Journal of Microbiology and Biotechnology
Vol. 3, No. 3, 156-160, 1993

Expression of Chimeric Chicken-Yeast-Chicken H2B Histone Gene

SON, SEUNG-YEOL* AND HO LEE

Department of Microbiology, College of Natural Sciences, Dankook University, Cheonan, 330-714, Korea
Department of Food Technology, College of Natural Sciences, Kyonggi University, Suwon 440-270, Korea

A chicken H2B histone gene was cloned and expressed in Rat 3 cell line. Its messenger RNA level was about 10 times higher during S phase than during G_1 phase. A chimeric chicken-yeast-chicken H2B histone gene was made to change some of wobble sequences of chicken H2B gene. When the chimeric H2B gene was transfected into the Rat 3 cell line, it showed a pattern of expression similar to that of the original chicken H2B gene. At least in this gene, it was concluded that the wobble sequences were not required for the cell-cycle regulated pattern of expression.

The histone proteins have been well preserved during the evolution. The five classes of histone proteins are encoded by multiple copies of genes. In higher eucaryotes, there are a number of nonallelic variants of each histone protein which are encoded by distinct histone genes organized in clusters. Most histone genes and mRNAs from various organisms have common structural features (2, 8). There are two major classes of histone proteins in cells, and one of them is cell-cycle regulated histones (11, 12). Cell-cycle regulated histones are synthesized exclusively during S phase of the cell cycle from nonpolyadenylated mRNAs (1). The mRNAs begin to be transcribed just before the start of S phase, accumulate to high levels during S, and then disappear rapidly after DNA synthesis stops (6, 7). As a result, there is little histone mRNA in the G_1 cell (3, 10). This pattern of cell cycle restriction ensures that the histone proteins are produced only when newly synthesized DNA is being packaged into nucleosomes. The mechanisms for coupling histone mRNA accumulation and DNA synthesis remain obscure, although it is generally thought that both transcriptional and post-transcriptional processes may be involved (7, 15, 17).

Grandy and Dodgson (5) found in a comparison of 7 of the 8 chicken H2B histone gene sequences that their internal coding region nucleotide sequences were highly conserved, more so than that levels of conservation needed merely to specify the same or similar polypeptide sequences. In other words, nucleotides at ambiguous sites (mostly 3′ or wobble sites) still showed a very high level of similarity. These authors proposed that a specific H2B mRNA secondary and/or tertiary structure play an important role in histone gene expression. In this report, a chicken H2B histone gene was cloned and expressed in Rat 3 cell line. In addition, a chimeric gene was made from this clone and tested whether it participates in cell-cycle regulation.

*Corresponding author
Key words: Histone, Gene expression, Chimeric gene

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 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 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 be easily characterized by their inability to grow in a selective medium contain-

ing 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 protocol has previously been described (18). Twenty four hours before transformation, 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 0.45 m*l* of sterile double distilled water (dd H_2O), and adjusted to a final concentration of 250 mM $CaCl_2$ by addition of 0.05 m*l* 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 10ml 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 culture cells as follows. Cells were washed twice with phosphate buffered saline (PBS) without calcium and magnesium. One m*l* 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℃ 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/m*l* solution of RNase-free DNase I were added, and the tubes were incubated at 37℃ 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℃. RNA was then pelleted in a microcentrifuge at 4℃ 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℃. Supernatants were discarded and the remaining pellets were resuspended in 100 microliters of TE and then ethanol precipitaed after the addition of 10 microliters of 20% sodium acetate. To determine the optical density, samples were spun down at 4℃ in a microcentrifuge for 15 minutes, drained, dried and resuspended in 200 microliters of RNase free dd H_2O. Five microliters of each sample was 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/m*l* of RNA.

S1 Nuclease Analysis

S1 nuclease protection assay was carried out according to standard techniques (16).

