	Type A
	met ser gly arg gly lys	

(C) Large Minor Species

Larva	...CGCTCCTGCTATC ATG TCT GGA CGC GGC AAA G

Figure 5. H4 leader sequences from different individuals of X. borealis. The sequences were from three adult frogs (1-3) and one set of sibling tadpoles. (A) Major species. Ov1 is the longest sequence shown in Fig. 4. In the same way all of the bands were sequenced for the tadpole RNA and since they varied in length, but not sequence, only the longest band is shown. For the other preparations bands 1-5 were pooled; the sequence of the terminal bases of the longer bands could not therefore be determined. The residue different from that in Ov1 is underlined. (B) Partial sequences of band 0. (C) Partial sequence of a long minor band in the tadpole. The residues in which type B differs from type A are underlined.

Table 1
Distribution of H4 leader types in different animals of X. borealis

Animal	Tissue	Major leader type	Minor leader type
Frog 1	Ovary	1	A
Frog 2	Ovary	2	B
Frog 3	Ovary and lung fibroblast	1	A + B
"Frog 4"	Sibling tadpoles	2	A

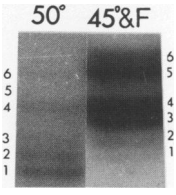
The nomenclature of the leader types is shown in Fig. 5. "Frog 4" was actually a group of sibling tadpoles.

Are the sequences described above restricted to oocytes and early developmental stages? To answer this question the complete set of extension bands (Fig. 2) derived from swimming tadpoles of a single mating were also sequenced. The major family were identical to each other, except in length, and differed by one base from the oocyte sequences shown in Fig. 4. However they are identical to the ovary H4 mRNA of a second animal (Frog. 2, Fig. 5). Presumably the tadpoles were the result of mating two animals with H4 genes like those of frog 2, although we cannot be sure that allelic exclusion does not occur.

To extend these observations to an adult somatic cell type we made a primary culture of adult lung. The RNA of these fibroblast-like cells yielded primer extension bands like those from ovary (Fig. 2). Those expected to contain major H4 leaders were pooled and sequenced. Fig. 5 shows that the sequence conformed to that of the oocytes of frog 1, and it was also the same as the ovary of the frog from which the fibroblasts were derived (frog 3). This was true of both the 50^{o} and 70^{o} products.

Partial sequences of the shortest 50^{o} hybridisation bands are shown in Fig. 5. It is clear that these bands are always different from the major band and that two related, but different sequences are present in different animals. These two sequences are not tissue or stage specific. In Fig. 5 the two kinds of sequence are designated 1 and 2 for the major bands and A and B for the minor. Their distribution is summarised in Table 1. A likely explanation of this distribution is that: (1) Bands of types 1/2 and A/B are the product of two sets of alleles (in the case of A and B there could be a single pair of alleles); (2) these sets are expressed throughout development; (3) the two classes of allele segregate independently.

These results demonstrate that in X. borealis the same H4 genes provide the major H4 mRNAs in oocytes, tadpoles and adult fibroblasts. Of course there is the possibility that different sets of genes are expressed, but that



NNTCACATAGTCGAGAGCTAGCTTCGAGATTTAAGTC ATG TCT GGA AGA GGC AAG G X. borealis Type I

NNNTCTTTCANGGTAATCCTTTGCGANTCCGTGAATC ATG TCT GGA AGA GGC AAG G Band 6
NNNCTCTCTCGATAANNACGCTTAGTGAGTGGAATC ATG TCT GGA AGA GGC AAG G Band 5
NNNTCTCTCGATAGCAACCTTTGCGCGTAGAATC ATG TCT GGA AGA GGC AAG G Band 4
NNNGNTTCGATAGGAACTTTGGGGGTAGAATC ATG TCT GGA AGA GGC AAG G Band 3 } 45° & F
NNNNTNCGATAGGAACCTTTGCGCGTAGAATC ATG TCT GGA AGA GGC AAG G Band 2
NNNTTCGATAAGGCATTAGTGNGTGAAATC ATG TCT GGA AGA GGC AAG G Band 1

