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# Expression of Replication-Independent Chicken H3.3 Histone Gene without Introns

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**Abstract:** We eliminated introns from replication independent chicken H3.3 histone gene using a H3.3 cDNA clone and a genomic H3.3 clone. After introduction into Rat 3 cells, we observed its pattern of expression by analyzing mRNA from different phases of the cell cycle. Even without introns, the H3.3 gene was expressed constitutively at a low level throughout the cell cycle. This indicates that the introns in the H3.3 gene are not responsible for the cell cycle-independent expression of the gene. This result contradicts previous reports that suggested their importance in cell cycle regulated expression. We believe that other regions of the gene, promoter, coding region, and/or 3'-end of the gene, are involved in its expression pattern.

**Key words:** cell cycle regulation, histone gene, intron

Histone proteins may be grouped according to timing of their synthesis (Zweidler, 1984; Son, 1993). Most histone protein synthesis occurs during S phase, coordinated with DNA replication. These histone gene transcripts are induced during S phase of the cell cycle, but they are degraded rapidly when DNA synthesis is halted. The transcripts for these replication-dependent histones are derived from genes without introns and are processed at a site between two conserved motifs to yield mature mRNAs which lack polyadenylate tract and which are processed at a highly conserved stem-loop or hairpin structure (Heintz, 1991; Osley 1991). The transcripts coding for these histones are most abundant in rapidly dividing tissues and are accumulated and degraded rapidly in concert with changes in the rate of DNA replication during S phase of the cell cycle (Heintz *et al*., 1983; Sittman *et al*., 1984; Bandyopadhyay *et al*., 1987). The rate of protein synthesis also affects their accumulation and degradation (Graves *et al*., 1987; Stimac *et al*., 1984; Wu *et al*., 1985). When DNA replication is inhibited by various means, these histone mRNA levels decrease 10- to 20-fold within 30 min because of a decreased transcription rate and a decreased mRNA stability. The 3'-terminal stem-loop structure plays an essential role in mRNA stability (Levine *et al*., 1987; Pandy *et al*., 1987).

The other group of histones is 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. They include two H2A subspecies, H2A.Z (Dalton *et al*., 1989; Hatch *et al*., 1990) and H2A.X (West *et al*., 1980), and one H3 subspecies, H3.3 (Wu *et al*., 1981; Wu *et al*., 1982). These genes have been sequenced from several species and shown to share common features. Unlike their replication-dependent counterparts they contain introns and lack the stem-loop and U7 binding motifs found in replication-dependent histone genes. They yield typical polyadenylated mRNAs. When two nonallelic chicken H3.3 histone genes were characterized, they shared a variety of properties which differ from the replication-dependent histone genes (Brush *et al*., 1985). One contains 3 or more introns and the other has 4 introns. But the location of the two introns within the coding regions of the two genes have been exactly conserved, whereas the intron positions in their respective 5' flanking regions differ. Both genes have a 5' untranslated leader segment spliced to the coding body of the mRNA. Both contain long 3' and 5' untranslated regions and are not linked to any other histone genes. Their predicted amino acid sequences are identical.

If these structural differences between replication-dependent histone genes and replication-independent histone genes play important roles in their pattern of expression, the appropriate modification of a replication-independent histone gene may result in cell cycle regu-

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lated expression. There could be two structural modifications possible. One is to eliminate introns from a H3.3 gene and the other is to introduce stem-loop structure at 3' end of the gene in place of polyadenylation signal. In this experiment, we eliminated introns from an H3.3 gene, introduced it into Rat 3 cells and observed its pattern of expression. Even without introns, the H3.3 gene was expressed constitutively at a low level throughout the cell cycle. This indicates that the introns in the H3.3 gene are not responsible for the cell cycle independent expression of the gene.

## Materials and Methods

### 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 protocol has previously been described (Son *et al.*, 1993). Twenty four hours before transfection, Rat 3 cells were plated to a density of $5\times 10^5$ cells per 100 mm tissue culture plate. Approximately 1 μg 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, the medium was removed and replaced with a HAT-containing medium. The medium was replaced with a HAT-containing medium every 3~4 days until HAT resistant colonies were clear (about 2 weeks). When transfected cells were selected in HAT medium, they were allowed to grow together in the same plate for a mass culture or individual colonies were transferred into other plates.

### RNA isolation

Total RNA was isolated from transfected cells as described (Choi *et al.*, 1995).

### S1 nuclease analysis

S1 nuclease analysis was performed as previously described (Sittman *et al.*, 1983) except using a probe labeled at Pvu II site from pBH6b-2.3 (Fig. 1).

