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Transcription of the Histone H5 Gene Is Not S-Phase Regulated

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Levels of the tissue-specific linker histone H5 are elevated in mature erythroid cells as compared with levels in dividing cells of the same lineage. We examined levels of H5 mRNA in relation to the cell cycle in early erythroid cells transformed by avian erythroblastosis virus to determine whether the gene for this unusual histone is S-phase regulated. Northern blotting analyses revealed that during the cell cycle steady-state levels of H5 mRNA remained relatively constant in contrast to levels of the major core and H1 mRNAs which increased approximately 15-fold during S phase. In vitro pulse-labeling experiments involving nuclei isolated from synchronized cells at various stages of the cell cycle revealed that transcription of the H5 gene was not initiated at any particular stage of the cell cycle but was constitutive. In contrast, transcription of the H2A gene(s) initiated in early S phase, was present throughout the DNA replicative phase, and was essentially absent in G1 and G2 phases.

The H5 histone gene is unusual in several ways. It is a single-copy gene which is not closely linked to the clustered core and H1 genes in the chicken genome (23). It is expressed only in cells of the erythroid lineage (27, 29), and its mRNA is polyadenylated (22). H5 proteins gradually replace H1 histones during maturation of erythroid cells, although fully mature erythrocytes still contain significant levels of H1 proteins. In this work we investigated the regulation of H5 gene transcription in relation to other chicken histone genes during the cell cycle.

A large body of evidence indicates that synthesis of core and H1 histones is coincident with DNA synthesis during the cell cycle (11, 35), although Groppi and Coffino (17) showed that histone synthesis in S49 mouse lymphoma cells can also occur in G1. Nevertheless, the general consensus is that histone protein synthesis is temporally coupled to DNA replication to facilitate the coordinated assembly of nucleosomes.

The controlling factors governing histone protein levels in eucaryotic cells are not clearly understood. The molecular cloning of histone genes from a range of eucaryotes has made experiments investigating the content and metabolism of the major histone (H1, H2A, H2B, H3, and H4) mRNA species possible. Studies with synchronous populations of tissue culture cells obtained by treatment with aphidicolin (19) or hydroxyurea (38), serum deprivation (9), use of cell-cycle mutants (2), and centrifugal elutriation (1) have shown that levels of the major histone mRNA species increase by approximately 10- to 20-fold during S phase. Furthermore, treatment of synchronized cells in S phase with inhibitors of DNA synthesis rapidly reduces levels of the major histone mRNAs (3, 16, 34), indicating a coupling between histone gene expression and DNA replication. Further data indicate that elevated histone mRNA levels in S phase result from an increased rate of transcription and increased mRNA stability (1, 19, 38).

We show in this study that these parameters are not valid for the histone H5 gene.

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MATERIALS AND METHODS

Suppliers. Aphidicolin was a generous gift from the Natural Products Division, National Cancer Institute. All radiolabeled precursors were obtained from Biotechnology Research Enterprises S. A. Pty., Ltd., except for [^{3}H]thymidine which was purchased from New England Nuclear Corp.

Recombinant DNA clones. All chicken histone gene recombinants were isolated and characterized in this laboratory. For core and H1 histone genes see reference 8; for H5 gene sequences see references 22 and 23.

Cell culture and synchronization. Cells used in this study were kindly provided by T. Graf (Institute for Virus Research, Heidelberg, Federal Republic of Germany). Designated ts34 A6 L1 (LSCC HD2), but abbreviated in this paper as ts34, the clonal cell line consisted of early chicken erythroblasts transformed by avian erythroblastosis virus (AEV-RAV 2) (5, 15). Extensive studies of cell-specific markers such as surface antigens, heme, hemoglobin, carbonic anhydrase, and H5 (4, 5, 12) characterized the cell line as early erythroid. Specifically, the HD2 subline was chosen for this study because it is incapable of differentiating to erythrocytes at elevated temperatures (42°C), even though other ts34 cell lines can be induced to do so under special culture conditions (4).

