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Regulated expression of mammalian histone H4 genes *in vivo* requires a *trans*-acting transcription factor

(cell cycle/histone gene regulation)

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ABSTRACT **Mouse L cells containing integrated copies of a human histone H4 gene have been obtained by cotransfection with the herpesvirus thymidine kinase gene. Nuclease S1 assays of RNA from several independent cell lines show that the expression of the introduced H4 gene is regulated during the cell cycle. One of these cell lines (line 6-8) contains more than 60 human H4 gene copies per haploid genome and does not express the endogenous mouse histone H4 mRNA. In contrast, the expression of the mouse H2a and H3 mRNAs in this cell line is not perturbed. In cell revertants that have lost the majority of the human H4 gene copies, the expression of the mouse H4 mRNA is restored, demonstrating that the mouse genes remain functional although not expressed. The rate of transcription of the histone H4 genes in clone 6-8 is at least 10-fold greater than that of the parental cell line and it is regulated during traversal of the cell cycle. These results show that the expression of mammalian histone H4 genes involves both a *trans*-acting transcriptional regulatory factor and an H4-specific activity. We propose that cell cycle regulation of histone gene expression may be effected through subtype-specific transcriptional regulatory proteins.**

The expression of most histone gene variants is coupled to DNA synthesis during the eukaryotic cell cycle (1–4). The steady-state concentration of these histone mRNAs increases ≈20-fold during the S phase, because of both an increased rate of synthesis and a decreased rate of decay (4). Our interest is in defining the molecular mechanisms controlling these levels of regulation of histone gene expression. In initial studies, the expression of a human histone H4 gene was analyzed (4, 5) because its complete nucleotide sequence is known and has been shown to encode a major cell cycle-regulated H4 mRNA in HeLa cells (6, 7). In addition, Heintz and Roeder (8) have demonstrated that this gene is maximally transcribed *in vitro* in nuclear extracts from synchronized S-phase cells. Furthermore, Hanly *et al.* (9) have shown that efficient transcription in the S-phase extracts requires distal promoter elements between 70 and 110 nucleotides upstream from the H4 initiation site. These results indicate the involvement of both an H4-specific transcription factor and distal promoter elements in the *in vitro* transcription of this gene and suggest that these components may be important for cell cycle regulation of this gene *in vivo*. To determine whether this is indeed the case, we have initiated a series of studies of the regulation of the H4 gene *in vivo* after transfection into cultured mammalian cells.

Preliminary experiments in which this gene was introduced into mouse L cells and assayed during transient expression indicated that the human H4 gene is accurately transcribed in mouse cells and, in agreement with previous *in vitro* results (9), that maximal expression requires the promoter elements distal to the H4 gene (data not shown). Similar transient-expression assays have been used to demonstrate accurate transcription of a mouse histone H4 gene in monkey COS cells (10). However, this type of transient assay cannot be used to definitively demonstrate that the exogenously introduced gene is transcriptionally regulated during S phase. To investigate whether such regulation can occur, we have established mouse L-cell lines that carry the pHu4A histone H4 gene integrated into their genomes. In this study, we show that the integrated H4 genes can be transcriptionally regulated during the cell cycle and that this regulation requires an H4-specific *trans*-acting factor.

MATERIALS AND METHODS

Cell Culture and Synchronization. Mouse LTK$^-$ (thymidine kinase-deficient) cells were grown in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum. Synchronization at the G_1/S boundary was achieved by using indomethacin and aphidicolin in a double-blocking protocol. Cells at $\approx 2.5 \times 10^4$ per cm^2 were arrested in G_1 by growth in 0.4 mM indomethacin for 24 hr (11) and released from the block by two washes in fresh medium. Three hours later they were reblocked with aphidicolin at 5 μg/ml (12). The cells were allowed to collect at the G_1/S boundary for an additional 12 hr and were released into S phase by washing three times with fresh medium. DNA synthesis was routinely monitored after release from the aphidicolin block by incorporation of [^{3}H]deoxycytidine into DNA (13). Cells were considered synchronous when incorporation of this precursor increased at least 15-fold after release from the aphidicolin block, and when the duration of increased incorporation was <9 hr.

