Chicken histone H5: selection of a cDNA recombinant using an extended synthetic primer

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ABSTRACT

We describe the use of a synthetic primer to select a cDNA recombinant clone containing H5 coding sequences. The strategy used was as follows: 1. Prepare oligo(dT) cellulose-bound mRNA from chicken reticulocytes and select 11S-18S material from sucrose gradients. 2. Use this RNA fraction both to prepare a cDNA library and as a template for H5-specific cDNA synthesis using a synthetic primer. 3. Screen out most globin cDNA recombinants with oligo(dT)-primed globin cDNA. 4. Search for H5 recombinants using H5 specific cDNA and verify the identity by DNA sequencing. Our screening suggests an H5 mRNA abundance of about two parts per thousand in chicken reticulocyte poly(A)-containing RNA. The isolation of an H5 cDNA recombinant clone is an initial step in the study of H5 genes and their relationship to H1 and core histone genes.

INTRODUCTION

A characteristic of eukaryote chromosomes is the presence of highly basic histone proteins. While it is generally thought that core histones in the form of nucleosomes occur throughout the chromosome, it is not clear whether their distribution is random. There are suggestions that site-specific non-histone proteins may dictate location of nucleosomes and that local regions of DNA such as origins of replication or promoters may be devoid of histones, (reviewed in reference 2). Histone variants particularly H1, H2A and H2B arise during development, but their significance is not clear.

The H5 histone, selectively expressed in erythroid cells of birds, amphibia and reptiles is a striking example of a major tissue-specific histone. During maturation of the cell series there is a progressive though incomplete replacement of H1 with H5 histone. The H1 group of histones, of which H5 is a particular member, certainly influence chromatin conformation and this has implications for transcription and possibly cell division. As H5 is present in dividing erythroblasts, its initial presence
does not prevent RNA or DNA synthesis. However, accumulation of H5 may be responsible for progressive chromatin condensation and consequent cessation of nucleic acid synthesis in mature red cells.

MATERIALS AND METHODS

Preparation of RNA

Total cytoplasmic RNA was prepared from reticulocytes isolated from the blood of anaemic chickens. After two rounds of oligo(dT)-cellulose chromatography the polyadenylated RNA was fractionated by sucrose gradient centrifugation and the 11S-18S material collected.

Construction of cDNA recombinants and colony screening

A library of cDNA recombinants was prepared from the 11S-18S reticulocyte RNA fraction using basically the methods of Chang et al.\(^9\). S1 nuclease-treated, double-stranded cDNA was fractionated by sucrose gradient centrifugation and the highest molecular weight fractions (20% of the cDNA) collected. A portion with an average of 10 dC residues per 3' terminus added by terminal transferase\(^10\), was annealed with an equi-molar ratio of PstI-digested, dG-tailed pBR322. A yield of 400 recombinants per ng of cDNA was obtained. Ampicillin-sensitive transformants were screened with either globin cDNA or synthetic primer-extended cDNA by standard colony screening procedures\(^11\).

Construction of the synthetic primer

The undecanucleotide d(G-C-G-A-T-C-A-T-C-T-C) was chemically synthesised by the solid phase phosphotriester method on a polyamide support and purified by ion-exchange and reversed-phase high performance liquid chromatography\(^12\).

Preparation of \(^32\)P-labelled extended primer

In analytical reactions cDNA was copied from 1 µg of 11-18S RNA by reverse transcriptase. A range of primer concentrations from zero to 4,000-fold molar excess was used (assuming H5 RNA was present at 0.5 percent abundance). Primer was pre-incubated with RNA in 0.5 M KCl (20°C, 30 min) and other reagents then added to a final volume of 20 µl containing 50 mM Tris-C1, pH 8, 50 mM KCl, 7 mM MgCl2, 1 mM β-mercaptoethanol and 600 µM of dCTP, dGTP and dTTP and 4 µCi of \(^32\)P-dATP at 5 µM. Reverse transcriptase (kindly provided by Dr. J. Beard, Florida, U.S.A.) was added (28 units), and the incubation continued for 1 h at 37°C. The cDNA was recovered after alkaline hydrolysis and neutralisation of the reaction mixture and electrophoresed on 8% 7 M urea polyacrylamide gels (see Fig. 2).
For preparative purposes, a similar reaction containing 2.5 µg of RNA was incubated with 1,000 fold molar excess of primer and 60 µCi of dGTP (400 Ci/m mole). The resulting cDNA (5 x 10^5 d.p.m.) was used for colony screening (Fig. 3 and text).

