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Isolation of a genomal clone containing chicken histone genes

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ABSTRACT

We have used enriched chicken histone cDNA to select genomal clones from a chicken library. Because the cDNA probe also contained other sequences, a further screening of positive plaques with negative probes eliminated most non-histone gene clones. One 'positively-selected' genomal clone, λCH-O1, hybridised with cloned sea-urchin histone genes and also detected histone genes in EcoRI-digested genomal sea-urchin DNA. Limited DNA sequencing of HaeIII fragments identified two sequences within the coding region of chicken histone H2A. A third fragment predicted an amino acid sequence with strong homology to an H1 histone sequence.

INTRODUCTION

The histone genes represent an excellent system for investigating structure-function relationships within a family of related proteins.

The organisation of histone genes in sea-urchins (1-4) and *Drosophila* (5) has been described. In lacking intervening sequences (6) the histone genes differ from other well-characterised eukaryotic genes. Histone gene transcripts also appear to be atypical. The mRNAs have a short half-life (7) and are not generally polyadenylated (8). The clustering of these genes also makes polycistronic transcription theoretically possible.

Because histones play a unique role in packaging eukaryotic chromosomes, they are ubiquitous in higher organisms. Amino acid sequences of the 'core' histone proteins have been highly conserved during evolution and any mutation in their genes would be expected to be lethal. However, variants do appear during development and differentiation (9). Little is known of the function of these variants or the events which lead to their

expression.

We have chosen to study the chicken histone genes for two reasons. First, the structure of histone genes in higher eukaryotes is not known. Although a repeating unit containing one each of the five histone genes is present in both sea-urchins and *Drosophila*, the internal ordering and polarity of the genes is different in these two organisms (5). Previous evidence (10) suggests that chicken histone genes are also clustered.

Secondly, red blood cells of birds express a special histone, H5. It has been suggested that histones H5 and H1 arose from a common ancestral gene (11), so it is of immediate interest to examine the relationship between the normal complement of chicken histone genes and H5 genes.

We report here the identification of a clone, selected from a chicken genomal library, which contains genes coding for chicken histones.

MATERIALS AND METHODS

Isolation of RNAs

Histone mRNA was prepared from 5-day-old chick embryos (10) and globin mRNA from chicken reticulocytes (12) as previously described. rRNAs (28S, 18S, 5.8S, 5S) and tRNAs (4S) from chick scale tissue were kindly donated by S. Wilton. Synthesis of cDNA from these RNAs was by random priming (13).

Transfer of DNA to Nitrocellulose and Hybridisation Protocol

DNA was transferred from agarose gels to nitrocellulose filters by the technique of Southern (14) as modified by Wahl, Stern and Stark to facilitate transfer of large fragments (15). Hybridisation of ^{32}P-labelled probes to filter-bound DNA was as described by Wahl *et al.* (15).

For screening recombinant plaques (16) the following modifications to the hybridisation procedure were made. Filters were pre-treated with a mixture containing Denhardt's solution (ref. 17; 10 x Denhardt's, 2 x SSC, 0.1% sodium dodecylsulphate) at 65°C for 4 hours and pre-hybridised for 1 hour at 42°C in 50% formamide, 5 x SSC, 1 x Denhardt's and 200 µg/ml denatured, sonicated *E. coli* DNA. The hybridisation mix contained the same ingredients and in addition, 10% Dextran sulphate (Pharmacia,

500) and 1-4 x 10^6 d.p.m. of ^{32}P-probe. After hybridisation (16 hours, 42°C), filters were rinsed in 2 x SSC, 10 x Denhardt's, 0.1% sodium dodecylsulphate at room temperature then washed with several changes of the same buffer at 65°C.

Autoradiograms, set up at -80°C using Ilford Fast Tungstate intensifying screens, were developed after 1-4 days exposure.