Cell Transfection and Selection. DNA was transfected into cells by the procedure of Graham and van der Erb (14), as modified by Wigler *et al.* (15), using 20 ng of pHSVTK106, 2 μg of pHu4A, and 18 μg of high molecular weight LTK$^-$ cell genomic DNA as carrier. TK$^+$ clones were selected in medium containing hypoxanthine, aminopterin, and thymidine (HAT medium) (16). TK$^-$ revertant clones were selected by growth in 5-bromodeoxyuridine (BrdUrd) after a single passage in the absence of aminopterin.

Isolation of RNA, Nuclease S1 Mapping, Southern Blot Hybridization, and Nuclear Run-on Transcription Assays. These were done according to established procedures (7, 17, 18). Quantitation of autoradiographic bands was done with a Beckman DU-8 spectrophotometer equipped with a gel scanning accessory. Multiple exposures of each film were separately quantitated to ensure accuracy.

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Abbreviations: TK, thymidine kinase; HAT, hypoxanthine/aminopterin/thymidine; BrdUrd, 5-bromodeoxyuridine.

RESULTS

Preparation and Analysis of Murine Cell Lines Containing the pHu4A Human Histone H4 Gene. We have employed the mouse LTK$^-$ cell line as recipient in this study for the following reasons: cotransfection with the herpesvirus TK gene provides a positive selection for introduction of the H4 gene, as well as the possibility of subsequently selecting for loss of the integrated genes in the presence of bromodeoxyuridine (19); our preliminary studies indicated that L cells can be effectively synchronized by use of indomethacin and aphidicolin; and it is possible to simultaneously measure the expression of the endogenous mouse and integrated human H4 genes by using a single probe in the nuclease S1 mapping procedure.

Seventeen individual TK$^+$ cell lines were isolated after cotransfection of the herpes TK and human H4 genes and selection in HAT medium. Fig. 1 shows nuclease S1 mapping of total RNAs isolated from a representative subset of cell lines. The endogenous mouse histone H4 mRNA protects the human DNA probe from the labeled 3′ end (produced by *Bst*EII digestion) (7) through the conserved coding region of the mouse mRNA and results in three major protected fragments (Fig. 1*B*, lanes 13 and 14). It seems probable that these bands represent distinct mouse H4 mRNA species that are protected to various extents by the human H4 probe, since increasing the nuclease S1 concentration does not change the relative intensities of these bands. The introduced homologous human H4 mRNA, as expected, protects a unique DNA fragment that includes both the coding sequences and the 3′ noncoding region of the human mRNA. Densitometric quantitation of the data from the experiment represented in Fig. 1*B* revealed that histone H4 mRNA concentration in these cell lines can vary dramatically. One particularly interesting cell line (6-8; see lanes 3 and 4) expresses very high levels of the exogenously introduced histone H4 mRNA and exceedingly small amounts of the endogenous mouse histone H4 mRNAs. Furthermore, the total histone H4 mRNA concentration in these cell lines varies by a factor less than two, suggesting that, as in the case of yeasts (20), mammalian histone mRNA production may be dosage-compensated.

To assess whether there is a correlation between the concentration of human H4 mRNA and the copy number of the introduced genes in these cells, we have measured the number of integrated human H4 genes in each cell line. As shown in Fig. 2*A*, Southern blot analysis using an enzyme (*Hin*dIII) that cleaves at a unique site in the pHu4A plasmid reveals that the introduced genes have been integrated into the genome mainly in a tandem array and that the copy numbers are highly variable. To accurately measure these copy numbers, "slot blot" hybridization analysis of the various clones was performed (Fig. 2*B*). Comparison of RNA S1 mapping and genomic DNA blots indicates that there is a general correlation between the number of human H4 genes integrated into the genome and the level of human H4 mRNA expressed, although there are some exceptions to this pattern. This becomes evident from Fig. 2 because the blot in *A* has been organized so that the individual clones express progressively more human histone H4 mRNA as one proceeds from clone 4-1 to clone 6-8. Furthermore, clone 6-8, in which the mouse H4 mRNA is minimally expressed, contains the largest number of integrated human H4 genes (≈60 copies

