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Cell Cycle Regulated Expression of Subcloned Chicken H3 Histone Genes and Their 5′ Flanking Sequences

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We subcloned two chicken H3 histone genes and transfected them into Rat 3 cell line. One contains 300 bp 5′ to its cap site and the other contains 130 bp 5′ to its cap site when cloned into plasmids. Both of them showed S phase specific expression of their mRNA about 8 fold higher (during S phase) than during G1 phase. This means that only 130 bp 5′ to its cap site was enough to confer cell cycle regulated expression of the latter gene. The DNA sequences of their 5′ flanking region did not reveal any particular homologies or subtype-specific sequences. The DNA sequence data also showed that even though the protein coding regions of the histone genes have been conserved exceptionally well throughout evolution, their 5′ untranslated regions have not been conserved as well.

Histone proteins may be grouped according to timing of their synthesis (6). The major class is that of the replication-dependent histones. The transcripts coding for these histones are most abundant in rapidly dividing tissues. These histone gene transcripts are induced during S phase of the cell cycle and synthesized coordinately with DNA replication. But when DNA synthesis is halted, they are degraded rapidly. The other group of histones is that of the replication-independent histones or replacement variants. Their synthesis is not affected by the inhibitors of DNA replication. They are expressed constitutively at a low level throughout the cell cycle.

Except for the TATA box and CCAAT box related sequences, no other principal homologies 5′ to the coding sequences of all histone genes are found. But a few histone gene families have subtype-specific sequences of their own. One example is the H1-box located about 100 bp upstream from the cap site and considered to play an important role in cell cycle regulation of H1 gene. Another example is an octamer sequence (ATTTGCAT) which is an H2B subtype-specific consensus sequence. This element is also important in cell cycle regulation of H2B gene by interacting with a regulatory protein. Two H4 subtype-specific sequences are also known (4). But, no such sequences have been found for H2A and H3 histone genes. We cloned two chicken H3 histone genes and tested their patterns of expression. They were both replication-dependent histones. Then we sequenced their promoter region to see any homologies between them.

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MATERIALS AND METHODS

Enzymes, Plasmids and Cell Line

Restriction enzymes, calf alkaline phosphatase, T4 DNA ligase, T4 polynucleotide kinase, RNase-free DNase I, S1 nuclease, and RNasin were obtained from the following sources: Bethesta Research Laboratories, USB (United States Biochemical Corporation), IBI (International Biotechnologies, Inc.), Promega Biotec, New England Biolabs or Boehringer Mannheim. T3 RNA polymerase was purchased from Stratagene. The plasmid containing human thymidine kinase (TK) cDNA, and Rat 3 cells were gifts from Dr. Susan E. Conrad (Michigan State University).

Cell Culture

Rat 3 cells, which lack the cytoplasmic thymidine kinase, were maintained in Dulbecco's Modified Eagle's Medium supplemented with 10% calf serum. These cells grow well in normal media but can easily be characterized by their inability to grow in a selective medium containing HAT. This medium contains 110 μM hypoxanthine, 20 μM thymidine and 2 μM aminopterin. Aminopterin inhibits dihydrofolate reductase and causes a block in the main pathway of thymidine phosphate

and purine nucleotide synthesis. In the presence of an exogenous source of thymidine kinase, Rat 3 cells can grow normally in HAT medium.

For synchronization, the medium was removed after the cells reached confluence, and it was replaced by a medium containing 0.1% calf serum. Cells were allowed to incubate for 48 hours to obtain synchrony in G_0/G_1. For serum stimulations, fresh medium containing 10% calf serum was added. At various times after the stimulation with serum, cells were harvested for RNA analysis.