.....CTTCGGCCAATTGACCNCAGTC ATG TCT GGA AGA GGC AAG G Band 6
NNNCAGCCCGTTCGTACCGAACTCTACTNGCGTCCGATC ATG TCT GGA AGA GGC AAG G Band 5
NNTGGTTTGTANNGAATCTCTTCANANCTTCNGATC ATG TCT GGA CGC GGC AAA G Band 4
NNNNCCTTTGACGGTCCGGCGCNCCNNNTGGNNC ATG TCC GGA CGC GGC AAA G Band 3 } 50°
NNNTCGTTGTGATCNAAGCACTGACAGGAA ATG TCT GGA CGC GGC AAA G Band 2
NNNTTTGTGATCNAAGCACTCGGNAGGAA ATG TCT GGA CGC GGC AAA G Band 1
met ser gly arg gly lys

Figure 6. H4 leader sequences from the ovary of one X. laevis female. The 70° hybridization in aqueous medium was replaced by hybridization at 45° in 50% formamide. The results of the two methods are indistinguishable, except that there is less thermal degradation at the lower temperature.

these sets have the same sequence. This seems unlikely, and is made more unlikely by the fact that the leader sequences in the ovary and fibroblast of frog 3 are the same, but different from certain other animals. We also cannot exclude the possibility that there are rare, tissue or stage-specific H4 mRNAs, hidden behind the background of ubiquitous abundant mRNAs.

Sequences of H4 Leaders in X. laevis

When the H4 extension products of X. laevis ovary were analysed two facts were immediately apparent; that the different extension bands from one animal had diverse sequences, and that each band was heterogeneous. The latter heterogeneity hindered the complete sequencing of many of the bands. The sequences obtained are shown in Fig. 6. It is clear that the 70° sequences, although heterogeneous, show considerable similarity, and that they are not unlike the major type of X. borealis leader. Part of the heterogeneity must come from the fact that a given mRNA would be expected to give two primer extension bands. This would be a particular problem for the 50° hybridization bands which are even more diverse than the 70° bands. In contrast the coding

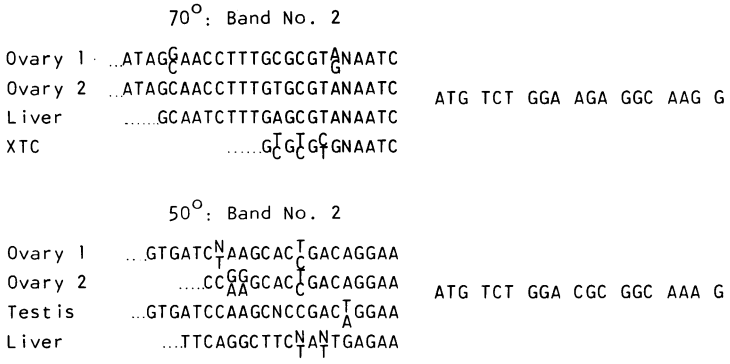


Figure 7. Comparison of the first parts of two H4 primer extension bands (see Fig. 6) from the ovaries of two frogs, and two other adult organs of X. laevis. All RNA was from different animals. XTC is a permanent cell line.

regions showed only two sequences, which are identical to the major and minor sequences of X. borealis. In all RNA samples examined the first arginine and lysine codons conform exactly to the pattern shown in Fig. 6. The other codons are invariant, except that slight heterogeneity of the GGA codon was observed in one band in one set of tadpoles.

When other X. laevis cell types were examined, different leader sequences were found. In the case of the 50° hybridization bands these differences were particularly pronounced. Selected data obtained for several comparable bands are shown in Fig. 7. This shows that similarities in the lengths of leaders can be misleading in terms of sequence. The differences could be characteristic of different organs or of different animals (the RNA preparations were from different animals, and the 70° bands from two females differed, Fig. 6). Since considerable labour would have been involved in sorting this problem out by sequencing, we adopted a short-cut for certain bands.

