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Role of the Promoter Region of a Chicken H3 Histone Gene in Its Cell Cycle Dependent Expression

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We fused the promoter region of an H3.2 chicken histone gene, whose expression is dependent on the cell cycle, to the 5′ coding region of an H3.3 chicken histone gene, which is expressed constitutively at a low level throughout the cell cycle. This fusion gene showed a cell cycle-regulated pattern of expression, but in a different manner. The mRNA level of the fusion gene increased during the S phase of the cell cycle by about 3.7-fold at 6 h and 2.7-fold at 12 h after the serum stimulation. The mRNA level of the intact H3.2 gene, however, increased by an average of 3.6-fold at 6 h and 8.7-fold at 12 h. This different expression pattern might be due to the differences in their 3′ end region that is responsible for mRNA stability. The 3′ end of the H3.2 mRNA contains a stem-loop structure, instead of a poly(A) tail present in the H3.3 mRNA. We also constructed a similar fusion gene using a H3.3 histone gene whose introns had been eliminated to rule out the possibility of involvement of the introns in cell cycle-regulated expression. The expression of this fusion gene was almost identical to the fusion gene made previously. These results indicate that the promoter region of the H3.2 gene is only partially responsible for its expression during the S phase of the cell cycle.

Keywords: Cell cycle regulation, Histone, Promoter, Transcriptional regulation.

Introduction

Histones are a complex family of proteins responsible for organizing the nucleosomal structure of eukaryotic chromatin. The five major classes of histone proteins, based on their electrophoretic mobility, are encoded by multiple copies of genes in all species examined. In higher eukaryotes, there are a number of nonallelic variants of each histone protein which are encoded by distinct histone genes organized in clusters (Isenberg, 1979). Histone variants can be classified according to their expression characteristics. Most histone genes and mRNAs from various organisms have common structural features, including the absence of introns and the presence of a 3′ terminal stem-loop structure in place of the usual polyadenylated terminus (Hentschel and Birnstiel, 1981; Maxon *et al.*, 1983; Marzluff and Graves, 1984). The expression of these replication-dependent histone genes is coupled to DNA synthesis and is coordinately regulated at both the transcriptional and post-transcriptional levels (Birchmeier *et al.*, 1983; Price and Parker, 1984; Son, 1993). The mRNAs for these variants begin to accumulate near the G1-S border of the cell cycle, reach maximum levels at mid-S phase, and then rapidly decline as DNA synthesis is completed (Alterman *et al.*, 1984; Artishevsky *et al.*, 1984). In addition to these replication-dependent histones, there are histone variants expressed constitutively at a low level throughout the cell cycle as well as in quiescent and terminally-differentiated cells. Although these replication-independent histones constitute only 5–10% of histone protein synthesis during the S phase, they are the predominant histones synthesized in nondividing cells. The genes that encode these histones somehow escape cell cycle regulation (Wells and Kedes, 1985). One of the replication-independent variants is the chicken H3.3 histone gene, which has introns and encodes polyadenylated mRNA (Brush *et al.*, 1985).

Despite similar modes of expression during the cell cycle, no principal homologies 5′ to the coding sequences of all replication-dependent histone genes are found except for the TATA box and CCAAT box. But a few histone gene families have subtype-specific, *cis*-acting elements responsible for their own replication-dependent expression,