DNA Transfection and HAT Selection

The transfection protocols has previously been described (5). Twenty four hours before transfomation, Rat 3 cells were plated to a density of 5×10^5 cells per 100 mm tissue culture plate. Approximately 1 microgram of a plasmid containing human TK cDNA and 10 micrograms of histone plasmid were ethanol precipitated along with 10~20 micrograms of high molecular weight carrier DNA (Rat 3 DNA). The DNA was resuspended in 4.5 *ml* of sterile double distilled water (dd H_2O), and adjusted to a final concentration of 250 mM $CaCl_2$ by adding 0.05 *ml* of 2.5 M $CaCl_2$. The DNA/$CaCl_2$ mixture was rapidly added to an equal volume of 2% HBS (Hepes-buffered saline; 280 mM NaCl, 50 mM Hepes, 1.5 mM Na_2HPO_4, pH 7.05~7.15). The DNA-calcium phosphate precipitate was allowed to form for 20~30 minutes at room temperature. One *ml* of this mixture was added to a 100 mm plate containing 10 *ml* of the medium. After about 16 hours, this mixture was removed and replaced with a fresh medium without HAT. After an additional 24 hours, the medium was removed and replaced with a HAT-containing medium. The medium was replaced with fresh HAT-containing medium every 3~4 days until HAT resistant colonies were clear (about 2 weeks).

RNA Isolation

Total RNA was prepared from the tissue cultured cells as follows. Cells were washed twice with the phosphate buffered saline (PBS) without calcium and magnesium. One ml of lysis buffer (100 mM Tris-HCl, pH 7.5; 12 mM EDTA; 150 mM NaCl; 1% sodium dodecyl sulfate) containing 200 micrograms per *ml* of proteinase K was added to each plate. DNA in the cell lysate was sheared by passage through a 22-gauge needle and the lysate was transferred to a tube. This solution was incubated at 37°C for 45 minutes and then extracted with 50 : 50 v/v phenol : chloroform. Sodium acetate was then added to 0.3 M and the solution was ethanol precipitated. Samples were spun in a Sorvall RC-2 centrifuge at 10,000 RPM for 20 minutes, the ethanol poured off and pellets allowed to air dry. The pellets were resuspended in 400 microliters of RNase-free TE buffer (10 mM tris-HCl, pH 7.5; 1 mM EDTA) and transferred to Eppendorf tubes. Four microliters of 1 M $MgCl_2$, 100 units of RNasin and one microliter of a 1 mg/*ml* solution of RNase-free DNase I were added, and the tubes were incubated at 37°C for 30 minutes. Next, 16 microliters of 0.5 M EDTA and 20 microliters of 20% sodium acetate were added, and this mixture was extracted twice with 50 : 50 v/v phenol : chloroform, and the aqueous phase was ethanol precipitated at −70°C. RNA was then pelleted in a microcentrifuge at 4°C for 15 minutes and pellets were dried in a vacuum pump dessicator. The pellets were then resuspended in 150 microliters of 20% sodium acetate and spun for 10 minutes in a microcentrifuge at 4°C. Supernatants were discarded and the remaining pellets were resuspended in 100 microliters of TE and then ethanol precipitated after the addition of 10 microliters of 20% sodium acetate. To determine the optical density, samples were spun down at 4°C in a microcentrifuge for 15 minutes, drained, dried and resuspended in 200 microliters of RNase-free dd H_2O. Five microliters of each sample were diluted into 500 microliters of dd H_2O and its optical density was read at 260 nm. One O.D. is equivalent to 50 μg/*ml* of RNA.

S1 Nuclease Analysis

DNA probes for different histone genes were used to analyze the RNA samples obtained. For example, pCH1a-SH4 was used to analyze the RNA transcribed from one of the H3 histone genes (Fig. 1). In this case, the SalI restriction enzyme site usually present at tenth codon was used. Twenty micrograms of the plasmid pSH4 was digested with 20 units of SalI for 4 hours at 37°C, then the terminal phosphates were removed by incubation with 2 units of calf alkaline phosphatase at 37°C for 1 hour. This DNA was radioactively labeled by treatment with [γ-^{32}P]ATP and 3 units of T4 polynucleotide kinase at 37°C for 1 hour. The DNA was then digested with 20 units of the restriction enzyme *Hin*dIII to remove the unwanted labeled end. The DNA was