Two 70° primer extension bands from oocyte RNA were hybridized to a number of other RNA preparations. The product was then digested with S1 nuclease to see if identical, or at least very similar, sequences were present (Fig. 8). In all cases some degree of complete protection was observed, and the relative lack of shorter bands was surprising (it may be partly because the RNA was in considerable excess and the correct hybrid was favoured). The amount of protected band was very small when liver and fibroblast RNA were used. This probably simply reflects their lower histone mRNA content. Thus different animals and tissues contain leaders which are very similar to those

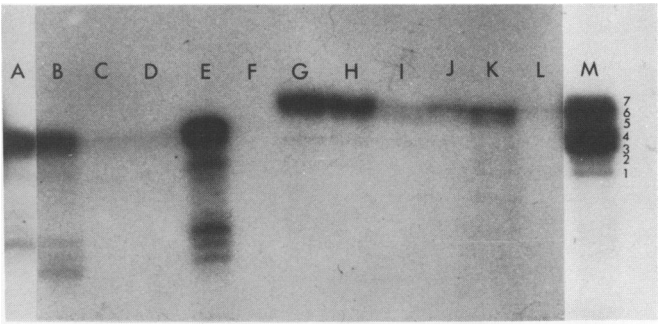


Figure 8. S1 nuclease analysis of H4 primer extension bands. Track M shows the product of primer extension on ovary RNA after hybridization at 70°. Bands 6 and 4 were isolated and hybridized to a variety of RNAs, before being subjected to S1 nuclease digestion. The protected hybrids were run on a standard sequencing gel, together with the original extension product. (A-F) band 4, (G-L) band 6, (M) the original product. (A,G) ovary 1 RNA, (B,H) ovary 2 RNA; these RNAs are those shown in Figs. 6 and 7. (C,I) XTC tissue culture cell RNA. (D,J) adult liver RNA. (E,K) late neurula RNA. (F,L) *E. coli* tRNA control.

in a particular ovary. Apparently the same or related mRNAs are always present, but at different abundances; it is always the most abundant mRNAs that are detected by sequencing.

Thus it seems likely that the same main H4 mRNAs are found at all developmental stages in *X. laevis*, just as in *X. borealis*.

Analysis of the 3' Ends of H4 mRNAs

The results described above have been confined to the 5' leaders of H4 mRNAs and to their adjacent coding regions. Information on the 3' ends was obtained using a probe from pcXbH4W1 (26), an H4 cDNA made from *X. borealis*. This probe contained coding region, the 3' trailer, 14 residues of a poly(A) tail and part of a linker added during cloning. An S1 protection experiment was performed using *X. borealis* ovary, tadpole and tissue culture RNA (Fig. 9). The only difference between the S1 products is that the ovary product has some longer bands than the other samples. This is because the ovary H4 mRNA contains polyadenylated species (33,34), whereas other tissues do not. However the ovary cDNA is protected to the start of the poly(A) tract by all of the RNA preparations. Thus, in *X. borealis* the 3' ends of the main H4 mRNAs are the same, or nearly the same, throughout development. Because the probe does not extend beyond the normal end of an ovarian H4 mRNA this experiment does not exclude the possibility that other tissues contain longer

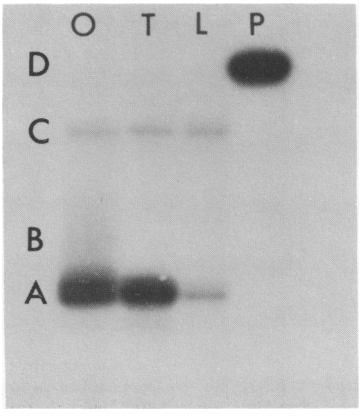


Figure 9. S1 analysis of the 3' ends of X. borealis H4 mRNAs. The 3' portion of the cDNA clone pcXbH4W1 was labelled within the coding region using T4 polynucleotide kinase (see text). The undigested probe was run in track P. The strand-separated fragment was hybridised to ovary (O), tadpole (T), and lung primary culture cell (L) RNAs. The part resistant to S1 nuclease was analysed by gel electrophoresis, as shown. (A) is the fragment protected up to the start of the poly(A) tract; B is the poly(A) tract, present only in ovary RNA; (C) is a band produced by trace contamination with the complementary strand; (D) is the undigested probe.

mRNAs. Experiments using probes from a number of other X. laevis histone genes have shown this not to be the case (Ballantine, unpublished data).