**DNA-Sequence Analysis**

DNA-sequencing was carried out by the chain-termination method of Sanger et al. 13.

**RESULTS AND DISCUSSION**

**Synthetic primer selection**

The amino acid sequence of chicken H5 is known 14, allowing a search for a region which on back-translation, would permit construction of a suitable primer. The region around the single methionine residue in H5 (met-31, Fig. 1) was examined as only one codon exists for this amino acid. As shown in Fig. 1a, if the central base of the alanine codon marks the 5' end of the primer, it was possible to construct a primer of 11 bases corresponding to the region glu-met-ile-ala in which 9 of the 11 bases would be correct. The only choices to be made were the third bases of ile and glu codons. Nucleotide sequences for chicken α 15 and β 16 globins and limited sequences for chicken histones H2A and H2B 1, indicated the third base codon usage for ile and glu shown in Fig. 1b. The overwhelming preference for AUC and GAG respectively made the decision on the primer sequence straightforward. Therefore, the final choice for the primer molecule was the undecanucleotide d(G-C-G-A-T-C-A-T-C-T-C). The primer sequence was matched against the sequence of chicken α and β globin mRNAs as these are major constituents of the mRNA preparation from which we hoped to obtain H5 sequences. Homologies were found in the 3' untranslated region in each case (see Fig. 1c). For a primer of 11 bases seeking out a low abundance (H5) mRNA it was possible that considerable priming might occur on both globin mRNAs. The major concern was the 3' untranslated α-globin gene region in which 7 bases in a row on the 3' end of the primer showed precise base-pairing with the RNA (Fig. 1c).

**Synthesis and analysis of primer-extended cDNA**

Cytoplasmic RNA from chicken reticulocytes, twice bound to and eluted from oligo(dT)-cellulose, was fractionated on sucrose gradients. Because H5 mRNA sediments at 13S 17, the 11-18S RNA was collected and used to construct a cDNA recombinant library and also as a template for synthetic primer-directed cDNA synthesis. This size fractionation excluded the majority of the globin mRNA which sediments at 9S. With limited amounts of available RNA,
**Figure 1. Strategy for Selection of H5-Specific Synthetic Primer.**

a. Amino acid sequence and possible codons around the single methionine residue of chicken H5 protein (Met-31 is contained in H5 protein of 189 amino acids). Starting at the central base of the alanine codon, a complementary 11-base sequence with only two ambiguities can be constructed. The third base of ile and glu codons were selected from other data (see Fig. 1b). The bases complementary to these are shown in italics in the primer.


c. Complementarity between the synthetic primer and 3' untranslated regions of α15 and β16 chicken globin mRNAs. Asterisks show mismatches.

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**Nucleic Acids Research**

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extensive characterisation of the reaction was not carried out. (See Materials and Methods for conditions). Some cDNA synthesis in the absence of primer was seen, possibly due to self-priming reactions, and increasing levels of incorporation of radioactivity into high molecular weight products occurred when levels of primer were increased from 20 to 200 times molar excess (assuming H5 mRNA was 0.5% of the RNA). Further increases in the ratio of primer to template RNA had little qualitative or quantitative effect,
although at the highest levels some changes were seen (Fig. 2c).