### RNase protection assay

A DNA fragment of pBH6b ΔI digested with PvuII and BamHI containing the 5' portion of the H3.3 histone gene and some of its flanking region (Fig. 1) 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 RNA polymerase. The *in vitro* transcription was done essentially same as Jeong and Kang (Jeong *et al.*, 1995). The reaction mixture includes transcription buffer (40 mM Tris-Cl,

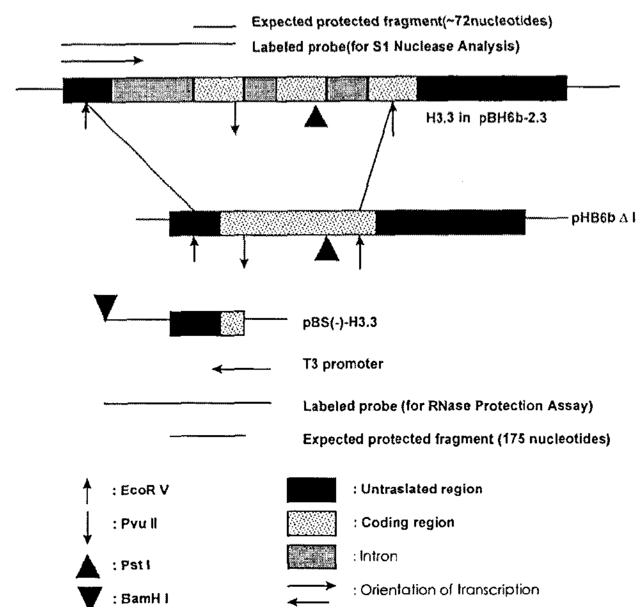


**Fig. 1.** Construction of intronless H3.3 histone gene. The probe for the RNase protection assay was made from pBS(-)-H3.3. The probe for the S1 nuclease analysis is also shown (see Materials and Methods).

pH 8.0; 10 mM $MgCl_2$; 2 mM spermidine; 50 mM NaCl), 1 μg 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 μl of 800 Ci/mM, 10 mCi/ml[$\alpha$-$^{32}$P]rUTP, and 10 units of T3 polymerase in a final volume of 25 μl. The reaction mixture was incubated at 37°C for 30 minutes. Following the RNA synthesis reaction, 1 μl of 1 mg/ml RNase-free DNase I was added to remove the DNA template followed by incubation at 37°C for 15 minutes. Extraction with an equal volume of a phenol:chloroform mixture (50:50 v/v) and ethanol precipitation followed. The pellet was then resuspended in 100 μl of 0.15 M sodium acetate, precipitated with ethanol again, and resuspended in 50 μl of DEPC (diethyl aminoethyl pyrocarbonate)-treated dd $H_2O$. The labeled RNA transcript was mixed with the RNA isolated from Rat 3 transformants and both were ethanol precipitated. The pellet was resuspended in 30 μl 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 RNAs. The sample was allowed to hybridize at 55°C for 12-16 hours. Following the hybridization, 300 μl of RNase buffer (0.3 M NaCl, 10 mM Tris, 5 mM EDTA, pH 7.5) containing RNase A (40 μg/ml) and RNase T1 (2 μg/ml) were added, and the reaction was incubated at 37°C for 1 hour. The RNase digestion was terminated by the addition of 20 μl of 10% SDS and 50 μl of 10 mg/ml proteinase K and followed by an additional incubation at 37°C 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.

### Densitometric analysis

Since it is critical to compare the level of mRNA of interest in different stages of the cell cycle, 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 lanes and peaks were compared.

## Results and Discussion

### Constitutive expression of transfected chicken H3.3 histone gene in Rat 3 cells

Before the transfection of the chicken H3.3 histone gene without introns into Rat 3 cell line, we wished to confirm that the intact chicken H3.3 histone gene with introns was properly expressed in Rat 3 cell line. Only if it is expressed constitutively throughout the cell cycle in Rat 3 cell line as in chicken, could we go further and test the gene without introns. The result is shown in Fig. 2. From the previous experiment with chicken cells, we already knew that the probe would give a protected band of 72 bases long. Because the Rat 3 cells start DNA synthesis 6~8 hours after the serum stimulation and the S phase lasts almost 10 hours, total RNA was prepared from cells 12 hours after the serum stimulation and from unstimulated cells. The difference in expression of H3.3 mRNA at 12 hours after serum stimulation (S phase, lane 6) and in the absence of stimulation ($G_0/G_1$ phase, lane 5) is less than 1.2 fold as monitored with a densitometer. The other transfected cell line also showed a similar result (lanes 3 and 4). This is less than the increase in the control gene, GAPDH (glyceraldehyde 3-phosphate dehydrogenase) gene which has been thought to be expressed constitutively throughout the cell cycle, as demonstrated previously. Thus, normal H3.3 expression was observed in transfected cells. (The smaller bands may represent the endogenous rat H3 histone mRNA because their amino acid sequences are conserved pretty well.)