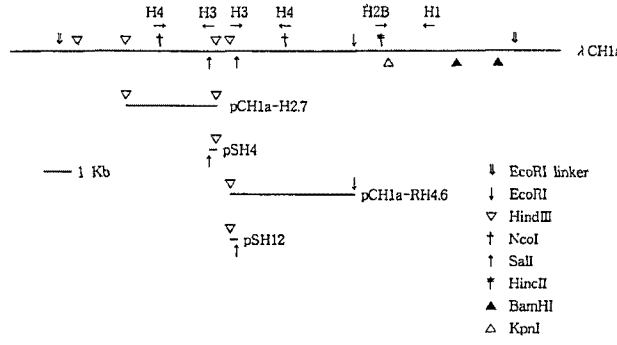

Fig. 1. Restriction map of λCH1a and its subclones.
The direction of transcription is shown by the horizontal arrows.

separated on a 1.2% agarose gel, and the desired fragment was isolated. The labeled DNA fragment was mixed with the RNA being studied and both were ethanol precipitated. The pellet was resuspended in 20 microliters of hybridization buffer (80% formamide; 0.4 M NaCl; 0.04 M Pipes, pH 7.25). The sample was heated at 90°C for 5 minutes to denature both the RNA and the probe. The sample was allowed to hybridize at 55°C for 12 hours. After the hybridization was completed, 300 microliters of S1 buffer (0.03 M sodium acetate, pH 4.5; 0.25 M NaCl; 4 mM $ZnSO_4$; 50 μg/ml denatured, sheared salmon sperm DNA) was added to stop the reaction. The sample was then split into two tubes and to each tube 100 and 200 units of S1 nuclease were added. The reaction was allowed to proceed for 15 minutes at 37°C, then was stopped by extraction with an equal volume of 50 : 50 v/v phenol : chloroform mixture. The supernatant was ethanol precipitated. The reaction products were analyzed on a 6% denaturing polyacrylamide gel. An LKB 2222-010 UltraScan XL Laser Densitometer (Bromma, Sweden) was used to measure the level of mRNA quantitatively. Protected bands of expected size were monitored in a radiogram along the lanes, and peaks were compared.

DNA Sequencing

pCH1a-SH4 and pCH1a-SH12 were sequenced by the chemical degradation method of Maxam and Gilbert (3). Both plasmids were digested with *Eco*RI to linearize the circular DNA, and the DNA was labeled as described above for S1 probe preparation. The labeled DNA was digested with *Sall*, and the desired fragment was gel isolated. The end-labeled DNA fragment was treated as described previously, and the reaction product was run on an 8% denaturing polyacrylamide gel followed by autoradiography of the gel.

RESULTS AND DISCUSSION

Expression of Two Chicken Histone Genes

λCH1a had previously been isolated from a chicken DNA library (2). The restriction map of λCH1a is shown in Fig. 1. Since both chicken H3 genes were previously shown to be regulated during the cell cycle when λCH1 a phage DNA was transfected into Rat 3 cells (about 8~10 fold increase), smaller DNA fragments containing these genes were separately tested. The plasmid pCH1 a-RH4.6 contains the H3 histone gene with about 300 base pairs 5′ to the cap site. Several colonies were isolated after cotransfecting with pCH1a-RH4.6 and the human TK cDNA plasmid. When these isolated cell lines were tested for the expression of the chicken H3 histone gene by S1 nuclease analysis, two of the cell lines (I-a and I-b) showed measurable expression. The probe

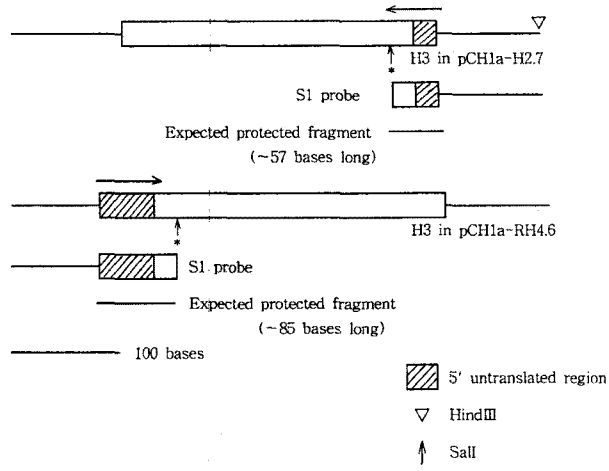

Fig. 2. S1 probes for two chicken H3 histone genes. The direction of transcription is shown by the horizontal arrows.