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the H1 box and the octamer sequence of H2B as examples (Perry *et al.*, 1985; Osley *et al.*, 1986). But no such sequences have been found for H2A and H3 histone genes. We had previously cloned two chicken H3 histone genes and tested their pattern of expression. They were both replication-dependent histones. Then, we sequenced their promoter regions and compared them to reveal no particular homologies or subtype-specific sequences (Son and Tae, 1994). We also tested the replication-independent chicken H3.3 histone gene. One of the most noticeable structural differences between the replication-dependent H3.2 histone genes we cloned and the replication-independent H3.3 histone gene is the presence of introns in the H3.3 gene. We eliminated all introns from the H3.3 histone gene and tested its mode of expression during the cell cycle by a DNA-mediated gene transfer experiment (Son and Hong, 1997). Its mRNA was synthesized and maintained constitutively at a low level throughout the cell cycle. This proved that the introns in the H3.3 histone gene are not involved in its expression pattern. In that report, we raised two other possibilities that might contribute to their different patterns of expression according to the cell cycle between the H3.2 and the H3.3 genes. One was their promoter regions and the other was their 3′ end regions. DNA sequences in their promoter regions are totally different and their 3′ end regions are different in that the H3.2 gene produces mRNA with a stem-loop structure while the H3.3 gene produces mRNA with a poly(A) tail.

To confirm these possibilities, we constructed fusion genes that consisted of the promoter region of the H3.2 gene and the 3′ region of the H3.3 gene. We fused the promoter region of the H3.2 gene to the 5′ end of the coding region of either the original H3.3 gene or the intronless H3.3 gene we had made previously. These fusion genes showed cell cycle-dependent expression. They were expressed similarly at a higher level during the S phase, but not as much as the H3.2 gene. This means that the promoter region of the H3.2 gene is partially responsible for its expression during the S phase. Furthermore, their patterns of expression were different from that of the H3.2 gene. The mRNA levels of the fusion genes were elevated early in S phase and slightly decreased late in S phase, while that of the H3.2 gene was continuously increased during S phase. This result might be due to the differences in the stability of the mRNAs. Since mRNAs of the H3.2 gene are considered to be more stable because of the stem-loop structure at the 3′ end, they can accumulate late in the S phase. We hope to confirm this possibility in the near future. From this experiment, it can be concluded that the promoter region of the H3.2 histone gene is not totally responsible for its elevated expression during the S phase. Increased mRNA levels of the H3.2 gene are partially due to the promoter region and possibly due to the stem-loop structure at its 3′ end.

Materials and Methods

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

DNA transfection and HAT selection The transfection protocol has previously been described (Son and Hong, 1993). Twenty-four h before transfection, Rat 3 cells were plated to a density of 5×10^5 cells per 100 mm tissue culture plate. Approximately 1 μg of a plasmid containing human TK cDNA and 10 μg of histone plasmid were ethanol-precipitated along with 10–20 μg of high molecular weight carrier DNA (Rat 3 DNA). The DNA was resuspended in 4.5 ml of sterile double-distilled water (ddH_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, 50mM Hepes, 1.5 mM Na_2HPO_4, pH 7.05–7.15). The DNA-calcium phosphate precipitate was allowed to form for 20~30 min at room temperature. One ml of this mixture was added to a 100 mm plate containing 10 ml of the medium. After about 16 h, 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 to other plates.

RNA isolation Total RNA was isolated from transfected cells as previously described (Choi *et al.*, 1997).

RNase protection assay A DNA fragment of pCH1a-H2.7 digested with *Pvu*II and *Bam*HI containing the 5′ portion of the H3.2 histone gene and some of its flanking region (Fig. 1) was cloned into a Bluescript vector (obtained from Stratagene, La Jolla, USA) 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 using essentially the same procedure as Lee *et al.* (1999). The reaction mixture included transcription buffer (40 mM Tris·Cl, pH 8.0; 10 mM $MgCl_2$; 2mM 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[^{32}P]rUTP, and 10 units

of T3 RNA 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 min. 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 ddH_2O. The labeled RNA transcript was mixed with the RNA isolated from Rat 3 transformants and both were ethanol-precipitated. The pellet was then 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 min to denature both RNAs, then allowed to hybridize at 55°C for 12–16 h. Following the hybridization, 300 μl of RNase buffer (0.3 M NaCl; 10 mM Tris; 5 mM EDTA, pH7.5) containing RNase A (40 μg/ml) and RNase T1 (20 μg/ml) were added, and the reaction was incubated at 37°C for 1 h. The RNase digestion was terminated by the addition of 20 μl of 10% SDS and 50 μl of 10 mg/ml proteinase K, followed by an additional incubation at 37°C for 15 min. 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. In parallel control experiments, we used the rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene, whose mRNA levels are consistent throughout the cell cycle, but the data is not shown.