AEV-transformed ts34 cells were grown in Dulbecco modified Eagle medium (GIBCO Laboratories) supplemented with 10% fetal calf serum (Flow Laboratories) and 2% chicken serum (Commonwealth Serum Laboratories). Synchronization of cells (approximately 2×10^6/ml) at the G1/S boundary was achieved by the addition of aphidicolin (5 μg/ml in dimethyl sulfoxide) for 20 h. Cells were released from the cell cycle block by washing three times in fresh media (minus serum) at 37°C and were then resuspended (2×10^6 cells per ml) in fresh medium plus serum.

DNA synthesis. Relative rates of DNA synthesis were monitored by the incorporation of [^{3}H]thymidine in trichloroacetic acid (10%, wt/vol)-precipitable material. The percentage of nuclei labeled with [^{3}H]thymidine was monitored by autoradiography as described by Rogers (37).

Extraction of cytoplasmic RNA. Cells were washed three

times with ice-cold phosphate-buffered saline before lysis in buffer containing 10 mM Tris hydrochloride (pH 8.0), 10 mM EDTA, 10 mM KCl, and 0.5% (vol/vol) Nonidet P-40. Lysates were centrifuged for 10 min at 5,000 × *g* at 4°C, and the supernatants were phenol-chloroform extracted and then reextracted with chloroform. After ethanol precipitation, the RNA pellet was suspended in water and stored as an ethanol precipitate at −20°C.

Agarose gel electrophoresis and Northern blot hybridization. Cytoplasmic RNA (10 μg) from ts34 cells was suspended in deionized formamide and heat denatured in the presence of deionized glyoxal. Samples were electrophoresed through 1.5% agarose gels at 3 to 4 V/cm, and resolved RNA species were transferred to GeneScreen membrane filters. Prehybridization, hybridization, and washing were done as recommended by New England Nuclear Corp. Nick-translated probes were prepared by the method of Maniatis et al. (24).

Isolation of nuclei. Cells were washed three times with ice-cold phosphate-buffered saline and were lysed in Nonidet P-40 lysis buffer. Lysates were then layered over 2 volumes of 0.6 M sucrose containing 0.5% (vol/vol) Nonidet P-40 and centrifuged for 10 min at 500 × *g* at 4°C. Nuclei were suspended at 3×10^8/ml in storage buffer (50 mM Tris hydrochloride [pH 7.9], 25% glycerol, 0.1 mM EDTA, 10 mM 2-mercaptoethanol, 5 mM $MgCl_2$), snap frozen in liquid nitrogen, and stored at −80°C as described by Alterman et al. (1).

In vitro transcription and RNA isolation. Nuclear transcription reactions were performed as described by Marzluff (25). Briefly, in vitro labeling of nuclear RNA was carried out by incubating nuclei (1.3×10^8/ml) in a 150-μl volume which contained 50 mM Tris hydrochloride (pH 7.9), 1 mM $MnCl_2$, 5 mM $MgCl_2$, 1 mM dithiothreitol, 1 mM ATP, 0.5 mM GTP, 0.5 mM CTP, 0.15 M KCl, and 100 μCi of [α-^{32}P]UTP for 15 min at 25°C. The reaction was terminated by the addition of sodium dodecyl sulfate to 0.5% (vol/vol) and adjusted to 100 mM NaOAc (pH 5.2)–1 mM EDTA. The mixture was phenol-chloroform extracted, brought to 0.2 M NaOAc (pH 5.2), and the RNA was ethanol precipitated. Incorporation of radiolabel into RNA species was determined by the number of counts present in 10% (wt/vol) trichloroacetic acid-insoluble material. All in vitro transcription reactions were carried out in duplicate.

DNA filter hybridization. Single-stranded M13 DNA (ssM13, 5 μg), with plus or minus H2A or H5 histone gene inserts, was heated for 5 min at 100°C, cooled on ice, and spotted onto GeneScreen filter membranes mounted in a Schleicher and Schull dot blot manifold. Aliquots of DNA were filtered by gravity after the filter membranes had been initially washed in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate). Filters were then washed twice under suction with 6× SSC and baked at 80°C for 2 to 3 h.