used in this experiment was a 0.4 Kb fragment from pSH12 which was radioactively labeled at the *Sall* site (Figs. 1 and 2). Our previous experiment had shown that this probe protected a fragment of about 85 nucleotides in length. Total RNA isolated from serum stimulated cells (lanes 9 and 11) showed much higher expression than total RNA from resting cells (lanes 8 and 10 in Fig. 3) as judged by the 85 nucleotide band protected. Thus with only 300 nucleotides of 5′ franking sequences, this chicken H3 histone gene appears to be appropriately expressed in transfected Rat 3 cells. Total RNA from untransfected Rat3 cells was used as a negative control and total RNA from chicken red cell was used as a positive control.

The other chicken H3 histone gene on λCH1a contains only 130 nucleotides 5′ to its cap site when it is cloned into plasmid pCH1a-H2.7 (Fig. 1). This plasmid DNA and the plasmid containing human TK cDNA were transfected and several colonies were isolated by HAT selection. Cell lines II-a and II-b were selected. The probe for the S1 nuclease analysis was a 0.2 Kb fragment from plasmid pSH4 which was end-labeled at the SalI site (Figs. 1 and 2). This probe protected a fragment of about 57 nucleotides from cells transfected with λCH 1a. Lanes 2 and 4 of Fig. 3 show a protected band of about 57 nucleotides long which are about 8 times darker than the ones in lanes 1 and 3, respectively. This means that this H3 histone mRNA level was increased about 8-fold in the S phase in comparison to the G_0/G_1 boundary. Artishevsky *et al.* (1) have shown that a 32 nucleotide region, located about 150 nucleotides upstream of the TATA box, contains a crucial control signal for the cell cycle regulation of the hamster H3 histone gene when the promoter region of the hamster H3 histone gene conferred cell cycle regulation on a