Densitometric analysis Since it is critical to compare the level of mRNA of interest in different stages of the cell cycle, an LKB2222-010 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

There are a couple of convenient restriction sites that are useful in making chicken H3 histone fusion genes. A *Pvu*II site and a *Pst*I site are located at exactly the same sites relative to the coding regions of both genes at the 20th codon and at the 93rd codon, respectively. The H3.3B chicken histone gene on pBH6b-2.3 as described by Brush *et al.* (1985) and the H3.2 gene on pCHla-H2.7 were used to make fusion gene pFHR-1.6, and the intronless H3.3B gene on pBH6bI and the same H3.2 gene were used to make another fusion gene, pFHR ΔI. A 1.05 kb fragment of pBH6b-2.3 cut with *Bam*HI and *Pvu*II was replaced by a 0.55 kb fragment of pCHla-H2.7 cut with *Bam*HI and *Pvu*II (Fig. 1). The *Bam*HI site is located on the vector and cannot be seen in this figure. Note that this 0.55 kb fragment contains about 330 bp of plasmid pBR322 vector DNA, 130 bp of the 5′ flanking region of the H3.2 gene, and 87 bp of the 5′ untranslated and coding regions of the gene. The newly constructed plasmid, pFHR-1.6, contains

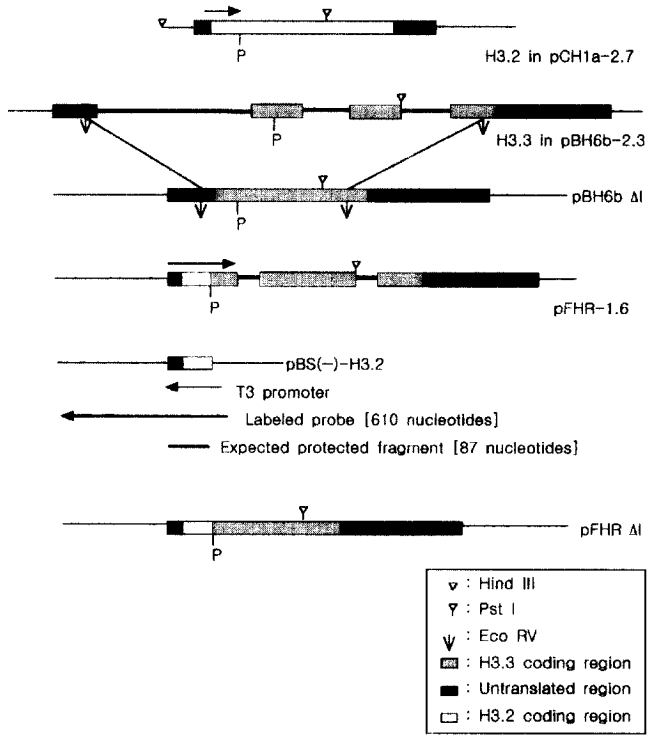

Fig. 1. Restriction map of fusion genes.

the 5′ flanking region and the first 20 amino acid codons from the H3.2 histone gene and the remainder (from codon 21) of the H3.3B gene. The same approach was applied to make a plasmid pFHR ΔI which is exactly the same as pFHR-1.6, except that pFHR ΔI does not contain any introns from the H3.3 gene. Each plasmid was cotransfected with the human TK cDNA into Rat 3 cells and the transfected cells were selected in HAT medium. Three different mass cultures for each plasmid were grown to confluence and kept in HAT medium containing 0.1% calf serum for 48 h. Total RNAs were then prepared from cells at 0, 6, and 12 h after serum stimulation. Because Rat 3 cells enter the S phase between 6 to 8 h after serum stimulation, these three time points should indicate the expression pattern of the gene at the RNA level. The 0.55 kb fragment of pCHla-H2.7 cut with *Bam*HI and *Pvu*II was inserted into the multiple cloning site of the pBS vector to make a probe for RNase protection assays. Using pBS(-)-H3.2 cut with *Bam*HI, a uniformly-labeled probe of 610 nucleotides was made (Fig. 1). (Because the *Pvu*II-cut end of the fragment was ligated to the *Eco*RV site in the pBS vector which is 60 bp downstream from the transcription initiation site, the probe is 60 nucleotides longer than the 0.55 kb fragment.) This probe should be able to protect RNA from the H3.2 gene transcription initiation site to the *Pvu*II site, which is 87 nucleotides in length. The result of the RNase protection assay of the three different mass cultures transfected with pFHR-1.6 and other three mass