Prehybridization of filters was carried out overnight at 52°C in a mixture containing 50% (vol/vol) deionized formamide, 5× SSC, 10 mM Tris hydrochloride (pH 7.5), 1 mM EDTA, 0.1% sodium dodecyl sulfate, 10 μg of denatured *Escherichia coli* tRNA per ml, 0.4% polyvinylpyrrolidone, and 0.4% Ficoll (38). The filters were then hybridized with 3×10^6 cpm of the appropriate RNA in the prehybridization mixture for 36 h at 52°C. Filters were washed twice at room temperature for 10 min in 2× SSC–0.1% sodium dodecyl sulfate followed by two 2-h washes at 65°C in 0.5× SSC–0.1% sodium dodecyl sulfate.

Hybridization of labeled RNA to filter-bound ssM13 (with insert) was carried out three times and detected by autoradiography. Subsequent quantitation of the amount of RNA bound to dots was made by scintillation counting, and pooled data were expressed as the mean ± the standard error of the mean after background counts from minus-strand insert dots had been subtracted. Background levels of hybridization (minus strand) never exceeded 20% of the H5/H2A plus-strand signal.

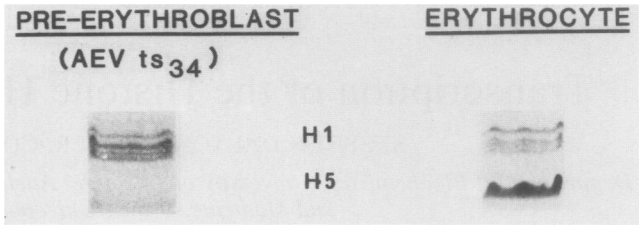

FIG. 1. Analysis of H5 (lower band) and H1 (upper bands) levels in dividing AEV ts34 cells and in mature erythrocytes by electrophoresis through a Triton X-100–acetic acid–urea–polyacrylamide gel. Levels of protein loaded were chosen such that the amount of H1 protein in each track was equivalent. H5 is barely detectable in the preparation from AEV ts34 cells.

Isolation and electrophoresis of histones. Isolated nuclei were suspended in 2 M NaCl and agitated gently for 30 min at 4°C. HCl was added to a final concentration of 0.25 M, and agitation was continued for an additional 30 min. The nuclear lysate was centrifuged at 15,000 rpm in a Beckman JA20 rotor for 10 min at 4°C, and the supernatant was dialyzed against bidistilled H_2O and freeze-dried. Protein preparations were suspended in water (2 mg/ml) and stored at −20°C. Histones from ts34 cells and mature erythrocytes were electrophoresed on Triton X-100–acetic acid–urea–polyacrylamide gels (6 mM Triton X-100, 5% acetic acid, 6 M urea, 15% acrylamide) essentially as described by Zweidler (42) followed by staining with amido black.

RESULTS

H5 levels in dividing and nondividing erythroid cells. Levels of H5 in erythroid cells increase during maturation, displacing a significant proportion of H1 histone (29). We compared H1 and H5 histones from ts34 cells and mature erythrocytes by gel electrophoresis (Fig. 1). The protein loads were chosen so that the amount of H1 protein in each track was approximately equivalent. Clearly, the ratio of H5 to H1 in erythrocytes was much greater than in dividing ts34 cells in which H5 was barely detected. The factors which contribute to elevated H5 levels in erythrocytes are likely to be associated at least in part with the rate of H5 gene transcription and with H5 mRNA stability. The results reported here address these factors.

ts34 cell synchronization with aphidicolin. The ts34 cell line used in these studies was an AEV-transformed early erythroid cell line which expresses the H5 gene in addition to the major H1 and core histone genes. Previous attempts to effectively synchronize these cells with hydroxyurea and thymidine or by isoleucine starvation were unsuccessful. The successful application of the antibiotic aphidicolin in synchronizing HeLa cells (19, 32) suggested this approach for ts34 cells. With a single application of aphidicolin and subsequent release of ts34 cells from the block, over 90% of the cells were found to enter S phase in synchrony (Fig. 2).

After addition of aphidicolin (5 μg/ml) to randomly growing, unsynchronized cells, incorporation of [^{3}H]thymidine into DNA rapidly decreased to a minimal level within 1 h

(Fig. 2). Throughout the period of aphidicolin exposure (20 h), the basal rate of DNA synthesis remained relatively constant. The effects of the antibiotic on DNA synthesis were shown to be clearly reversible after the washing procedure by a sharp increase in [^{3}H]thymidine incorporation. Pulse-labeling data indicated that the S phase proceeded for approximately 6 to 7 h after which the rate of DNA synthesis was close to the prerelease rate (Fig. 2). In addition, the increase in the rate of DNA synthesis was paralleled by an increase in the percentage of [^{3}H]thymidine-labeled nuclei indicating that a high degree of cell synchronization was achieved.