Experiments Involving Recombinant DNA Techniques

All experiments involving the screening and amplification of recombinants containing chicken DNA in λ Charon 4A 'phage (18) were carried out in a certified C_3 safety laboratory in Adelaide as recommended by ASCORD (Australian Academy of Science Committee on Recombinant DNA).

Chicken Genomal Library

The chicken library was a generous gift of Dr. J. Dodgson. It was constructed by J. Dodgson, D. Engel and R. Axel (California Institute of Technology) from a partial HaeIII and AluI digest of chicken DNA, subsequently ligated to EcoRI "linkers" and inserted into a λ Charon 4A vector as described by Maniatis *et al.* (19).

Growth of Recombinants

Recombinant 'phage were adsorbed to *E. coli* K-12 (strain LE 392, originally from L. Enquist, provided by D. Kemp) mixed with plating agar (1% Bacto-tryptone, 0.5% NaCl, 0.7% agar) at 45°C and poured on to LG plates (1% Bacto-tryptone, 1% NaCl, 0.5% yeast extract, 0.1% glucose, 1.5% agar, adjusted to pH 7). Subsequently 'phage were diluted in 'phage storage buffer, PSB (10 mM Tris-Cl, pH 7.5, 0.1 M NaCl, 10 mM $MgCl_2$, 0.05% gelatin).

For large scale growth, plate stocks of 'phage were added to liquid cultures (37°C) of LE 392 at $O.D._{600}^{1\ cm}$ of 1.0 using an m.o.i. of 1. After complete lysis (usually about 2 hours), cultures were saturated with chloroform (15 min) and bacterial debris removed by centrifugation (10,000 x g, 15 min). The clarified lysates were adjusted to 0.3 M NaCl and 10% polyethylene glycol (Carbowax 6000, Union Carbide); 'phage were allowed to flocculate (16 hours, 4°C) and were collected by centrifugation (10,000 x g, 10 min). Pellets were resuspended in PSB (5 ml per 500 ml culture) and treated with RNA'ase A and DNA'ase I (100 μg/ml and 20 μg/ml, respectively) at 0°C for 4 hours.

Purified 'phage were isolated after two cycles of centrifugation (80,000 x g, 4°C) in 43% (w/w) CsCl. Purified DNA was isolated from 'phage after Proteinase K digestion and phenol extraction.

DNA Sequencing

All procedures were carried out as described by Maxam and Gilbert (20). DNA fragments, de-phosphorylated with bacterial alkaline phosphatase (Worthington), were labelled at 5' termini with γ-^{32}P-labelled ATP (1,500 Ci/mmole) using polynucleotide kinase (Boehringer). Fragments were separated on 6% polyacrylamide gels, detected by autoradiography and DNA electro-eluted. Denatured DNA fragments were electrophoresed on 6% polyacrylamide gels under conditions which optimise single-strand resolution (20). Clearly resolved bands were electro-eluted, subjected to the appropriate chemical degradation reactions required for DNA sequencing, and analysed on 10% or 20% polyacrylamide gels (20).

RESULTS AND DISCUSSION

We have previously isolated mRNA from 5-day-old chick embryos highly enriched for histone mRNA sequences (10). Here we use cDNA from these preparations to detect histone genomal clones (on filters) by the Benton and Davis plaque-screening procedure (16).

It has been noted that histone genes could be identified by inter-species cross-hybridisation (2). For example, those of *Drosophila* were detected in a population of recombinant plasmids by their ability to hybridise with sea-urchin histone mRNA (5). It might have been feasible, therefore, to screen filters of chicken recombinants with cloned sea-urchin histone genes as probe. However, in practice others have found that sea-urchin histone genomal clones detect sequences other than histone coding regions (K. Murray, K. Gross; personal communications). Consequently, we chose to use the homologous chicken cDNA as probe and to employ a sea-urchin histone gene clone, "λ clone 55" (10), for subsequent verification.

A total of 10^5 plaques from a chicken genomal library were screened (16) in duplicate with histone cDNA. Positives appearing in duplicate were re-plated for single plaques.