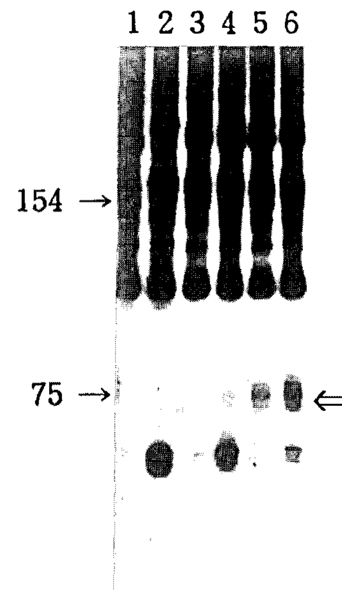


**Fig. 2.** Expression of transfected chicken H3.3 histone gene in Rat 3 cells. Fifty micrograms of RNA and 1000 u/ml S1 nuclease were used in all assays. Samples were assayed with a fragment of the H3.3B gene (Fig. 1) labeled at PvuII site. RNAs tested were: Untreated Rat 3 cells, quiescent (lane 1) or serum-stimulated (lane 2); Rat 3 cells transfected with the chicken H3.3 gene, isolated cell lines 2-1 (quiescent, lane 3; serum-stimulated, lane 4) and 2-2 (quiescent, lane 5; serum-stimulated, lane 6) (Arrows: size marker, open arrow: protected band)

**Expression of a chicken H3.3 histone gene without introns**

To test whether the introns in the chicken H3.3 histone gene are responsible for the constant expression of the gene, a chicken H3.3 histone gene without introns was made from a H3.3 cDNA clone and the genomic H3.3 clone, pBH6b-2.3, both of which were made from a plasmid pUC19. Because one EcoR V site each is located in the first (leader) exon and the last exon of the gene, the internal EcoR V fragment of pBH6b-2.3 was cut out and replaced by the analogous cDNA EcoR V fragment. The resulting plasmid, pBH6b ΔI (Fig. 1), is exactly the same as pBH6b-2.3 except for the absence of all three introns. After the cotransfection and HAT selection, cells were grown to confluence, incubated in 0.1% serum-containing media for 2 days, and stimulated with serum. Total RNAs were prepared from cells at 0, 6, and 12 hours after the stimulation. Since the S1 nuclease analysis in the previous experiment (Fig. 2) gave too much background, we performed RNase protection assay by using a uniformly labeled RNA probe that was transcribed *in vitro* from pBS(-)-H3.3 (Fig. 1). We expected a protected fragment of 175 bases long. Results of RNase protection assay using one isolated cell line and one mass culture are shown in Fig. 3. These cells expressed the H3.3 gene without introns at almost the same rate at 0, 6, and 12 hours after the serum stimulation (lane 1, 2, and 3 for the mass culture and lanes 4, 5, and 6 for the isolated cell line, respectively). Lane 7 is a negative control that contains RNA from Rat 3 cells and lane 8 is a positive control represented by anemic chicken red cell RNA. A densitometer scanning indicated that there was less than 20% fluctuation in the amount of the H3.3 mRNA when the cell went from $G_0/G_1$ phase to the later stage of S phase. Replication-dependent chicken H3 histone gene usually shows about 8~10 fold increase during S phase. This result suggests that the introns in the chicken H3.3 histone gene are not responsible for the constant expression of the gene throughout the cell cycle (The larger bands in lanes 4 through 8 may be caused by the secondary structure of the probe).

Seiler-Tuyns and Patterson (Seiler-Tuyne *et al*., 1986) showed that the introduction of artificial introns (globin gene intron) could eliminate the cell cycle regulation properties of a replication dependent histone gene. Wells and Kedes (Wells *et al*., 1985) also suggested that the differences in the structures of replication-dependent and replication-independent histone gene are responsible for their expression patterns. According to our results, however, elimination of introns from the replication-independent histone gene did not change its expression pattern. This is contradictory to previous reports. Our result obviously shows that the introns in the replication independent H3.3 histone gene are not responsible for their constitutive expression throughout the cell cycle. There could be two other regions of the gene that could be involved in its expression: Promotor region and 3'-end region. Either one or both may be responsible for its expression pattern by controlling the transcription rate and/or the mRNA stability. In addition, there also exists a possibility that the coding region may be involved. We'll pursue these possibilities in the near future.

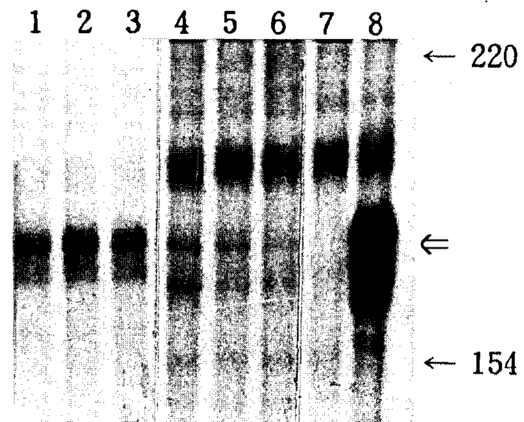


**Fig. 3.** Expression of a chicken H3.3 histone gene without introns. One mass culture (lanes 1-3) and one isolated cell line (lane 4-6) were grown from Rat 3 cells transfected with pBH6bΔI. Total RNAs were isolated at 0 h (lanes 1 and 4), 6 hr (lanes 2 and 5) and 12 h (lanes 3 and 6) after the stimulation. Thirty micrograms of RNA were used in each RNase protection assay. The probe used is shown in Fig. 1. Lane 7 represents RNA from stimulated Rat 3 cells (negative control) and lane 8 represents RNA from anemic chicken red cells (positive control) (Arrows: size marker, open arrow: protected band).

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