Analysis of H5 mRNA levels during the ts34 cell cycle. To monitor the steady-state levels of specific histone mRNA species during the cell cycle, cytoplasmic RNA was prepared from cells before, during, and after treatment with aphidicolin. RNA (10 μg) was resolved electrophoretically, and analysis of the individual histone mRNAs was carried out by Northern blot hybridization. An H2A probe was used routinely as an indicator of S-phase-controlled histone gene expression, and results for H5 are therefore shown in relation to H2A. In other experiments, expression from H1 and other core histone genes behaved in the same way as expression from H2A. Relative amounts of histone mRNA species detected on autoradiographs were quantitated by densitometric scanning. Results are expressed as relative increases over prerelease mRNA levels (Fig. 3).

It is apparent that there were dramatic increases in the steady-state levels of H2A mRNA as the cells progressed through S phase (Fig. 3A). Immediately after release from the aphidicolin block, H2A mRNA levels increased reaching a maximal steady-state level 3 to 4 h postrelease. At this stage there was approximately 15-fold more H2A mRNA present than before release or after S-phase completion (8 h postrelease). After the peak in histone mRNA accumulation in S phase, levels declined in parallel with DNA synthesis as monitored by [^{3}H]thymidine incorporation, reaching a minimum level at 6 to 7 h postrelease. Increases in the accumulation of H1 and H2B (Fig. 3B) and H3 and H4 (data not shown) mRNAs during S phase were similar to that observed for H2A mRNA, indicating that the major histone genes are

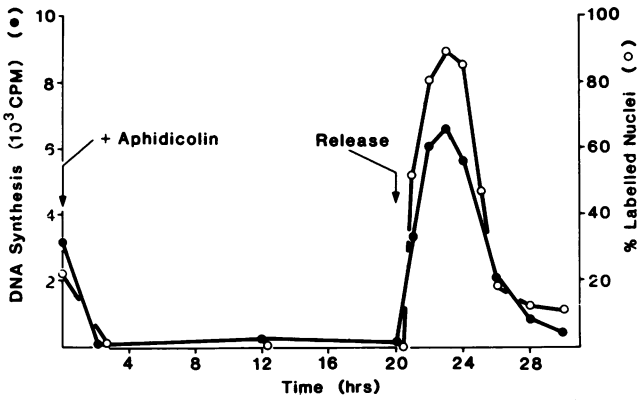

FIG. 2. Kinetics of DNA synthesis in AEV ts34 cells. Where indicated, aphidicolin (5 μg/ml) was added to randomly growing cultures of cells and was removed by washing in fresh media after 20 h. Cells were pulse-labeled with [^{3}H]thymidine for 30 min. The rate of radioactivity incorporation into trichloroacetic acid-insoluble material, indicating the rate of DNA synthesis, and the percentage of labeled nuclei, indicating the percentage of cells in S phase, are shown.