The cDNA used to screen the cDNA library (Fig. 3) was derived from a reaction using 2.5 μg of 11-18S RNA and 1,000-fold molar excess of primer. Although the products of the reaction appeared complex it was encouraging that a vast excess of primer resulted in the incorporation of only $5 \times 10^5$ d.p.m., while routine cDNA synthesis on a similar sample of RNA, but using oligo(dT) as primer yields at least 100-fold greater level of incorporation. The synthetic primer therefore appeared to show selectivity and it seemed

Figure 2. Synthesis of cDNA from 11-18S Chicken Reticulocyte RNA Using A Primer Designed to Detect H5 Sequences. In the analytical reactions shown here, each track represents cDNA copied from 1 μg of RNA by reverse transcriptase and electrophoresed on an 8% polyacrylamide gel containing 7 M urea. The arrow indicates the position of a single-stranded marker of 134 bases. If H5 represents 0.5% of the RNA, the molar excess of primer is (a) 200 fold, (b) 1,000 fold, (c) 4,000 fold. Asterisks mark differences in banding pattern at the highest concentration of primer.
Figure 3. Colony Screening of Recombinant Plasmids. Colonies were grown on nitrocellulose filters and prepared for nucleic acid hybridisation. Each 8 cm filter was hybridised to cDNA using the procedure of Wahl et al. and after incubation the filters were washed in 0.5 x SSC/0.1 per cent sodium dodecylsulphate, dried and exposed to X-ray film at -80°C in the presence of an intensifying screen.

(a) Filter probed with 5 x 10^5 d.p.m. of primer specific cDNA (Fig. 2 and Materials and Methods) and exposed for 100 hours.

(b) Duplicate filter probed with 6 x 10^6 d.p.m. of globin cDNA and exposed for 18 hours.

Clone pCH5-1 is circled.

possible that some of the products seen on gels may have been inter-related.

Screening cDNA recombinants

The construction of a cDNA library from 11-18S RNA was by standard procedures. About 400 ampicillin-sensitive colonies were screened with globin cDNA and 100 of these were classified as strongly positive for globin sequences. The remaining 300 colonies were re-picked onto two filters and screened with the synthetic primer-extended cDNA. Several positives (15-20) were evident on one filter (Fig. 3a). Re-screening this filter with globin cDNA showed that most of these were globin-containing sequences which came through the first round screening (Fig. 3b). Significantly, one colony strongly positive with the synthetic primer-extended cDNA, did not hybridise with globin cDNA (circled in Fig. 3a).
Identification of a recombinant containing H5 mRNA sequences

Plasmid DNA from the colony circled in Fig. 3a contained a PstI-excisable insert of 350 base pairs. An HaeIII fragment derived from the insert was ligated with Smal digested M13 mp83 DNA and after transformation of JM101 cells, a single transformant (white) plaque picked. The DNA sequence of this insert was determined by the dideoxy sequencing technique and is presented in Fig. 4. In one reading frame the sequence unambiguously predicts the amino acid sequence of a portion of chicken H5. The sequence shows that the predicted codon usage for ile and glu in the synthetic primer (Fig. 1a) was correct.

The use of a synthetic primer as a means of selecting low abundance sequences in a pool of cDNA recombinants has been vindicated several times. Examples include interferon and relaxin. The H5 recombinant, pCH5-1 is only 350 base pairs long, starting about 100 bases to the 3' side of synthetic primer sequence (around met-31 in the amino acid sequence) and extending well into a 5' untranslated region. It was therefore derived from very extensive first-strand cDNA synthesis and inefficient (or S1-sensitive) second strand synthesis. We have used pCH5-1 to re-screen further cDNA.

Figure 4. DNA Sequence Analysis of pCH5-1. An HaeIII fragment derived from the pCH5-1 insert was sub-cloned into the Sma site of M13 mp83 vector DNA and the sequence determined by the dideoxy chain termination procedure. In one reading frame the predicted amino acid sequence corresponds to chicken histone H5 protein sequence determined by Briand et al. The sequence complementary to the synthetic primer is underlined.
recombinants and from this data estimate the abundance of H5 to be about 0.2 per cent of avian reticulocyte mRNA. The isolation of clones containing H5 mRNA sequences will permit an investigation of the structure of H5 mRNA and of genomal H5 sequences.

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REFERENCES