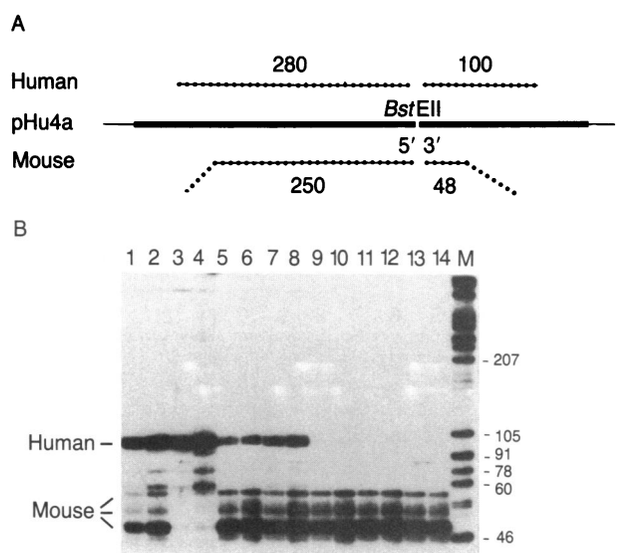

FIG. 1. (*A*) General strategy of nuclease S1 mapping of mammalian H4 histone mRNAs. Numbers on the left refer to the expected size of protected fragments from human (top) or mouse (bottom) H4 histone RNAs, when a 5′ end labeled probe is used. Numbers on the right refer to the expected size of protected fragments when a 3′ end labeled probe is used. (*B*) S1 mapping of total RNA from transfected cell lines. The probe used was *Bst*EII-digested pHu4a, labeled at the 3′ end. Each RNA sample was hybridized with two different amounts of probe, to show that the hybridization was performed with an excess of DNA. Lanes: 1 and 2, clone 6-3; 3 and 4, clone 6-8; 5 and 6, clone 6-9; 7 and 8, clone 8-2; 9 and 10, clone 8-3; 11 and 12, clone 8-4; 13 and 14, LTK$^-$ cells. Numbers at right refer to the length (in base pairs) of marker DNAs.

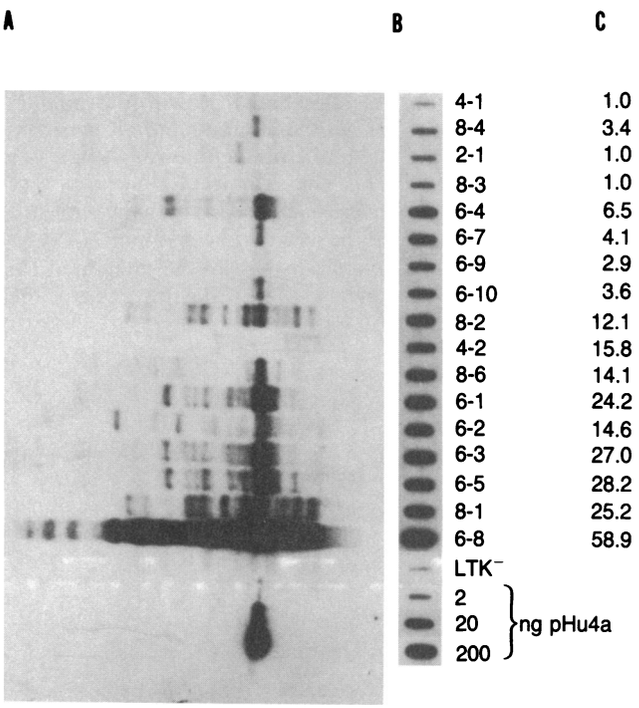

FIG. 2. Analysis of DNA from transfected cell lines. (*A*) High molecular weight DNAs were isolated from 17 TK$^+$ clones, digested with *Hin*dIII, and electrophoresed in a 0.8% agarose gel. After blotting to a nitrocellulose filter, DNAs were hybridized with a nick-translated probe prepared from the *Eco*RI–*Hin*dIII insert of pHu4a (7). DNA from clone 4-2 did not transfer completely to nitrocellulose filter, thus resulting in a weaker signal. (*B*) One microgram of each of the same DNAs was spotted on nitrocellulose filter and hybridized as in *A*. At right are the names of the cell lines that correspond to the spots and to the lanes in *A*. Control lanes and spots contained 2, 20, and 200 ng of the pHu4a insert DNA, corresponding to 1, 10, and 100 gene equivalents per microgram of genomic DNA, respectively. (*C*) Calculated copy numbers of integrated human H4 genes in each cell line per mouse haploid genome (see *Materials and Methods*).