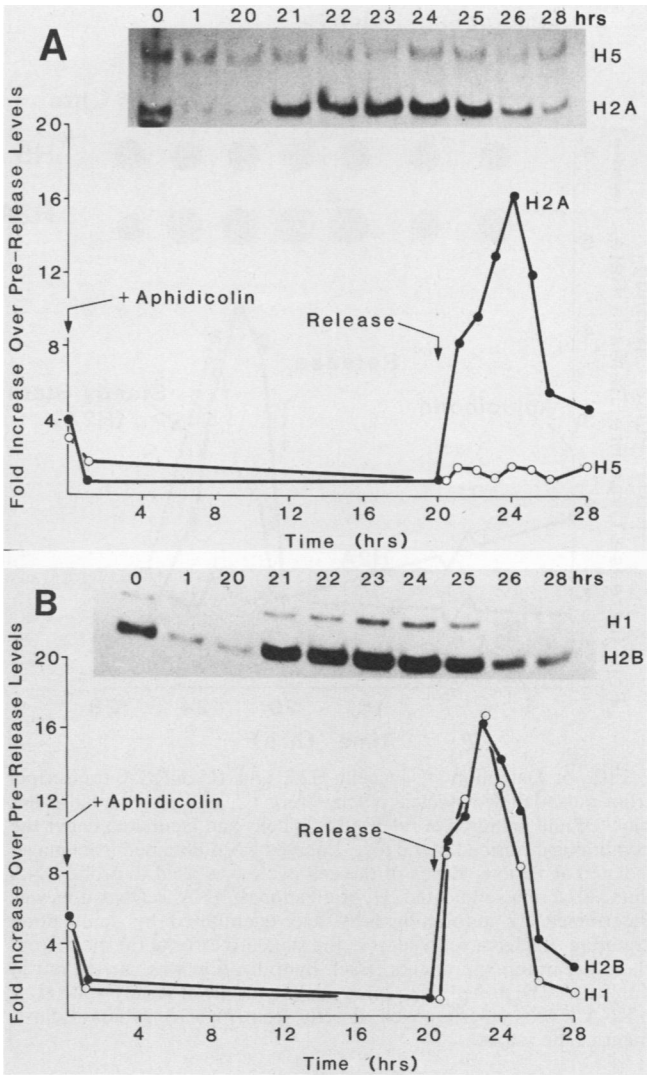

FIG. 3. Steady-state levels of individual histone mRNAs during the cell cycle. Cytoplasmic RNA samples (10 μg) prepared at the times indicated were resolved on 1.5% agarose gels after denaturation, and levels of different histone mRNAs were detected by Northern blot hybridization with specific gene probes. Densitometric quantitation of the autoradiograms shown in each panel is also represented. (A) Symbols: ●, H2A mRNA; ○, H5 mRNA. (B) Symbols: ●, H2B mRNA; ○, H1 mRNA.

S-phase regulated. In contrast, H5 mRNA levels did not change significantly after release from the aphidicolin block. The results illustrate the difference between an S-phase-regulated histone gene (H2A) and the H5 gene (Fig. 3A). Clearly, no distinct accumulation of H5 mRNA occurred after release from the aphidicolin block. Furthermore, when synchronized ts34 cells were allowed to progress through another cell cycle after aphidicolin release, H5 mRNA levels remained constant in G1, S, and G2, whereas H2A mRNA levels were elevated during S phase only (data not shown). Therefore, H5 gene expression appears to be independent of DNA synthesis (and probably other stages of the cell cycle).

Transcription of H5 and H2A genes. Although results presented thus far show that levels of H5 mRNA do not significantly fluctuate during the cell cycle, it has still not been determined whether transcription of the H5 gene is initiated at a particular stage of the cycle. One possibility is