RNase Protection Assay

A DNA fragment containing the 5′ portion of a histone gene and some of its flanking region was cloned into a Bluescript vector (obtained from Stratagene, La Jolla, CA.) which has multiple cloning sites in between T3 and T7 promoters. The RNA probe was made by *in vitro* transcription using T3 or T7 RNA polymerases. The *in vitro* transcription was done according to the manufacturer's recommendation. The reaction mixture includes a transcription buffer (40 mM Tris-HCl, pH 8.0; 10 mM $MgCl_2$; 2 mM spermidine; 50 mM NaCl), 1 microgram of restricted, proteinase K-treated DNA template, 0.4 mM rATP, 0.4 mM rCTP, 0.4 mM rGTP, 30 mM DTT (Dithiothreitol), 25 units of RNasin, 5 microliters of 800 Ci/mM, 10 mCi/m*l* [α-^{32}P]rUTP, and 10 units of T3 or T7 RNA polymerases in a final volume of 25 microliters. The reaction mixture was incubated at 37℃ for 30 minutes. After the RNA synthesis reaction, 1 microliter of 1 mg/m*l* DNase I was added to remove the DNA template followed by incubation at 37℃ for 15 minutes. Extraction with an equal volume of a 50:50

v/v phenol : chloroform mixture and ethanol precipitation followed. The pellet was then resuspended in 100 microliters of 0.15 M sodium acetate, precipitated with ethanol again, and resuspended in 50 microliters of DEPC-treated dd H_2O. The labeled RNA transcript was mixed with the RNA isolated from Rat 3 transformant and both were ethanol precipitated. The pellet was resuspended in 30 microliters of the hybridization buffer (80 % formamide; 0.4 M NaCl; 0.04 M Pipes, pH 7.25). The sample was allowed to hybridize at 55℃ for 12~16 hours. Following the hybridization, 300 microliters of the RNase buffer (0.3 M NaCl, 10 mM Tris pH 7.5, 5 mM EDTA) containing RNase A (40 micrograms per ml) and RNase T1 (2 micrograms per ml) were added, and the mixture was incubated at 37℃ for 1 hour. The RNase digestion was terminated by the addition of 20 microliters of 10% SDS and 50 microliters of 10 mg/ml proteinase K and followed by an additional incubation at 37℃ for 15 minutes. The reaction mixture was extracted with an equal volume of phenol : chloroform (1:1) and the ^{32}P-labeled RNA was precipitated with ethanol (sometimes with the addition of carrier tRNA). The pellet was washed with 70% ethanol, dissolved in a loading buffer containing 90% formamide and analyzed by denaturing polyacrylamide gel electrophoresis. An LKB 2222-010 UltraScan XL Laser Densitometer (Bromma, Sweden) was used to measure the level of mRNA quantitatively. Protected bands of expected size in a radiogram were monitored along the lanes and peaks were compared.

RESULTS AND DISCUSSION

Expression of Chicken H2B Histone Gene in λCH1a DNA

λCH1a had previously been isolated from a chicken DNA library (4). The restriction map of λCH1a is shown in Fig. 1. To test the cell cycle regulation properties of the H2B histone gene, S1 nuclease analysis was performed. This phage DNA was introduced into Rat 3 cells with a plasmid containing human TK DNA. After the HAT selection, the cells were grown to confluence and then incubated in a medium containing 0.1% calf serum for 2 days. Because the Rat 3 cells start DNA synthesis 6~8 hours after the serum stimulation and because the S phase lasts almost 10 hours, total RNA was prepared from cells 12 hours after the serum stimulation and from unstimulated cells. The probe used in this experiment was made in the following manner (Fig. 1). Since this H2B histone gene was sequenced (5) and the BstEII site was found at codon number 17, the plasmid pRB10a-3.5 was cut with BstEII and end-labeled with ^{32}P at this site. The linearized, labeled plasmid DNA was digested with *Eco*RI and a 0.6 Kb fragment was gel-isolated to be used as an S1 probe. As shown in Fig. 2, the expression of this H2B histone gene is appropriately regulated during the cell cycle. During the S phase of

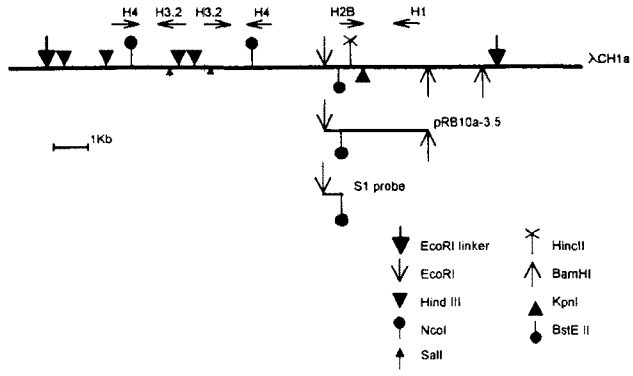

Fig. 1. Restriction map of λCH1a and its subclones. The direction of transcription is shown by the horizontal arrows.