cultures transfected with pCH1a-H2.7 indicated that in all cases the expression of this fusion gene or the intact H3.2 gene was stimulated during the S phase of the cell cycle (Fig. 2), but their expression patterns were different. The mRNA levels of the H3.2 gene at 6 h (lanes 2, 5, and 8) and at 12 h (lanes 3, 6, and 9) increased by an average of 3.6-fold and 8.7-fold, respectively, compared to 0 h levels (lanes 1, 4, and 7). Meanwhile, mRNA levels of the fusion gene pFHR-1.6 at 6 h (lanes 11, 14, and 17) and 12 h (lanes 12, 15, and 18) increased by an average of 3.7-fold and 2.7-fold, respectively, compared to 0 h levels (lanes 10, 13, and 16). Similar results were obtained from the RNase protection assay of the three different mass cultures transfected with pFHR ΔI. An average 3.9-fold and 3.1-fold increase in mRNA levels was observed at 6 h and at 12 h, respectively (Fig. 3). Exogenous fusion gene mRNAs were induced at 6 h after stimulation or even earlier. This means that the 220 bp H3.2 fragment at the 5′ end of the fusion gene is, at least, partially responsible for the cell cycle regulation of the gene, perhaps due to transcriptional regulatory elements in the 130 bp of the 5′ H3.2 flanking region present in this construct. One thing that is noticeable in this experiment is that the mRNA level of the fusion genes at 6 h was a little higher than that at 12 h after stimulation. It is generally considered that 5′ regions of the cell cycle-dependent histone genes are responsible for regulation at the transcriptional level and the 3′ regions are responsible at the post-transcriptional level. Because these fusion genes contain the 5′ region of the H3.2 histone gene and the 3′ region of the H3.3 histone gene, the transcriptional stimulation may begin slightly prior to the S phase, but is normally ineffective since mRNA with an H3.3 3′ end is unstable until sometime further into the S phase.

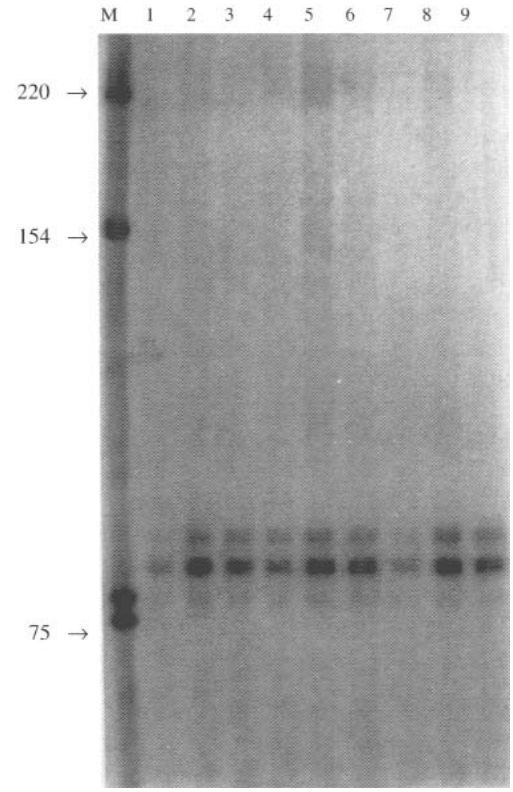

Fig. 3. Cell cycle regulation of a fusion gene pFHR ΔI. Three mass cultures (D-MA, lanes 1–3, D-Mb, lanes 4–6, and D-Mc, lanes 7–9) were grown after Rat 3 cells were transfected with pFHR ΔI. Total RNAs were isolated at 0 h (lanes 1, 4, and 7), 6 h (lanes 2, 5, and 8), and 12 h (lanes 3, 6, and 9) after stimulation. See Fig. 2 for details.