Filters from these were screened with a "combined" cDNA probe made from RNAs considered likely to contaminate histone mRNA preparations. These were rRNA (28S, 18S, 5.8S, 5S), globin mRNA and 4S RNA (Materials and Methods). This "negative" screening eliminated most of the non-histone gene recombinants selected initially. Those filters which were "silent" with the negative probe were re-screened with histone cDNA to verify the original positive and allow selection of the appropriate single plaque. Of four recombinants selected, one which gave the strongest positive hybridisation signal was re-screened and characterised

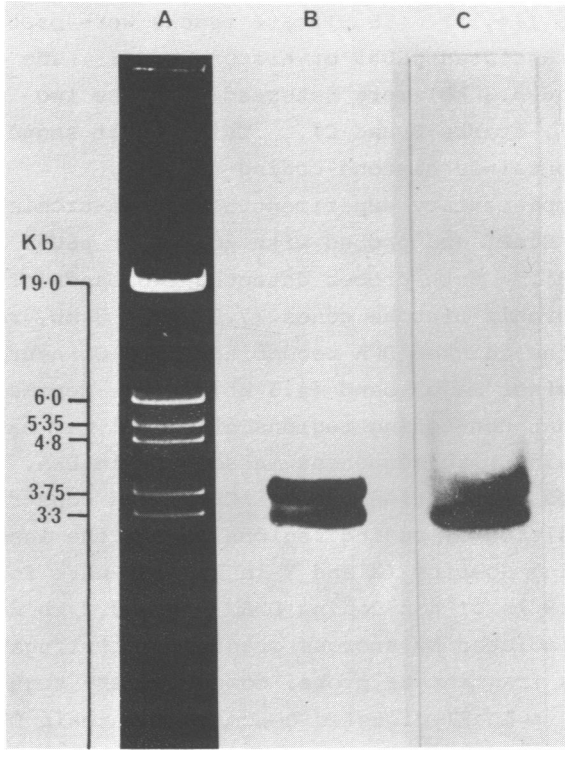

Figure 1. Detection of histone coding sequences in λCH-01. HindIII/EcoRI digests of λCH-01 DNA were electrophoresed on 1% agarose gels detected with ethidium bromide and transferred to nitrocellulose filters (14,15). The sizes of bands were calculated from the migration of markers run in the same gel. Track A, DNA bands detected with ethidium bromide (small fragments have run off the bottom of the gel - see Fig. 3). Track B, autoradiogram of filter hybridised with pSU55. Track C, autoradiogram of filter hybridised with histone cDNA.

further. We have since designated this clone λCH-O1.

Cross-reaction of λCH-O1 Insert with Sea-urchin Histone Genes

We have previously used "λ clone 55", which contains the entire sea-urchin (*Echinus esculentus*) histone gene repeat to characterise chicken histone mRNA (10). For the purposes of this study, we have re-cloned the sea-urchin gene insert into the EcoRI site of the plasmid pBR325 to form the recombinant pSU55. This avoids vector cross-reaction during screening procedures.

DNA isolated from λCH-O1, double-digested with EcoRI and HindIII was electrophoresed on an agarose gel and transferred to nitrocellulose (14,15). Duplicate tracks were probed with either nick-translated pSU55 or histone cDNA. The same fragments (3.75 and 3.3 kb) were detected with the two ^{32}P-labelled probes (Fig. 1, tracks B and C). This result suggested that these bands contained histone-coding regions.

In a complementary experiment total sea-urchin DNA was digested with EcoRI and probed with λCH-O1 or pSU55 (Fig. 2, tracks A and B). Both probes detected two bands of the expected size for sea-urchin histone genes (7.0 and 6.3 kb, respectively). In addition, the chicken DNA recombinant λCH-O1 hybridised with at least one other major band (4.5 kb) in the genomal DNA. It is possible that non-coding regions of λCH-O1 may have been cross-hybridising with sequences in sea-urchin DNA.