per haploid genome). Additional Southern blot experiments using *Kpn* I, which does not cleave the pHu4a plasmid, resulted in a very high molecular weight series of bands containing the human gene which failed to be resolved in the agarose gel, providing additional evidence that these genes have integrated as a large tandem array (data not shown).

We next wished to examine whether the production of histone mRNA in each of these cell lines was regulated during the cell cycle. Unfortunately, our attempts to synchronize some of these cell lines have been unsuccessful. The reasons for this are not understood. However, several cell lines could easily be synchronized by the indomethacin/aphidicolin blocking procedure. Hence, we have analyzed the steady-state concentration of histone H4 mRNA in several of these clones as they traverse the cell cycle. As shown in Fig. 3, the concentrations of human and mouse histone H4 mRNA in these cell lines are directly proportional and increase during S phase. An exception to this general rule is provided by clone 6-3, in which the level of human H4 mRNA remains elevated relative to the endogenous mouse H4 message at the end of S phase. Although these results suggest that the integrated human genes may be correctly regulated in mouse cells, they do not address whether this regulation occurs at the transcriptional and/or posttranscriptional level.

Detailed Analysis of Clone 6-8. To determine whether the pHu4A histone gene is transcriptionally regulated in mouse L cells and to understand why the endogenous mouse H4 genes are not expressed in clone 6-8, we have analyzed this clone in detail. The first question we wished to answer was whether the endogenous mouse H4 genes are still functional in cell line 6-8 but are not expressed due to competition with the large number of copies of the human H4 gene. As demonstrated by Kucherlapati and co-workers (19), selection of TK^- revertant clones from TK^+ transfected cells by growth in bromodeoxyuridine frequently results in loss of both the TK gene and the cotransfected gene due to their integration at a single site in the genome. Hence, TK^- revertants of clone 6-8 were selected and five of them were analyzed for both the presence of the human H4 gene and the expression of H4 mRNA (Fig. 4). Three of these clones have lost all of the integrated H4 gene copies (Fig. 4*A*, lanes 4–6) and do not express the human H4 mRNA (Fig. 4*B*, lanes 7–12). In contrast, two of these revertants have retained several copies of the human H4 gene (Fig. 4*A*, lanes 3 and 7) and express low levels of the human histone mRNA (Fig. 4*B*, lanes 5, 6, 13, and 14). In all

