
Histone gene number in relation to C-value in amphibians

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ABSTRACT

We have compared the number of copies of sequences complementary to a cloned *Xenopus* histone H4 coding sequence in the genomes of *Xenopus*, *Triturus* and *Ambystoma*, amphibian species with widely different C-values (3, 23 and 38pg DNA/haploid genome respectively). Quantitative autoradiography indicates that H4 sequences constitute a greater proportion of the genome the larger that genome is. Measurement of the absolute copy-number by reassociation kinetic analysis indicated 47 ± 10 , 636 ± 21 and 2685 ± 349 copies per haploid genome each in *Xenopus*, *Triturus* and *Ambystoma* respectively. Whilst this confirms a trend of increasing copy-number with increasing C-value, the two are not directly proportional and some other factors must contribute to determining the number of copies of these genes.

INTRODUCTION

The histone genes are a family of moderately-repetitive DNA sequences which are ubiquitous in eukaryotes. The products of these genes have a uniquely intimate association with the genomic DNA, being responsible for its organization into nucleosomes and, with other factors, into the higher order chromatin structures (1).

The histone genes of the anuran amphibian *Xenopus laevis* are under intensive investigation by a number of groups. It has been estimated that there are some 20-50 copies of the gene for each of the core histones in this species (2,3). It can be argued that this number is around the minimum necessary to allow the accumulation of sufficient histone messenger-RNA and stored histone proteins during oogenesis to package the large amounts of DNA which are rapidly synthesised during early embryonic cleavage (4).

Xenopus has a relatively modest genome for an amphibian, with a C-value (haploid DNA content) of about 3pg (5). Urodele amphibians such as the newt *Triturus cristatus carnifex* and the axolotl *Ambystoma mexicanum* have very much higher C-values, of 23pg (6) and 38pg (7)

respectively. As the details of embryonic cleavage in these organisms do not appear to be very different from Xenopus it may be assumed that they need more stored histone and histone mRNA in their eggs to package the larger amounts of DNA/cell. Since these organisms, like Xenopus, have an annual breeding cycle, we suspected that, in order to produce enough histone message during oogenesis, the repetition of the histone genes might increase in proportion to the genome size.

The histone gene coding-sequences are unusually highly conserved (8) and so we have used a cloned Xenopus H4 coding sequence to investigate the relationship between C-value and copy-number of histone genes in the three species mentioned.

MATERIALS AND METHODS

Materials. Adult Xenopus laevis laevis, Triturus cristatus cristatus and Ambystoma mexicanum were obtained from Xenopus Ltd. and Gerard and Haig Ltd.. High molecular weight DNA was prepared from blood cells of individual animals by phenol:chloroform and methoxethanol extraction and RNase treatment (9).

RNA was prepared from ovaries by phenol:chloroform extraction (10) and the polyadenylated fraction isolated by oligodT-cellulose chromatography (11).

Restriction endonucleases were obtained from Boehringer-Mannheim or Bethesda Research Labs. and used in the conditions specified by the suppliers; DNA polymerase I was from Boehringer-Mannheim; S₁ nuclease was from Sigma; α -³²P-dCTP (>2000Ci/mMol) was from the Radiochemical Centre (Amersham) and nitrocellulose filters were Millipore HAWP.

Cloned Xenopus H4 coding sequence. The plasmid pcX1H4W1 was isolated from a library of Xenopus laevis ovary cDNA inserted into the BamHI site of pAT153 with BamHI linkers and propagated in E. coli HB101. The 382bp insert has been sequenced and contains an almost complete copy of an H4 coding sequence (Fig. 1). Details of the preparation and characterization of this plasmid will be presented elsewhere (P. C. Turner and H. R. Woodland, in preparation).

Supercoiled plasmid DNA was prepared by the method of Colman et al. (12). The insert was isolated from low melting-point agarose gels (13) following BamHI digestion of pcX1H4W1.

Plasmid DNA was labelled with ³²P-dC by nick-translation (14) to specific activities of 10⁶-10⁷ cpm/ μ g.

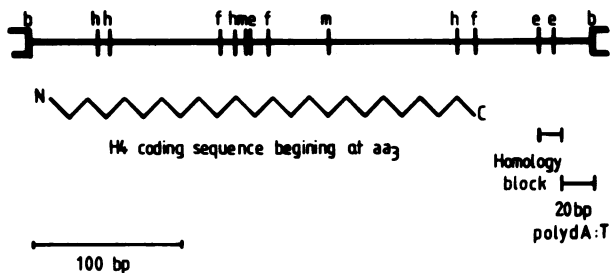


Figure 1. Characteristics of the *Xenopus* ovary cDNA insert in plasmid pcXlH4W1. Restriction sites shown are BamHI (b), HhaI (h), HinfI (f), MspI (m) and HaeIII (e).