A limited restriction map of λCH-O1 was constructed to define the position of coding regions within the insert (Fig. 3). Two major coding domains (X and Y in Fig. 3) were found separated by at least 4.9 kb of non-coding DNA. The 3.3 kb EcoRI fragment (Y) was isolated by sucrose gradient centrifugation. Utilising this fragment as probe, complementary sequences were again located in EcoRI digested genomal sea-urchin DNA (Fig. 2C). This probe detected only two major bands which were identical to those detected by pSU55.

DNA Sequence Data

Although our hybridisation data strongly suggested that λCH-O1 contained histone gene sequences, we sought absolute confirmation of this by limited DNA sequencing to detect coding regions. Fig. 4 shows a HaeIII digests of fragment Y which

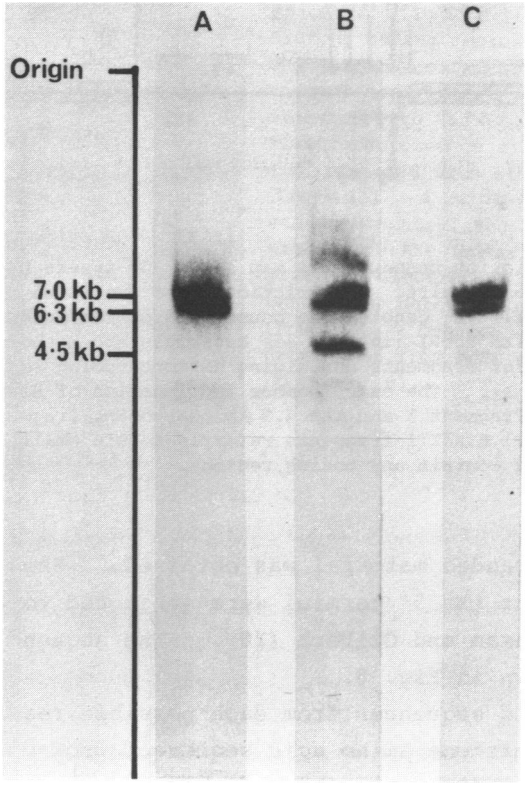

Figure 2. Detection of sequences in genomal sea-urchin DNA with three related probes. Sea-urchin (*Echinus esculentus*) DNA was digested to completion with EcoRI, electrophoresed on a 1% agarose gel (10 µg per track) and DNA transferred to nitrocellulose. Hybridisation was as follows: Track A, pSU55 probe, Track B, total λCH-O1 probe, Track C, the 3.3 kb EcoRI fragment Y of λCH-O1 as probe (see Figs. 1 and 3).

represents one of the major coding regions of the insert (see Fig. 3). A HaeIII digest of Y was de-phosphorylated and 5' labelled with polynucleotide kinase (20). The labelled fragments were resolved on a polyacrylamide gel (Fig. 4B), then cut from the gel and DNA electro-eluted. The larger fragments of the digest kinased poorly.

DNA from each band was denatured with DMSO and electrophoresed on gels appropriate for resolving single-stranded DNA (20). Excellent resolution of bands 3,7,8,9,11 and 12 (Fig. 4)

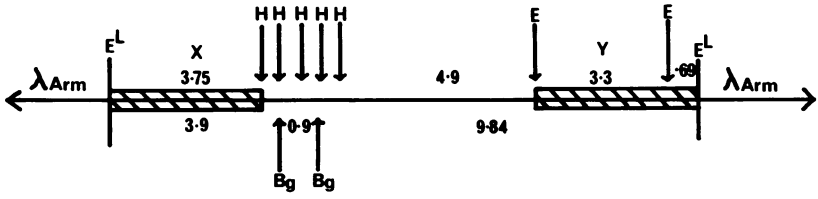

Figure 3. Restriction endonuclease map of λCH-O1 insert DNA. Map distances are shown in kilobase pairs. Restriction sites are shown as Bg = BglII, E = EcoRI, H = HindIII. E^L denotes the boundaries of the insert (the library was constructed with EcoRI linkers; see Materials and Methods). The blocked-in regions represent fragments containing histone coding regions (Fig. 1 and unpublished results). The exact number and location of HindIII sites situated between fragment X and the 4.9 kb non-coding fragment are not known. Because the several HindIII fragments referred to are small, it is not yet known whether they contain any coding regions.