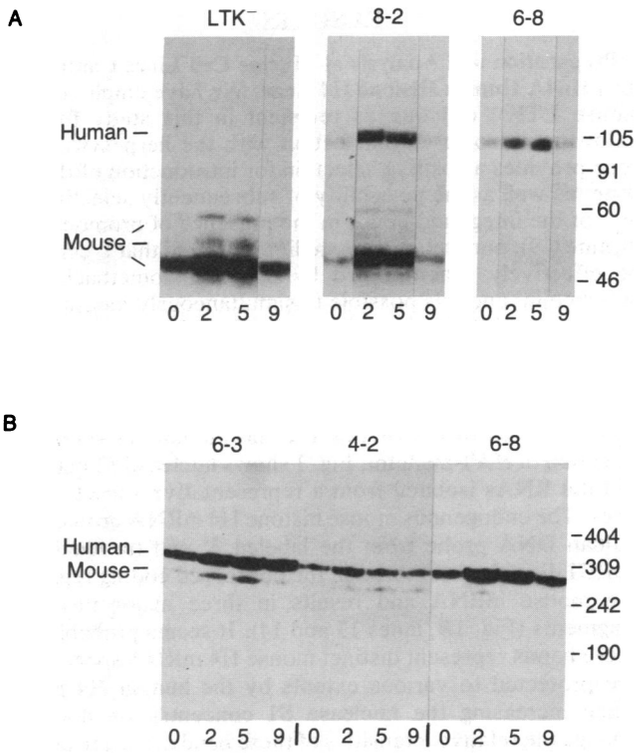

FIG. 3. Nuclease S1 mapping analysis of total RNA from synchronized cell lines. (*A*) RNA was hybridized with a 3′ end-labeled probe as in Fig. 1. (*B*) RNA was hybridized with a 5′ end-labeled probe (see Fig. 1*A*). Cell lines are indicated above the corresponding sets of lanes. Numbers below each lane refer to the time (in hr) after release of the cells from the G_1/S boundary. Numbers at right refer to the length (in base pairs) of marker DNAs.

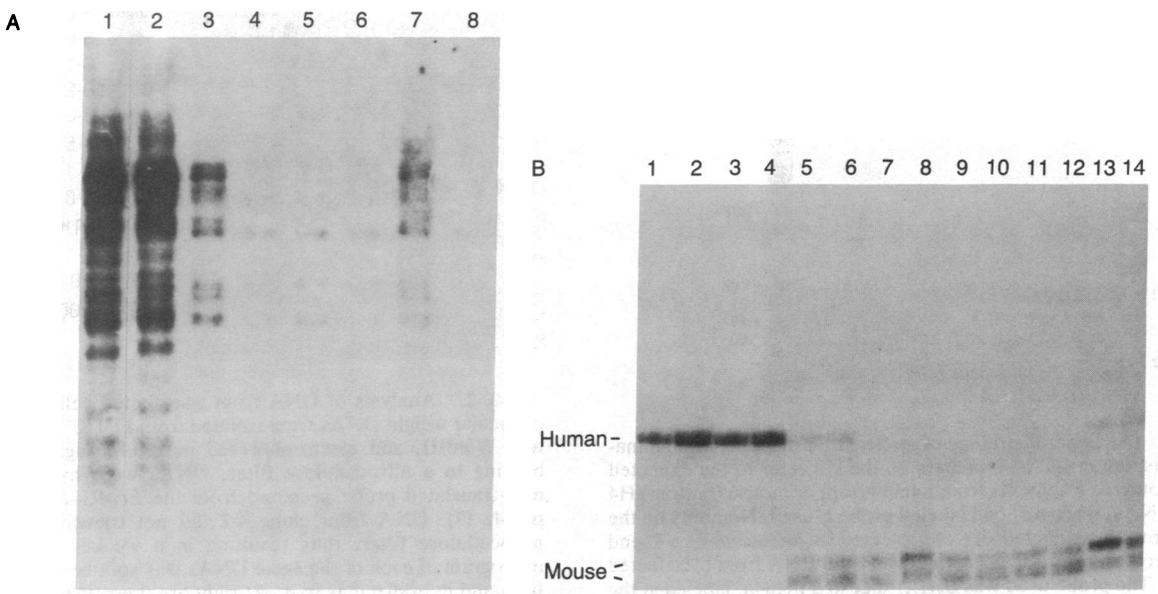

FIG. 4. (*A*) Southern blot analysis of DNA from revertant subclones of clone 6-8. DNA was analyzed as described in the legend to Fig. 2*A*. Lanes: 1, parental clone 6-8; 2, a HAT-resistant 6-8 subclone; 3–7, five independent BrdUrd-resistant 6-8 subclones; 8, LTK^- cells. (*B*) S1 mapping of RNA from BrdUrd-resistant subclones of clone 6-8. RNA from the same subclones analyzed in *A* was hybridized with 3′ end labeled pHu4a probe (see Fig. 1*A*). Each RNA sample was hybridized with two different amounts of probe. Lanes: 1 and 2, clone 6-8; 3 and 4, a HAT-resistant subclone; 5–14 BrdUrd-resistant subclones, in the same order as in *A*.