Blot hybridization. DNA was transferred from agarose gels to nitrocellulose as described by Southern (15). Filters were baked at 80°C for 2h and prehybridized in Denhardt's solution (3xSSC, 0.2% each bovine serum albumin, Ficoll and polyvinylpyrrolidone) containing 100 µg/ml sonicated, denatured calf thymus DNA at 65°C for 4h. Under these conditions, calf thymus DNA efficiently reduced background binding of the probe without apparently reducing the signal from hybrids. Hybridization with heat-denatured ³²P-labelled pcXlH4W1 was carried out in Denhardt's solution containing 0.1% SDS at 65°C for 18-24h. Filters were washed in 3xSSC at 65°C and exposed to Fuji RX film at -20°C using Ilford FF intensifying screens.

Polyadenylated RNA (10 µg/filter) was dot-blotted to nitrocellulose as described by Thomas (16). Filters were prehybridized overnight at 37°C in 50% deionized formamide, 5xSSC, 50mM phosphate buffer, pH 6.5, 0.2% each BSA, Ficoll and PVP, 100 µg/ml sonicated, denatured calf thymus DNA. Hybridizations were carried out in a similar solution with ³²P-labelled pcXlH4W1 replacing calf thymus DNA at 37°C for 24h. Filters were washed extensively in 0.1xSSC, 0.1% SDS at room temperature before the thermal elution in 0.1xSSC of the probe was monitored by Cerenkov counting.

Reassociation kinetics. The reassociation of identical amounts of heat denatured ³²P-labelled H4 insert from pcXlH4W1 was monitored at 65°C in 0.5M NaCl, 10mM Tris, 1mM EDTA, pH 7.4 in the presence of measured amounts of sonicated, heat-denatured pcXlH4W1 or genomic DNA. Samples were rapidly quenched in a solution to give a final concentration of 0.5mM ZnCl₂, 230mM NaCl, 30mM NaAc, pH 4.4 and 200 µg total DNA

(sonicated calf thymus DNA used as carrier) and digested with 100 units S_1 nuclease at 3°C for 1h. 10 μg BSA was added and the mixture made 0.5N PCA and left to precipitate at 0°C for 15 min. A sample of the supernatant was taken to determine PCA-soluble radioactivity, the pellet washed twice in 0.5N PCA and solubilized in 0.3N NaOH for determination of PCA-precipitable radioactivity by Cerenkov counting.

RESULTS

Sequence homology

As we were using a heterologous probe in the cases of Triturus and Ambystoma we considered it necessary to obtain some estimate of the extent of divergence between their H4 coding sequences and the cloned Xenopus H4 probe. We have examined the melting profiles of hybrids formed between ^{32}P -labelled pcX1H4W1 DNA and ovary polyadenylated RNA immobilized on nitrocellulose filters (Fig. 2). We used ovary polyadenylated RNA because it is a relatively rich source of histone message (17).

The $6-13^\circ\text{C}$ reduction in T_m between hybrids with homologous and heterologous RNA indicated that there is probably no more than 15%

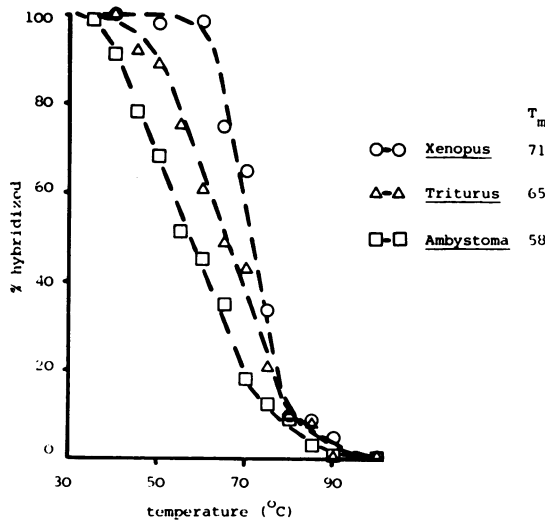


Figure 2. Thermal elution in 0.1 x SSC of ^{32}P -labelled pcX1H4W1 hybridized to ovary polyadenylated RNA immobilized on nitrocellulose filters.

sequence divergence between the cloned Xenopus H4 coding sequence and the H4 coding sequences expressed in the ovaries of Triturus and Ambystoma. All of these sequences should therefore, have formed stable hybrids at the criteria of reassociation which we used.

Relative copy-number of H4 genes by quantitative autoradiography

The use of quantitative autoradiography is becoming more widespread for determining the genomic copy-number of specific DNA sequences, including its use to measure histone gene reiteration in the American newt Notophthalmus viridescens (18). In our case, we were unable to generate restriction fragments containing all of the H4 coding sequences of the same size from each of the three species and the plasmid, so we estimated the relative abundance of the H4 sequence/unit weight of sheared genomic DNA from the three species.