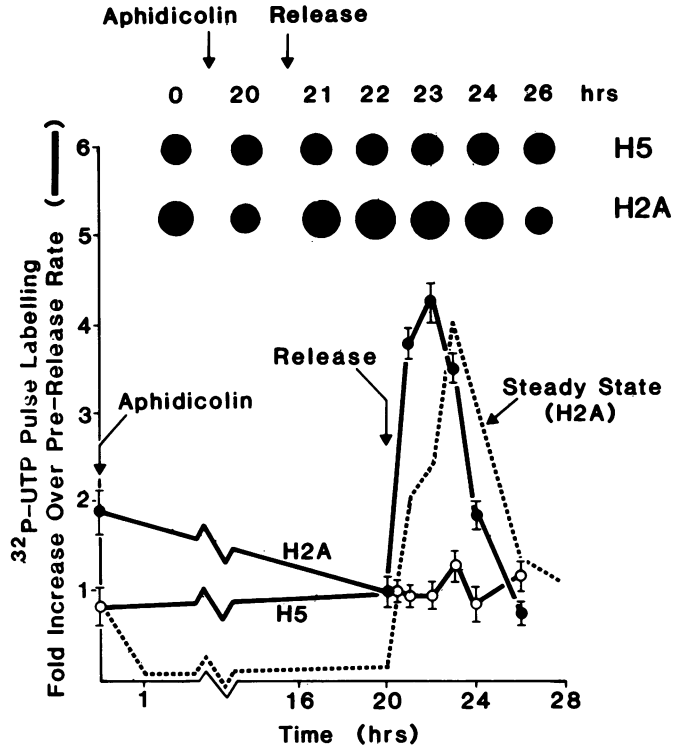

FIG. 4. Detection of nascent H2A and H5 mRNA transcripts from pulse-labeled nuclei. Nuclei were isolated from aphidicolin-blocked and aphidicolin-released ts34 cells and incubated under the conditions described in the text. Labeled RNA obtained from nuclei isolated at various stages of the cell cycle was used to probe DNA dots at the times indicated. Hybridization of RNA to DNA dots was determined by autoradiography and quantitated by scintillation counting. Each bar represents ± the standard error of the mean from three separate experiments. Each hybridization was carried out in duplicate. The dotted line represents the profile of steady-state H2A mRNA levels superimposed directly from Fig. 3A without adjustment of the *y* axis.

that H5 mRNA synthesis is initiated in S phase but that the polyadenylated transcripts are stable throughout the cell cycle, unlike the situation for other histone genes. To test this hypothesis, nuclei were prepared from aphidicolin-treated and aphidicolin-released (non-S-phase and S-phase, respectively) cells and pulse-labeled with [^{32}P]UTP. Labeled nascent nuclear transcripts were used to probe filters containing ssM13 with plus or minus inserts of H5 or H2A genes. As enhanced rates of transcription during DNA replication make a major contribution to the accumulation of histone mRNA from S-phase-regulated genes (16, 19, 38), it was of particular interest to see if the same was true for the H5 gene. Hybridization of labeled nuclear RNA to filter-immobilized ssM13 with inserts was detected by autoradiography and quantitated by scintillation counting of individual dots.

From the autoradiograph (Fig. 4) it is evident that transcription of H2A genes was relatively low during aphidicolin exposure, but after reversal of the effect of the antibiotic, a rapid increase in levels of nascent transcripts occurred. Maximum levels of nascent H2A transcripts occurred 1 to 2 h postrelease and thereafter declined for the duration of the cell cycle monitored. The maximal rate of H2A gene transcription therefore occurred before the peak steady-state level of H2A mRNA accumulation (Fig. 4, dotted line). This is consistent with previous observations involving transcription of the major histone genes in HeLa cells (19), mouse lymphoma cells (38), and hamster fibroblasts (2). Only background levels of radioactivity were detected on dots of ssM13 with inserts non-complementary to labeled message (data not shown). From these results it is evident that initiation of H2A gene transcription occurred in early S phase. Analysis of hybridization between labeled RNA and ssM13 with an H5 insert complementary to mRNA indicated that levels of nascent transcripts were similar in nuclei isolated throughout the cell cycle (Fig. 4). This apparent pattern of constitutive transcription contrasts to that of H2A and indicates that the H5 gene is not transcriptionally activated in coordination with DNA replication.

DISCUSSION

The cell cycle regulation of histone gene expression has been extensively studied in yeast cells (30), HeLa cells (19, 34), and mouse erythroleukemia cells (1). These investigations demonstrated that a distinct temporal coupling exists between DNA synthesis and histone gene expression. In our work, by analyzing steady-state mRNA levels at various stages of the cell cycle and by analyzing nascent RNA transcription, we have shown that expression of the H5 gene is not regulated in coordination with DNA replication. The cell line specifically chosen for these studies (see Materials and Methods) does not have the capacity to differentiate to nondividing erythrocytes even under ideal conditions (4). The cells contain H5 protein, an early marker for avian erythroblasts (5), but globin was not detected by radioimmunoassay (4), and the cells had been through many doublings in culture. Taken together, these facts render it extremely unlikely that the H5 mRNA detected in our experiments arose from a subpopulation of cells which was not under cell cycle control.

The mechanisms which activate core and H1 gene transcription in S phase do not apply to the H5 gene. Although absolute levels of H5 transcripts were not obtained, a comparison of RNA synthesis from H5 and H2A genes by pulse-labeling isolated nuclei clearly showed obvious differences in the activities of these genes during the cell cycle. It seems likely that the H5 gene is transcribed constitutively throughout the cycle and that this together with H5 mRNA stability (in comparison to core and H1 mRNAs), which is a result of its polyadenylated state, contributes to the dramatic increase in H5 protein levels during erythroid cell maturation (Fig. 1).