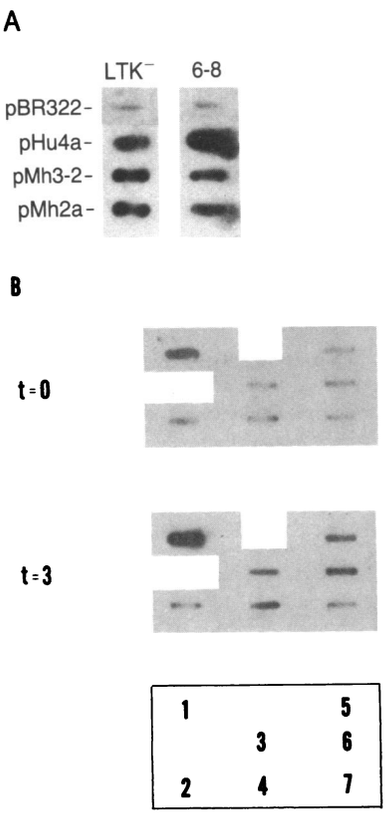

FIG. 5. Transcription rate of histone genes in clone 6-8. An equal amount (4.0×10^6 cpm) of RNA from nuclei that had been incubated with [^{32}P]UTP *in vitro* was hybridized with different plasmids (6 μg) spotted on nitrocellulose filters. (*A*) RNA was isolated from 6-8 and LTK$^-$ nuclei. pMh3-2 and pMh2a are plasmids carrying mouse H3 and H2a genes, respectively. (*B*) Nuclei were isolated from 6-8 cells either blocked at the G_1/S boundary (t = 0) or 3 hr after release from the block (t = 3). Positions of "slot blots" of plasmids are identified in the diagram at the bottom: 1, pHu4a; 2, pBR322; 3, plasmid carrying the mouse ornithine decarboxylase gene; 4, plasmid carrying the herpesvirus TK gene; 5 and 6, pHe7 and pHe27 [cDNA clones corresponding to two different abundant HeLa cell polyadenylylated RNAs (21)]; 7, 70-kDa heat shock protein cDNA clone (22). Nonrelevant portions of the autoradiograms have been removed.

of these clones, the endogenous mouse histone H4 mRNAs are expressed at normal levels. It is evident, therefore, that the endogenous mouse H4 genes in the 6-8 subclone remain functional but are not active. It is highly unlikely that the integration of the introduced genes into a particular site in the mouse genome is responsible for decreased levels of H4 mRNA, since revertant clones that retain some of the human H4 gene copies are able to express their endogenous H4 genes. Rather, these results suggest that the introduced human histone H4 genes compete with the endogenous mouse H4 genes for a factor that is required for H4 gene expression *in vivo*.

If these results reflect competition for a *trans*-acting transcriptional regulatory factor, then we would expect that the introduced human H4 genes should be transcribed at a very high rate relative to the mouse H4 genes in the parental LTK$^-$ cell line and that they should be transcriptionally regulated during the cell cycle. To determine whether this is the case, we have compared the rate of histone H4 gene transcription in the parental LTK$^-$ cell line and the 6-8 subclone by using the nuclear run-on transcription assay (17). Densitometric quantitation of the data shown in Fig. 5*A* indicates that the level of histone H4 transcription in the 6-8 cell line is at least 10-fold higher than that found in the parental LTK$^-$ cells. It is also evident from this experiment that the transcription of the mouse H2a and H3 genes is not affected by introduction of the human H4 genes. A similar nuclear run-on transcription assay was employed to ascertain whether the increased histone H4 transcription in clone 6-8 was regulated during the cell cycle. Comparison of the transcription of the H4 genes at the G_1/S boundary and 3 hr after release into S phase (Fig. 5*B*) clearly indicates that they are transcriptionally induced upon entry into S phase. Furthermore, densitometric scanning of these data indicates that the increase in transcription upon entry into S phase is $\approx$5-fold. This agrees very well with previous measurements of histone-gene transcriptional induction during the S phase of both murine and human cultured cells (4, 23). It is apparent, therefore, that the human histone H4 genes are both transcriptionally active and cell cycle-regulated in this mouse cell line. These results very strongly indicate that the expression of mammalian histone H4 genes *in vivo* requires a *trans*-acting transcriptional regulatory factor.