Genomic DNA was sheared to a modal size of 2.3-2.5Kbp and a range of amounts (1-20 µg) run on a 1% agarose gel. Sonication is not a very reproducible procedure so the relative amounts of DNA/track within a fixed size range (1.2-5.2Kbp) was checked from the intensity of ethidium bromide staining. The gel was 'Southern transferred' and hybridized to nick-translated pcX1H4W1. The relative amount of probe hybridized to DNA within the fixed size range was determined by scanning the autoradiograph with a Joyce-loebl microdensitometer.

There was a linear relationship between DNA amount and autoradiographic density for the five amounts of Xenopus DNA loaded. This was used as a standard against which the three tracks each of Triturus and Ambystoma DNA were measured (Fig. 3).

This gave us an estimate of the amount of Xenopus DNA which produced the same intensity of labelling as a known amount of Triturus or Ambystoma DNA. From this we calculated the relative number of copies complementary to the cloned Xenopus H4 sequence per unit weight of DNA. Allowing for the different sizes of the genomes we estimated the increase in the number of copies per haploid genome in Triturus and Ambystoma (Table 1).

Allowing for the potential sources of error in this method, we are confident that these results indicate that the histone sequences are significantly over-represented in the Triturus and Ambystoma genomes, even above an increase in proportion to the C-value.

Copy-number of H4 genes from DNA reassociation kinetics

In order to estimate the absolute copy-numbers of H4 sequences in

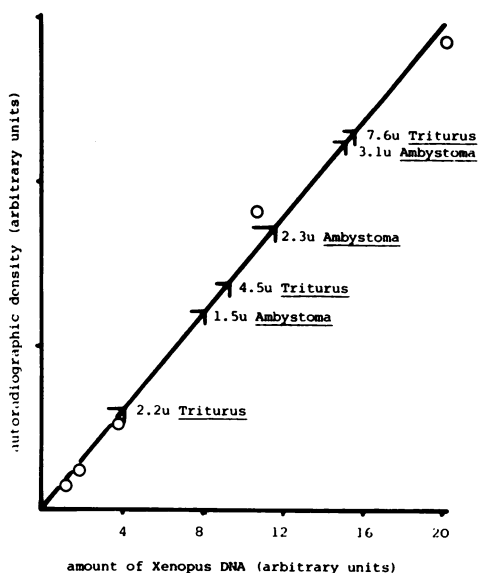


Figure 3. Calibration curve from quantitative autoradiography of pcX1H4W1 hybridized to sheared genomic DNA 'Southern transferred' from a 1% agarose gel.

the genomes of these three species, we measured the rates of re-association of tracer ^{32}P -labelled cloned H4 coding sequence in the presence of precisely measured amounts of pcX1H4W1 or genomic DNA. Representative reassociation curves are presented in Figure 4. The data were fitted to the equation $C/C_0 = (1 + kCot)^{-.44}$ (19) and from this the times to half reassociation ($t_{1/2}$) were calculated. The reciprocal of $t_{1/2}$ was linearly related to the amount of additional H4 derived from pcX1H4W1 in the reaction (Fig. 5). This was used as the standard curve

Table 1. Relative abundance of H4 coding sequences in the genomes of Xenopus, Triturus and Ambystoma determined by quantitative autoradiography.

	<u>Xenopus</u>	<u>Triturus</u>	<u>Ambystoma</u>
C-value (pg)	3	23	38
Relative C-value	1	7.7	12.7
Relative copy-number/unit DNA	1	1.96 \pm .15	4.71 \pm .36
Relative copy-number/genome	1	15.0	59.7