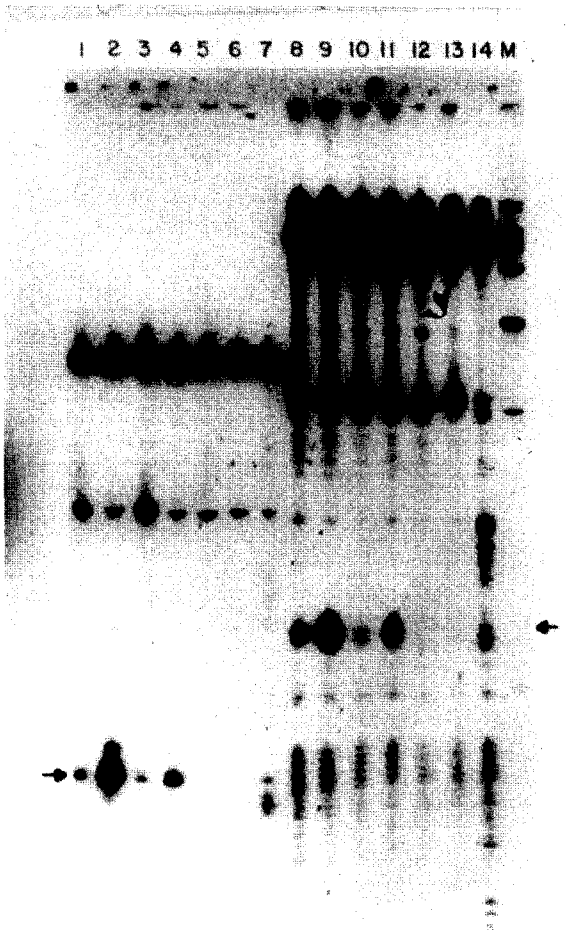

Fig. 3. Cell cycle-regulated expression of chicken H3 histone genes in λCH1a.
Total cellular RNA was isolated from cell line I-a (lanes 8 and 9), cell line I-b (lanes 10 and 11), cell line II-a (lanes 1 and 2), cell line II-b (lanes 3 and 4), or untransfected Rat 3 cells (lanes 5, 6, 12 and 13). Lanes 7 and 14 result from the assay of chicken red cell cytoplasmic RNA. RNA used for lanes 1, 3, 5, 8, 10, and 12 come from quiescent cells. RNA used for lanes 2, 4, 6, 9, 11, and 13 was isolated from serum stimulated cells (for 12 hours) in S phase. Lanes 1~7 were assayed for the levels of the H3 gene present on the pCH1a-H2.7 and lanes 8~14 for levels of mRNA from the other H3.2 gene on pCH1a-RH4.6. Probes used are shown in Fig. 2. Thirty micrograms of RNA and 1000 u/ml of S1 nuclease were used for each reaction.

bacterial neomycine resistance gene. This region is located at 180 nucleotides upstream from the cap site and hence, a total of at least 210 nucloetides 5′ to the cap site are needed for the cell cycle regulation of the hamster H3 histone gene. But in our experiment with a chicken H3 histone gene, it seems that no more than 130 nucleotides 5′ to the cap sites are sufficient to confer cell cycle-regulated expression on the gene.

DNA Sequencing of 5′ Flanking Regions of Two H3 Histone Genes

1. AAGCTTGTTT TCACTGCTTG CTAGTATCTG GCTTCTTCTC AGGTTAAATG
   AGGTGTGTGA AAATGCGATT TATTGCTGAA AGAAGACAAT GAGGGAAGAC
   AACTAGATAA AAAGAAGAAA GGCTTTATGA ATCCGTAGCA AACCGAAAAG
   AGAAACGCTG GGGTTTAACT ATTAAAGAGC AGCAGTAGGR ACAGCAGGAG
   ATTAACGCTG GTTTTTCAAA TTGAACCAAT AATATTCGTC CTTTCTTCAG
   CCAATGGCAA TGCAGCGTTC GGTATAAAAG CGAGTCAGGA ACGGCGCCAC
   CTCARATGCG GTTTTACGGG TCATTTGTGT AGTTGTGGGA AA

2. AAGCTTCTTT GCAAGGTGGG ACAGGCAGAA GGCTTAGAGT TAGCCAATTA
   AATTCATTGA TTTATTGACC AATCAGAGGC GAATGGGCGG GGTTTCATCT
   ACTATAAATA AGAGCCGCTG CAACGAGACC GCCTACTTTC GGTTGCAGAG
   CAGTTCTGCC AATGGCGCGT ACGAAGCAGA CGRCGYGT

Fig. 4. DNA sequences of 5′ flanking regions of chicken H3 histone genes on pCH1a-RH4.6 (1) and pCH1a-H2.7 (2).
Putative transcription initiation sites are marked by arrows. Possible TATA boxes and CCAAT boxes are underlined.

Because the two H3 histone genes in pCH1a-H2.7 and in pCH1a-RH4.6 showed cell cycle-regulated expression, their 5′ flanking regions were sequenced to see if any regions of significant homology existed. Fig. 4 shows the 5′ flanking sequences for the two genes. Except for the TATA box and the CCAAT box, no other significant homologies 5′ to the coding sequence of these two H3.2 genes were found.

It is interesting that even though the two chicken H3 histone genes are less than 1 Kb apart, the lengths of their 5′ untranslated regions are different by about 28 nucleotides. The H3 in pCH1a-H2.7 contains about 27 bp of 5′ untranslated region and the H3 in pCH1a-H4.6 contains about 55 bp of 5′ untranslated region. This further confirms that even though the protein coding regions of histone genes have been highly conserved throughout evolution, their 5′ untranslated regions have not been conserved as well.

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