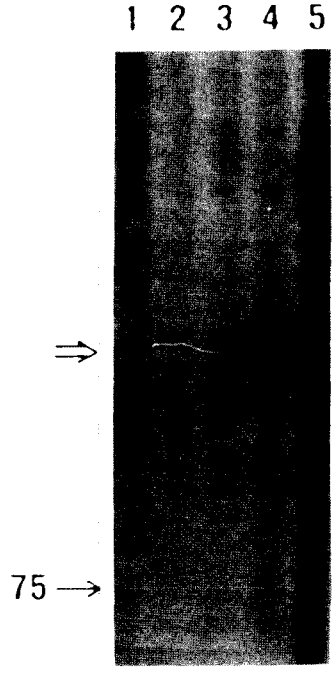

Fig. 2. Cell cycle regulation of the chicken H2B histone genes in a Rat 3 cell line transfected with λCH1a. A DNA fragment cut with *Eco*RI was end-labeled at the *Bst*EII site to be used as a probe.

Fifty micrograms of RNA and 670 μ/ml of S1 nuclease were used for all assays. RNAs tested were: 1. Untranslated Rat 3 cells, quiescent (lane 1) or stimulated (12 hr, lane 2). 2. Rat 3 cells cotransfected with λCH1a DNA for lane 3 (unstimulated) and lane 4 (stimulated). 3. Anemic chicken red cells (lane 5).

the cell cycle (lane 4), the level of specific H2B histone mRNA is about 9 times as much as the level of the H2B histone mRNA during resting period (lane 3). Lanes 1 and 2 are negative controls and lane 5 is a positive control. Because the protein coding regions but not the 5′ and 3′ untranslated regions of histone gene mRNAs are very well conserved, shorter fragments were also expected to arise from hybridization of the probe to endogenous rat H2B histone mRNAs (lane 1-4) and to chicken H2B histone mRNA from other genes (lane 5).

Expression of the H2B Histone Gene in pRB10 a-3.5

The plasmid pRB10a-3.5 contains about 550 base pairs 5′ to the cap site of the H2B histone gene (Fig. 1). Rat 3 cells were cotransfected with plasmid pRB10a-3.5 and the human TK cDNA plasmid, and the transfected cells were selected with HAT media. This mass culture was grown to confluence before changing the medium to 0.1% calf serum containing medium. Total RNAs were prepared before and 12 hours after serum stimulation, and subjected to an RNase protection assay. The radioactively-labeled RNA probe used in this experiment was made in the following manner (Fig. 3). An 0.8 Kb *Eco*RI-*Hin*cII DNA fragment from plasmid pRB10a-3.5 was cloned into the multiple cloning site of the vector plasmid pBS(−) at *Eco*RI and *Eco*RV site. This newly constructed plasmid, pBS(−)-H2B, was cut with *Eco*RI and subjected to *in vitro* transcription using [α-^{32}P]rUTP and T3 RNA polymerase. The resulting uniformly-labeled RNA probe was about 860 nucleotides long. The HincII site is located 239 bp downstream from the transcription start site, and thus the protected fragment from the exogenous pRB10a-3.5 H2B gene should be 239 nucleotide long. The result is shown in Figure 4. Lane 1 represents the H2B histone mRNA level prepared 12 hours after the serum stimulation and lane 2 represents the mRNA level before the stimulation. The protected band in lane 1 is about 10 times darker than the one in lane 2. This means that the chicken H2B histone gene in pRB10a-3.5 was expressed 10 times more during the S phase than during the resting stage at the RNA level. The H2B histone mRNA was increased by 9 fold during S phase when the complete λCH1a phage DNA was transfected (Fig. 2). These two stimulation levels are not significantly different. The H2B-specific octamer element (ATTTGCAT) which is known to be essential in cell-cycle regulation of H2B histone genes (14) was originally found in the λCH1a H2B gene (5). This gene also contains a 3′ end stem-loop structure (5), another sequence important in cell-cycle regulation of replication-dependent histone genes (13). Therefore it is not surprising, given our previous results, that the H2B histone gene on pRB