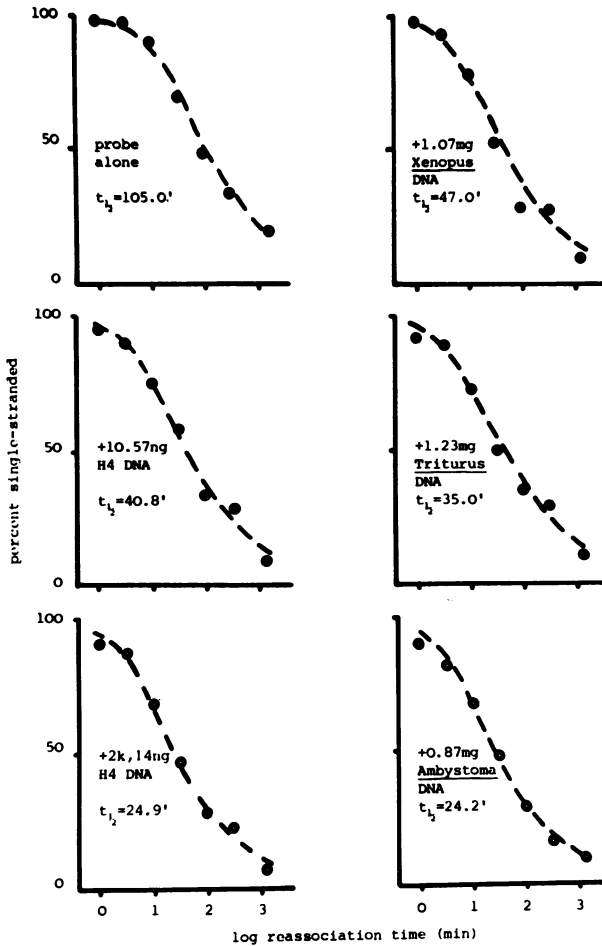


Figure 4. Reassociation curves of 32 P-labelled pcXCH4W1 insert DNA in the presence of various amounts of pcXCH4W1 (containing known amounts of H4 DNA) and genomic DNAs.

from which the amount of H4 coding sequence in the known amounts of genomic DNA was read, and hence the copy-number calculated. The figures were corrected for the fact that the polyA and Bam HI-linker regions in the insert are not likely to be present in genomic DNA, but not for the 3'-non-coding region. Blocks of homologous sequences downstream from the 3'-end are a feature of histone genes (20), but we do not know the full extent of such homology in this case. It is possible, therefore, that

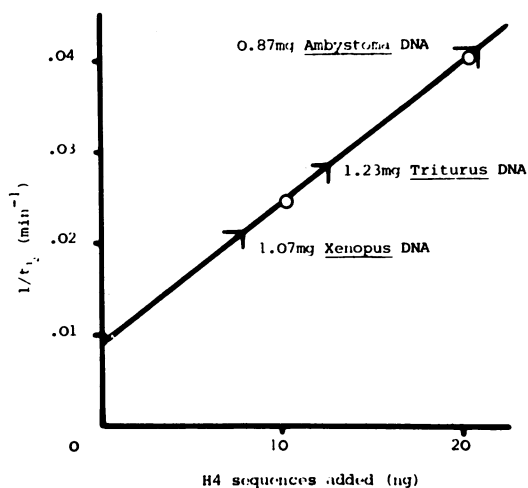


Figure 5. Calibration curve for determination of H4 gene copy-number from reassociation kinetics.

we underestimated the copy-number, particularly for Triturus and Ambystoma, by anything up to 15%.

The results (Table 2) confirmed the conclusion from quantitative autoradiography that the histone genes are over-represented in Ambystoma and Triturus even above the increase in C-value.

DISCUSSION

The remarkable conservation of primary structure of the histones is reflected in the limited divergence of histone gene coding sequences and suggests that these genes must be subject to very strong selective pressure. Because of the primary role of histones in the packaging of DNA into chromatin, we asked ourselves whether the number of copies of these genes is related to the size of the genome and probably also

Table 2. H4 gene copy-numbers from reassociation kinetics. Numbers are the means \pm accumulated S.E. of two experiments.

<u>Xenopus</u>	<u>Triturus</u>	<u>Ambystoma</u>
47 \pm 10	636 \pm 21	2685 \pm 349

subject to strong selective pressure.

The American newt, Notophthalmus, has some 15 times more DNA per cell than Xenopus and has some 12-16 times as many copies of the histone genes (18), supporting the idea that, amongst amphibians, the reiteration frequency of the histone genes is proportional to the C-value. We find, however, that Triturus, with a C-value 7-8 times that of Xenopus, has nearly 14 times as many copies of the H4 coding sequence, and Ambystoma, with a C-value nearly 13 times that of Xenopus, has almost 60 times as many copies. It can be seen, therefore, that the number of copies of histone genes per genome tends to increase with increasing C-value, but not proportionally and some other factors must contribute to determining the number of copies of these genes.

It is likely that the number of copies of these genes would be determined by the amount of histone message required at the time of maximum demand. In most organisms this is expected to occur during the rapid cell proliferation in early embryogenesis. It is clear, however, that a number of different strategies can be adopted to meet this demand.

It has been suggested that the very high reiteration frequency of histone genes in sea urchins is to satisfy this requirement for histones during cleavage (21). Mammals and birds have a much lower copy-number of histone genes (2, 22-24), but their development proceeds much more slowly, without the phase of rapid nuclear division which is characteristic of most groups of animals. Amphibians overcome the problem by the accumulation of stored histone and histone mRNA in the egg (4). Even in Xenopus, however, sufficient message can only be accumulated if several sets of histone genes are transcribed simultaneously throughout oogenesis (3). The high reiteration frequency of histone genes in Ambystoma and Triturus more than compensates for the large sizes of their genomes and suggests that this simple model of what determines histone gene number is not adequate. One major problem, to which there is no current solution, is that we do not know how many of these copies are truly functional genes, nor what proportion of them are active during oogenesis.

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