into single-stranded material was obtained. These fragments labelled only at the 5' termini were subjected to the sequencing reactions of Maxam and Gilbert (20). The sequencing ladders for band 9 are shown in Fig. 5.

Amino acid sequences from each possible reading frame were compared with histone amino acid sequences from chicken where these were known (21) and with sequences from calf, rabbit and trout (9) where chicken sequences were unavailable.

DNA sequencing data from bands 8,9 and 11 of Fig. 4 have been examined in detail and predicted amino acid sequences from one possible reading frame are shown in Fig. 6. Fragments 9 and 11 predict amino acid sequences which are identical with two separate regions of chicken histone H2A. This result confirms that λCH-O1 contains histone gene sequences.

In addition, fragment 8 predicts an amino acid sequence which has striking homology with a C-terminal portion of histone H1 from trout and rabbit (Fig. 6). (The C-terminal chicken H1 amino acid sequence is not yet available.) The two amino acid substitutions shown for H1 (Fig. 6) are not conservative but do result from single base changes in the DNA. Histone H1 is the most variable of all the histones (22) and tissue variants also occur within an organism (22). Proton NMR spectra suggest that the C-terminal portion of H1 histone adopts a random coil conforma-

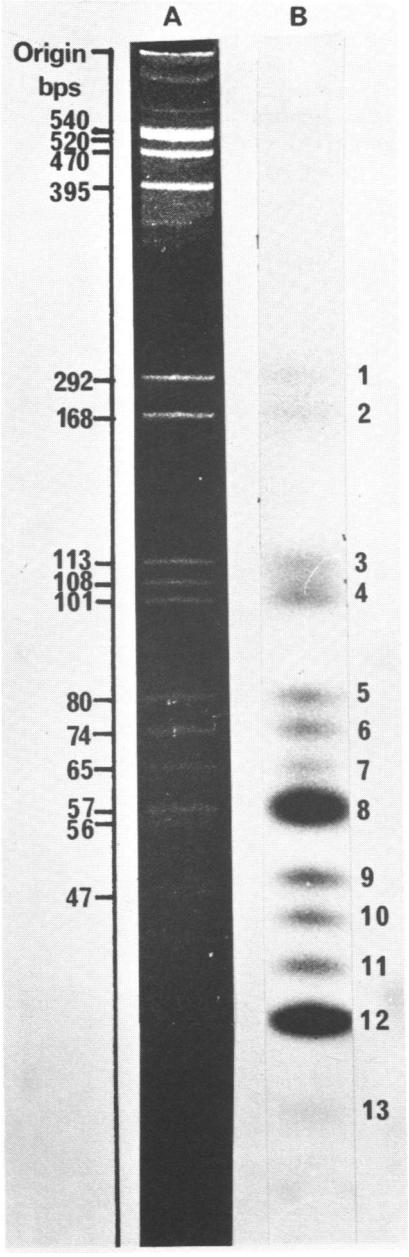

Figure 4. HaeIII digest of a major histone coding region (Y) of λCH-O1. The 3.3 kb EcoRI fragment Y of the histone gene insert (see Fig. 3) was digested with HaeIII, a sample electrophoresed on a 1% agarose gel and detected with ethidium bromide (Track A). 4 µg of the same digest was dephosphorylated and 5'-labelled with γ-^{32}P-ATP (Materials and Methods) prior to electrophoresis. Radioactive bands were detected by autoradiography (Track B). DNA sequences were determined from some of these bands (Figs. 5, 6).