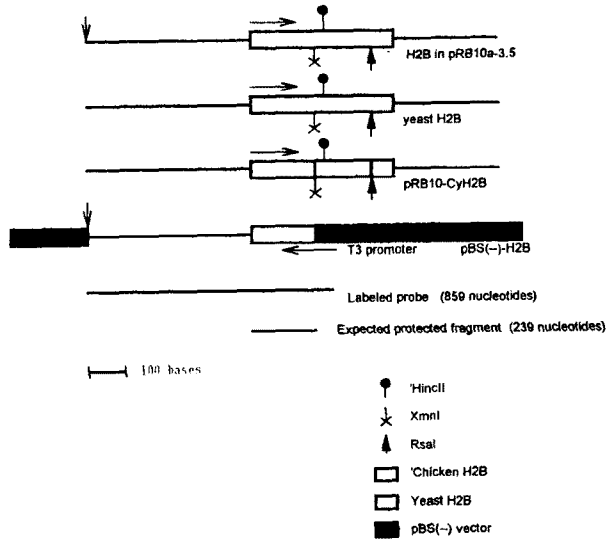

Fig. 3. Construction of pBR10-CyH2B and its probe for the RNase protection assay.

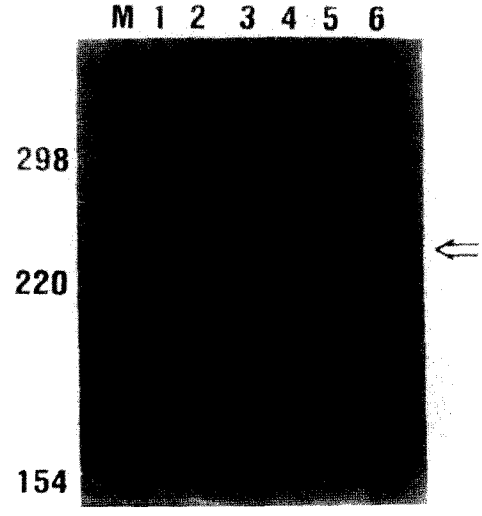

Fig. 4. RNase protection assay of a chicken H2B histone gene. Thirty micrograms of total RNAs were used in each reaction. The probe is made from pBS(−)-H2B, shown in Fig. 3.

Lane 1: stimulated cells transfected with pBR10a-3.5. Lane 2: unstimulated cells transfected with pBR10a-3.5. Lane 3: stimulated cells transfected with pBR10-CyH2B. Lane 4: unstimulated cells transfected with pBR10-CyH2B. Lane 5: stimulated Rat 3 cells. Lane 6: anemic chicken red cells.

10a-3.5 seems to contain all the sequences necessary for a proper cell-cycle regulated expression.

Expression of Chimeric Chicken-Yeast-Chicken H2B Histone Gene

To test whether the wobble sequence has a role in

the cell-cycle regulation of the chicken H2B histone gene, as Grandy and Dodgson (5) proposed, we took advantage of the fact that while all chicken (and other vertebrates) H2B histone genes examined to date have high levels of G:C base pairs in wobble sites, the yeast H2B genes have high levels of A:T base pairs in these sites.Yeast H2B histone genes are also regulated during the cell cycle. Even though the protein sequences of chicken and yeast H2B histones are similar, the third nucleotides of their amino acid codons are often different from each other as suggested above. To answer the question whether the third nucleotides of amino acid codons affect the expression pattern of the H2B histone gene, we replaced a part of the chicken H2B histone gene with the analogous portion of a yeast H2B gene, TRT-1 (9). The fact that an XmnI site at codon 68 and an RsaI site at codon 125 are conserved between chicken and yeast H2B genes made this reasonably straightforward (Fig. 3). The hybrid chicken-yeast-chicken H2B histone gene thus has a 57 codon (171 bp) yeast insert. The hybrid histone differs from the chicken H2B by only 8 of 126 amino acids, but within the 171 bp insert there is only 63.7% nucleotide sequence homology between the yeast H2B gene and the chicken H2B gene (differs in 62 of 171 bp). The hybrid H2B gene in plasmid pRB10-CyH2B was transfected into Rat 3 cells with human TK cDNA. A mass culture was grown after HAT selection. Using the same probe for mRNA from the H2B gene as described previously, an RNase protection assay was performed. Fig. 4 shows the result of this experiment. The protected band in lane 3 (12 hr after serum stimulation) is about 10 times darker than the one in lane 4 (from unstimulated cells). Lane 5 represents RNA from Rat 3 cells that were used as a negative control and lane 6 represent RNA from anemic chicken red cells that were used as a positive control. This compares well with the 9 fold stimulation observed previously from the H2B histone gene in λCH1a transfected cells. Therefore, the ambiguous nucleotides of amino acid codons in the chicken H2B histone gene sequence do not seem to be requred for cell-cycle regulated expression.