The evidence presented in this paper highlights the differences between the previously studied cell-cycle-regulated major core and H1 genes and the H5 gene, the expression of which is not temporally coupled to DNA replication. The mechanisms by which histone genes are expressed during S phase are not fully understood. Specific sequences which are proximal to histone genes and which facilitate the binding of transregulatory molecules present during S phase may be involved. A number of such regulatory factors operate in the expression of non-S-phase-regulated genes including those for simian virus 40 early promoter (13), β-globin (10), *Xenopus* 5S RNA (39), heat shock (31, 41), and RNA polymerase I (26). In addition, this idea is supported by the work of Heintz and Roeder (18) who demonstrated that a soluble factor(s) present in S phase nuclear extracts, but absent in non-S-phase extracts, can stimulate histone gene transcription in vitro. These factors would presumably not apply to the H5 gene.

Another means of controlling the periodic expression of histone genes could involve the dynamic three-dimensional structure/arrangement of the genes themselves. Attachment of histone genes or nearby regions of DNA to the nuclear matrix could possibly facilitate this. Recently, specific adenine-plus-thymine-rich nucleotide sequences between the H1 and H3 genes in the repeating histone gene units of *Drosophila* were found to be associated with the nuclear scaffold (28). This is a particularly interesting observation in view of other results which indicate that transcriptionally active β-globin (20), ovalbumin (6, 36), and vitellogenin II (21) genes are always associated with the nuclear matrix. Furthermore, fixed sites of DNA replication have been proposed which involve DNA replication complexes being anchored to the matrix. It therefore seems reasonable to speculate that attachment of histone genes to the nuclear scaffold may be involved in, or necessary for, their S-phase activation. This possibility is currently being explored.

A possible link between specific sequences, histone gene expression, and DNA replication was provided by Osley and Hereford (30) who demonstrated that deletion of a DNA sequence 3′ to a yeast H2B gene resulted in loss of S-phase regulation of the adjacent H2A gene. This deleted fragment is thought to contain an autonomously replicating sequence (*ars*) which has been implicated as an origin of DNA replication. Moreover, the S-phase-regulated *CDC9* gene (the structural gene for DNA ligase) in *Saccharomyces cerevisiae* has a sequence with weak *ars* activity located approximately 3 kilobase pairs upstream from the cap site (33).

Although the H5 gene has some recognizable remnants of H1 histone gene structure (7), we recently described a 5′ element which is ubiquitous to all H1 genes (H1 box) but is absent from the H5 gene. Intriguingly, this 10-base-pair sequence is completely homologous to an *ars* sequence of yeast (our unpublished data). Furthermore, a number of chicken histone gene clusters that were tested contained weak to moderate *ars* activity in yeast cells (G. Pure, personal communication), whereas this was not the case for a 16-kilobase DNA fragment containing the H5 gene. The lack of this sequence near the H5 gene may explain the absence of S-phase control. Evolution of the H5 gene from the H1 gene(s) may have resulted in the loss of putative elements involved in S-phase regulation and the concomitant gain of sequences responsible for erythroid-specific expression.

H5 histone has the capacity to displace H1 molecules from internucleosomal DNA and to condense chromatin (40). If H5 (and its mammalian counterpart H1° [14]) appeared only postmitotically in differentiating cell lines, a direct causal relationship between the presence of this histone and the cessation of DNA synthesis could be implied. However, H5 certainly appears in early erythroblasts which undergo a number of further cell divisions before maturation as erythrocytes (4). H1° is also present in dividing cells in vivo (14). These unusual histones accumulate to higher levels in nondividing cells, and their major biological role may be to condense chromatin in maturing cell lines so that sites for gene transcription or for origins of DNA replication are inaccessible for the enzymes and other factors involved in these processes. If this is the case, H5 (or H1°) synthesis is not required to maintain the normal nucleosome structure of dividing cells, and thus synthesis need not be linked to S phase. Instead, stable H5 mRNA is constitutively synthesized, and the stable H5 protein accumulates finally to preclude further RNA or DNA synthesis.

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