The Central Coding Region of H4 mRNAs

The impression gained from Figs. 5, 6 and 7 is that the leaders of H4 mRNAs are far more variable than the coding regions, even when only the third base positions of the coding region are considered. In X. laevis we have seen only two sequences for the first 19 residues of the coding sequence, and the same holds for X. borealis, except that in the minor gene type there was heterogeneity of the third codon.

We asked if this was a characteristic of other regions of the coding sequence by using primer B, shown in Fig. 1. The product was too large to be resolved into individual bands and was sequenced en bloc. It therefore gave an impression of the consensus coding sequence in the ovary of a given animal of each species, spanning the codons for the 63rd to the 81st of the 102 total amino acids. Fig. 10 shows that a clear consensus emerges for both X. laevis and X. borealis and that these differ in only a few nucleotides, all involving synonomous codon switches. Fig. 10 also compares these sequences with a cDNA clone from ovaries of each of the two species. These show very slight

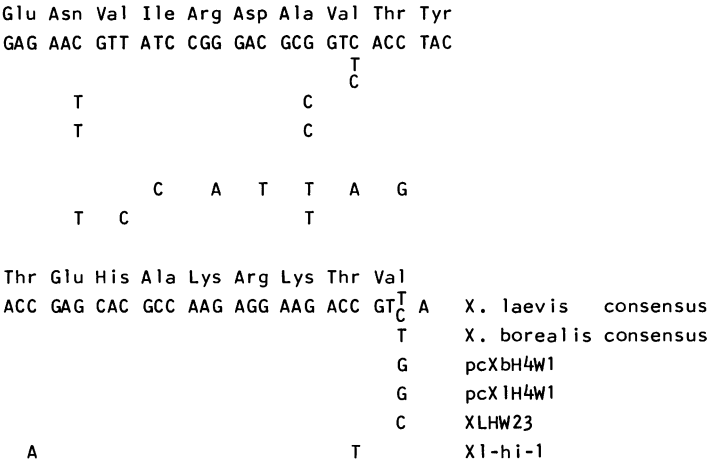


Figure 10. Partial sequences of a number of Xenopus H4 mRNAs, from the 63rd to the 81st codons. The consensuses were obtained by primer extension on total ovary RNA using primer B (Fig. 1). pcXbH4W1 and pCX1H4W1 are two ovary cDNA clones, from X. borealis and X. laevis respectively. XLHW23 is a genomic clone from a rare type of histone gene cluster in X. laevis (35), as also is X1-hi-1 (28).

differences from the appropriate consensuses, one base in the case of X. laevis and one base ambiguity in the case of X. borealis. This is consistent with the view that there is an overall consensus in the products of the many H4 histone genes, but that individual members diverge from this consensus slightly. However the clones and the consensuses were obtained from different animals, and therefore might represent polymorphisms.

Fig. 10 also compares the consensus sequences with two genomic clones which are similar in their 5' coding sequence to 50° hybridization bands, and whose leaders are very different from any mRNA we have analysed by primer extension (Fig. 6). They diverge greatly from the ovary consensus sequences (XLHW23 even has an amino acid change). This is consistent with the view that the 50° sequences represent highly diverged genes which are polymorphic and produce small amounts of product compared with the class of X. laevis genes which hybridise to the 5' primer only at 70°. However a probe from the 5' position of the H4 gene of XLHW23 is completely protected by ovary RNA from a number of frogs, so this type of gene seems to provide small amounts of mRNA in most or all animals (R. W. Old, personal communication).