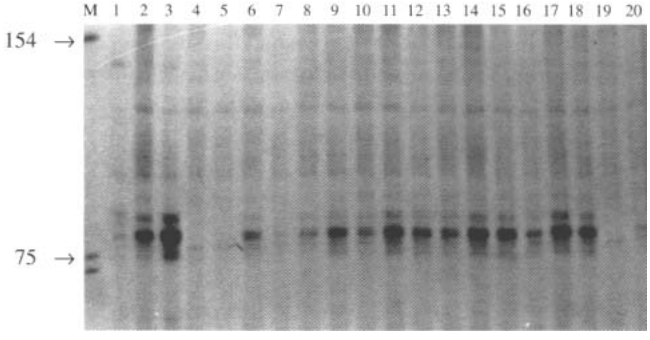

Fig. 2. Expression of the chicken H3.2 histone gene and its fusion gene mRNAs. Three mass cultures were grown from Rat 3 cells cotransfected with pCH1a-H2.7 (3-Ma, lanes 1–3, 3-Mb, lanes 4–6, and 3-Mc, lanes 7–9) or with pFHR-1.6 (C-Ma, lanes 10–12, C-Mb, lanes 13–15, and C-Mc, lanes 16–18). Total RNAs were isolated at 0 h (lanes 1, 4, 7, 10, 13, and 16), 6 h (lanes 2, 5, 8, 11, 14, and 17), and 12 h (lanes 3, 6, 9, 12, 15, and 18) after stimulation. Thirty micrograms of RNA was used in each RNase protection assay with a probe made from pBS(-)-H3.2, shown in Fig. 1. Lane 19 represents RNA from stimulated Rat 3 cells as a negative control and lane 20 represents RNA from anemic chicken red cells as a positive control.

Discussion

During the S phase there is a 15-fold increase in the levels of histone mRNA in HeLa cells resulting from both an increased rate of RNA synthesis and a lengthening of the half-life of histone mRNAs. To determine the degree to which transcription and post-transcriptional processes are responsible for histone mRNA accumulation, Heintz *et al.* (1983) measured both the rates of the synthesis and the half-life of histone mRNA during or in the absence of DNA synthesis. Quantitation of nascent histone mRNA synthesized during a 5-min *in vivo* pulse-labeling suggested that the rate of histone mRNA synthesis is 3- to 4-fold higher in cells at the point of maximal rate of accumulation of histone mRNA (2.5 h into S phase) than cells blocked at the G1/S boundary. Their studies of the half-life of the histone mRNA after a block in DNA synthesis (8 min) or during S phase (40 min) suggested

that the stability of histone mRNA might be increased as much as 5-fold during DNA synthesis. Transcription rate measurement indicated that the triggering of histone mRNA synthesis occurred in late G1 phase at a point prior to initiation of DNA replication, and that this mRNA was synthesized at its maximal rate 3 to 5 h before its peak of accumulation (Hereford *et al.*, 1982; Artishevsky *et al.*, 1984). Our results are in good accordance with these published results, because the transfected Rat 3 cells started to move into the S phase at 6 h and the peak of DNA synthesis seemed to be around 12 h. The mRNAs of the fusion genes or intact H3.2 gene were induced before 6 h after stimulation, and their levels at 12 h were different according to their 3' regions which should control their stability. Currently, we are constructing H3.3 genes with 3' stem-loop structure from the H3.2 gene.

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