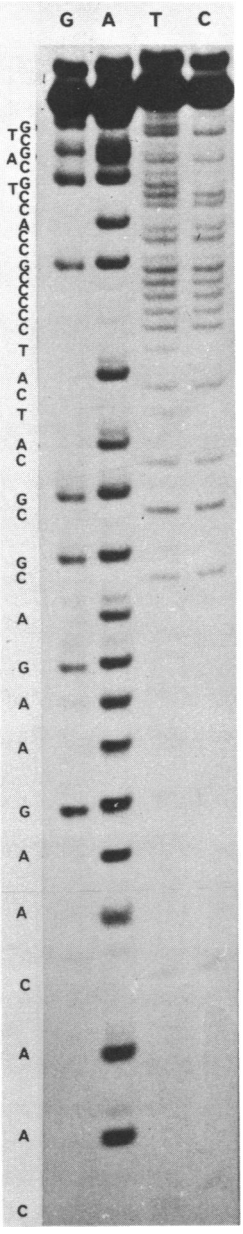

Figure 5. Autoradiogram of DNA sequencing gel from HaeIII fragment 9 (see Fig. 4). Single-stranded 5'-labelled fragments were sequenced by the method of Maxam and Gilbert (20) and bands resolved on 20% polyacrylamide gels (0.5 mm thick). The DNA sequence shown corresponds to a portion of the chicken H2A gene (Fig. 6).

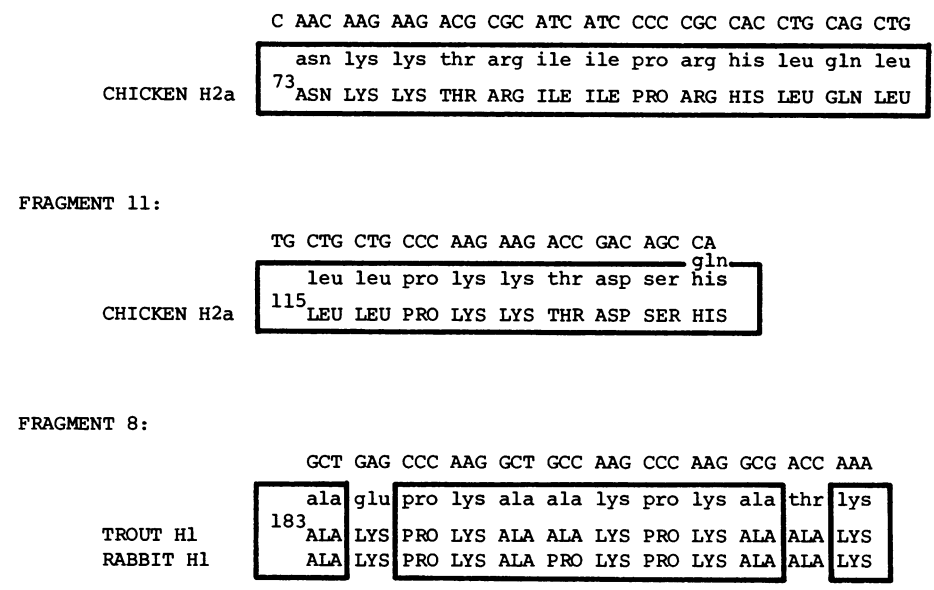

Figure 6. DNA sequence data from λCH-O1 fragments corresponding to histone coding regions. Fragments 9 and 11 (see Fig. 4) predict the precise amino acid sequence for portions of chicken histone H2A. The DNA sequence data from fragment 8 predicts an amino acid sequence with striking homology to histone H1 (see text).

tion both when free in solution and when bound to DNA (23). Because this region of the molecule is not subject to the same degree of structural constraints that apply for other histones, we believe that the sequence derived from fragment 8 (Fig. 6) represents a portion of an H1 gene.

The isolation of clone λCH-O1 now makes detailed investigation of vertebrate histone genes possible.

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