The Expression of Other Histone Genes

Fig. 11 shows primer extension bands using a probe made from an H3 gene.

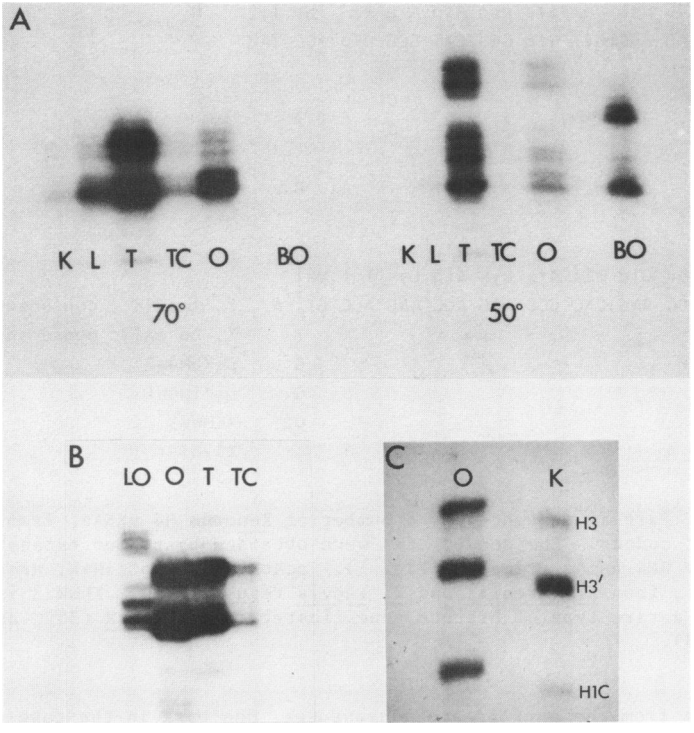


Figure 11. Analysis of H3 and H1 gene expression. All RNA samples were unfractionated. (A) Primer extension analysis using a probe from the H3 gene of XLHW23 (35), a minor X. laevis clone. Hybridizations were conducted at 50° and 70° as indicated. RNA was from X. laevis: K, adult kidney; L, adult liver; T, adult testis; TC, XTC-2 tissue culture cells; O, ovary. BO, X. borealis ovary RNA. (B) As in A, but 50° hybridisation and RNA from X. borealis: O, ovary; T, swimming tadpoles' TC, primary cultured lung cells; L,O was X. laevis ovary. (C) S1 nuclease of H3 and H1C genes. The probes were end-labelled fragments from XLHW8, a X. laevis histone gene quintet (35). Protection of the entire front portion of the H1 and H3 genes gives the bands marked. H3' is protection to the initiator ATG. The identity of these bands was established by careful comparison with molecular weight markers and from knowledge of the sequence (R. W. Old and personal communication). O, total X. laevis ovary RNA. K, total adult X. laevis kidney RNA.

The probe is derived from the genomic clone XLHW23, a minor histone gene cluster of X. laevis (35). As with H4, more product is observed at 70°. The products from ovary, testis, liver, kidney and a cultured cell line are ssentially identical. For some of the samples the signals at 50° are too weak to show on the figure but careful examination of the autoradiographs shows that the faint bands are identical to those in the other samples.

Fig. 11C also shows an S1 protection experiment using a probe, which is

from an X. laevis genomic quintet (XLHW8) containing an H1C gene (35). It detects H1C and H3 mRNAs. H1 is the most variable of the histone classes. Complete protection of the probes is seen with both ovary and adult kidney total RNA.

Thus preliminary experiments on histone genes other than H4 suggest that the major histone mRNAs encoding all histone classes do not change during development.