Neither the transcription rates (Fig. 5*A*) nor the steady-state concentrations (data not shown) of the mouse histone H2a and H3 mRNAs are perturbed in cell line 6-8. Thus, although the observed competition between the introduced and endogenous H4 genes is quite severe, it is specific to the H4 genes. There is present in mammalian cells, therefore, a subtype-specific activity that is involved in H4 gene expression.

DISCUSSION

We have analyzed the expression of a human histone H4 gene after introduction into the genome of mouse L cells. Measurement of the steady-state concentration and cell cycle modulation of the heterologous human histone mRNA in several independent cell lines indicates that expression of the introduced genes is largely independent of chromosomal position. In most cases the abundance of the heterologous human H4 mRNA is proportional to the endogenous mouse H4 message as the cells progress through the cell cycle. These results suggest that the mechanisms regulating histone mRNA abundance in mouse cells operate effectively on the introduced human H4 genes.

A detailed analysis of H4 gene expression in the clone 6-8, which contains $\approx$120 copies of the human H4 gene integrated at a single locus, leads us to several conclusions concerning cell cycle regulation of histone gene expression *in vivo*. First, that the introduced pHu4a histone H4 gene is transcriptionally induced upon entry into S phase demonstrates the role of *trans*-acting transcriptional regulatory proteins in the *in vivo* expression of this gene. Furthermore, the introduced H4 gene must carry all of the signals required for accurate transcription and regulation of this gene during the cell cycle. Thus, the regulatory sequences controlling expression of this gene *in vivo* are present within the 1.3 kilobase pairs of human genomic DNA contained within this plasmid. Second, although these experiments do not directly address whether regulation is dependent on integration next to an origin of DNA replication, it is clear that the regulated expression of this mammalian histone genes is not strictly dependent on chromosomal position. This is in contrast to the case in yeast, where the periodic expression of histone genes during the cell cycle requires close proximity to an origin of DNA replication (24). Third, the observed competition between the human and mouse H4 genes must reflect the presence of an H4-specific regulatory factor. Thus, the presence of the integrated human H4 gene copies in the 6-8 cell line severely depresses the expression of the endogeneous mouse H4 genes, although they are reexpressed in revertant cell lines that have lost the human H4 genes. Furthermore, the high rate of transcription of the human H4 genes has no effect on the expression of the mouse H2a and H3 genes. It seems likely, therefore, that the very high copy number of the

pHu4A histone genes in these cells can effectively sequester a limiting factor specific for histone H4 gene transcription. This interpretation is supported by the identification of subtype-specific conserved sequences in the 5′ flanking region of histone genes from a variety of organisms (25, 26). Alternatively, these results may reflect the presence of an H4-specific component in the posttranscriptional mechanisms regulating histone mRNA abundance. In either case, the fact that the mouse H4 mRNA level is depressed 20–30% in clone 6-3 (see Fig. 1*B*, lanes 1 and 2 and Fig. 2) suggests that the threshold for observing this competition is ≈30 pHu4A human histone H4 genes per mouse haploid genome.

The model for H4 histone gene regulation in mammalian cells that emerges from these studies is simply that transcription of these genes is dependent on a positively acting transcription factor whose activity is regulated during progression through the cell cycle. The processes that regulate the activity of this molecule remain obscure, although the tight and dynamic coupling of the expression of these genes to DNA synthesis suggests that a small molecular effector may be involved. We suggest, therefore, that the coordinate expression of replication-variant histone genes may be effected through a common mechanism for modulating the activity of subtype-specific transcription factors.

The *in vivo* results presented in this study strongly support the results concerning the regulation of histone H4 gene transcription *in vitro* (8, 9). In those studies, expression of the pHu4A gene in extracts from synchronized cells was shown to involve both a soluble transcription factor and distal elements in the H4 promoter. It seems quite reasonable to speculate that the histone H4 transcription factors detected by using these two very different approaches are identical. Demonstration of this identity will require precise localization of those sequences required for histone gene transcriptional regulation *in vivo*.

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