REFERENCES

1. **Adesnik, M., and J.E. Darnell.** 1972. Biogenesis and charicterization of histone messenger RNA in HeLa cells. *J. Mol. Biol.* **67**: 397-406.
2. **Birnstiel, M.L., M. Busslinger, and K. Strub.** 1985. Transcription termination and 3′ processing: the end is in site!. *Cell* **41**: 349-359.
3. **Detke, S., A. Lichtler, I. Phillips, J. Stein, and G. Stein.** 1979. Reassessment of histone gene expression during cell cycle in human cells by using homologous H4 histone cDNA. *Proc. Natl. Acad. Sci. UAS* **76**: 4995-4999.
4. **Dodgson, J.B., J.S. Strommer, and J.D. Engel.** 1979. Isolation of the chicken beta-globin gene and a linked embryonic beta-like globin gene from a chicken DNA recombinant library. *Cell* **17**: 879-887.
5. **Grandy, D.K. and J.B. Dodgson.** 1987. Structure and organization of the chicken H2B histone gene family. *Nucleic Acids Res.* **15**: 1063-1080.
6. **Groppi, U.E., Jr., and P. Coffino.** 1980. G1 and S phase mammalian cells synthesis histones at equvalent rates. *Cell* **21**: 195-204.
7. **Heintz, N., H.L. Sive, and R.G. Roeder.** 1983. Regulation of human histone gene expression: kinetics of accumulation and changes in the rate of synthesis and in the half-lives on individual histone mRNAs during the HeLa cell cycle. *Mol. Cell. Biol.* **3**: 539-550.
8. **Hentschel, C.C., and M.L. Birnstiel.** 1981. The organization and expression of histone gene families. *Cell* **25**: 301313.
9. **Hereford, L.A., M.A. Osley, J.R. Ludwig II, and C.S. McLaughlin.** 1981. Cell-cycle regulation of yeast histone mRNA. *Cell* **24**: 367-375.
10. **Hirschorn, R.R., F. Marashi, R. Baserga, J. Stein, and G. Stein.** 1984. Expression of histone genes in a G1-specific temperature-sensitive mutant of the cell cycle. *Biochemistry* **23**: 3731-3735.
11. **Old, R.W., and H.R. Woodland.** 1984. Histone genes: not so simple after all. *Cell* **38**: 624-626.
12. **Osley, M.A. and L. Hereford.** 1981. Yeast histone genes show dosage compensation. *Cell* **24**: 377-384.
13. **Pandey, N., and W.F. Marzluff.** 1987. The stem-loop structure at the 3′ end of histone mRNA is necessary and sufficient for regulation of histone mRNA stability. *Mol. Cell. Biol.* **7**: 4557-4559.
14. **Perry, M., G.H. Thomsoen, and R.G. Roeder.** 1985. Genomic organization and nucleotide sequence of two distinct histone gene clusters from Xenopus laevis: identification of novel conserved upstream sequence elements. *J. Mol. Biol.* **185**: 479-499.
15. **Plumb, M., J. Stein, and G. Stein.** 1983. Coordinate regulation of multiple histone mRNAs during the cell cycle in HeLa cells. *Nucleic Acids Res.* **11**: 2391-2410.
16. **Sambrook, J., E.F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
17. **Schumperli, D.** 1986. Cell-cycle regulation of histone gene expression. *Cell* **45**: 471-472.
18. **Son, S.Y. and H. Lee.** 1992. The cellcycle expression of chicken H4 histone gene in Rat 3 cells. *J. Inst. Biotechnol. Korea Univ.* **4**: 20-24.

(Received August 18, 1993)