DISCUSSION

Expression of H4 Genes in Development

The data presented here show that the main H4 mRNAs present throughout the development of X. borealis are products of a single set of genes. In early amphibian development histone synthesis is temporally and quantitatively uncoupled from DNA synthesis (Woodland, 1980), but the same genes and gene products are used in this pattern of regulation as are involved in the typical tight coupling to DNA synthesis typical of later development. In the latter case the coupling of histone synthesis to DNA synthesis is precise and involves several distinct processes: (1) Histone genes are transcribed only in late G1 and S. This has been most clearly demonstrated in yeast (36), but it seems largely to be true of vertebrate cells (37-40). Inhibition of DNA synthesis lowers the rate of transcription, though the extent may depend on the cell type and treatment (38,41). (2) Most histone mRNAs are translated only during the S-phase. Inhibitors which block DNA synthesis cause immediate cessation of histone synthesis (e.g. 1,38-46,50; however, in mammalian cells certain minor histone mRNAs are translated throughout the cell cycle, and when DNA synthesis is blocked (40,48). It is not known when these mRNAs are transcribed). (3) Most histone mRNA is degraded as soon as DNA synthesis stops, either naturally or through artificial inhibition (e.g. 1, 38, 40, 41, 49).

Although none of the selected studies quoted are of Xenopus there is no reason to believe that a process common to yeast and man is any different in amphibians. We do in fact know that inhibitors of DNA synthesis cause a great reduction of histone mRNA concentration in cultured Xenopus cells, exactly as expected (unpublished data).

Thus the reason that in oogenesis and early development H4 histone synthesis does not conform to the regulatory phenomena listed above is not that the genes transcribed are different. Nor are the transcripts from these genes different. Instead the reason that histone genes and their products show an unusual relation to the cell cycle in early amphibian development must

be that other components in their environment behave differently in these cells. For example oocytes accumulate considerable stores of histone mRNA even though they make no DNA. It is possible that the same factors that stimulate histone gene transcription in the S-phase of typical somatic cell, are present in non-S-phase oocytes. Thus the condition that brings about histone mRNA translation and stabilisation during the S-phase of adult cells would exist continuously in early embryonic cells. Alternatively histone gene transcription and translation might be regulated by a different complement of regulatory molecules in embryonic and adult cells.

The H4 mRNAs reported here differ very greatly in their leader sequences, particularly within the X. laevis species. The question arises as to whether thee leaders have a functional significance. An obvious possibility is that the mRNAs have different stability. We have treated X. laevis cells with hydroxyurea, which causes a large proportion of the H4 mRNA to be destroyed. However the stability of the mRNAs with different leaders is indistinguishable (Ballantine and Woodland, unpublished).

Evolution of Histone Genes

The histone genes of X. borealis are mainly present in a reiterated class of homogeneous quintets, whereas those of X. laevis are much more variable in organisation (20,21,24,28,35,51,52). Our analysis of the expressed H4 mRNAs indicates that this difference is consistent even at the level of transcribed sequences. There is a simple major X. borealis H4 sequence, and at least one minor class. Although there are slight differences between animals there is very little heterogeneity apparent within an animal, both at the 5' and the 3'end. In X. laevis, although there is a major class of H4 genes expressed, these are much more heterogeneous than those of X. borealis. They may derive from the cluster of 4 core histone genes identified in one X. laevis individual by Van Dongen et al. (21), though most animals contain several abundant types of histone cluster (24,35). There are also a series of fairly abundant H4 mRNAs that are quite different from the major type and from each other. In the first part of the coding region they are identical to the rare H4 mRNAs of X. borealis, just as in this region the major H4 mRNAs of the two species are identical. This suggests that the genes encoding these two H4 mRNA classes have a common evolutionary origin sufficiently long enough ago for the leaders to have become quite different.

X. borealis employs a smaller range of H4 sequences than X. laevis. This implies that the wider range of major and minor sequences found in X. laevis has no great fundamental significance (the two species are sufficiently

similar to form viable hybrids).

In the X. borealis inidividuals examined so far (3 females and the mixed progeny of one mating) each animal contained detectable amounts of only one of two types of H4 mRNA leader, differing by only one nucleotide. This is very surprising, since we expect the mRNAs responsible to be derived from many genes in each individual at least in the ovary (23). Further study is necessary to establish the basis of these observations.

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