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Faithful cell-cycle regulation of a recombinant mouse histone H4 gene is controlled by sequences in the 3′-terminal part of the gene

(cell-cycle mutant/post-transcriptional regulation/3′ termini)

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ABSTRACT **We have analyzed the expression of endogenous histone H4 genes and of a newly introduced H4 gene in 21-Tb cells, a mouse mastocytoma cell-cycle mutant. Endogenous H4 mRNAs were less abundant by a factor of 120–180 in G_1-arrested than in exponentially multiplying cells. However, H4 transcription rates were only decreased by a factor of 3 under these conditions, as determined by *in vitro* elongation of nascent transcripts. This indicates that post-transcriptional control of histone mRNA levels is important, in accord with published data. We introduced a mouse H4 gene, modified by a 12-base-pair (bp) insertion in its coding sequence, into 21-Tb cells by DNA-mediated gene transfer. The levels of transcripts from this gene were regulated in parallel with those of the endogenous genes. Moreover, fusion of the simian virus 40 (SV40) early promoter to a 463-bp fragment containing the 3′-terminal half of the mouse H4 gene, including 230 bp of spacer sequences, led to the regulated expression of SV40/H4 fusion RNA. However, a small proportion of SV40-initiated transcripts were not processed to histone-specific 3′ ends, but extended farther through the downstream *Escherichia coli* galactokinase gene to a SV40 polyadenylylation site. In contrast to the short SV40/H4 RNA, the levels of these longer transcripts were not reduced in G_1-arrested cells. These results show that sequences in the 3′-terminal part of the H4 gene can regulate gene expression in the cell cycle, presumably at the post-transcriptional level, as long as they are not positioned much more distant from the terminus than normal.**

Histone biosynthesis in most eukaryotic cells is tightly coupled to nuclear DNA synthesis such that translatable histone mRNAs are present in significant amounts only during the S phase of the cell cycle (1–3). Recent studies using synchronized murine or human cells and homologous histone genes as hybridization probes have shown that histone mRNA steady-state levels vary over a 10- to 50-fold range, and similar variations were also observed in cells treated with inhibitors of DNA synthesis (4–9). Where analyzed, histone gene transcription varied only 2- to 5-fold (6–9), suggesting a major contribution of some post-transcriptional mechanism(s) to regulation of histone gene expression during the cell cycle.

We are interested in defining the genetic information responsible for cell-cycle regulation of histone gene expression. To this end, we have transformed a temperature-sensitive mouse mastocytoma cell-cycle mutant with a recombinant mouse H4 gene. We describe the faithful regulation of this gene and present evidence indicating that sequences in the 3′ part of the H4 gene can effectively control RNA metabolism during the cell cycle.

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MATERIALS AND METHODS

Cell Growth and Transformation. The K21 line of P-815-X2 mouse mastocytoma and its heat-sensitive cell-cycle variant 21-Tb (10, 11) were cultured in suspension in medium I (12) supplemented with 10% horse serum (Amimed, Basel, Switzerland). Before transfection, $\approx 10^6$ cells were immobilized on 3.5-cm plastic tissue culture dishes by treatment with concanavalin A (10). Transfections were performed by a modification (13) of the standard DNA/calcium phosphate coprecipitation technique (14, 15) or by protoplast fusion (16, 17). Transfected cells were selected for expression of the *Escherichia coli* xanthine guanine phosphoribosyltransferase (*gpt*) gene present on these plasmids as described (18). Several transformed cell lines were obtained in each experiment. These were either analyzed individually or pooled for further analysis.

Monkey COS-1 (19) and hamster R1610 (20) cells were grown in monolayers in Dulbecco's modified minimal essential medium supplemented with 5% fetal calf serum (Amimed) and transfected by DNA/calcium phosphate coprecipitation (13).

RNA Purification and Nuclease S1 Analysis. Total cellular RNA was isolated by hot acid phenol extraction (21). Approximately 45 μg of RNA was hybridized overnight at 52°C with 0.05 pmol of end-labeled DNA probe. Hybridization, S1 nuclease digestion, and gel analysis were performed by published procedures (22, 23).

Elongation of Nascent RNA in Isolated Nuclei. Nuclei were isolated according to Carneiro and Schibler (24) except that 0.05% Triton X-100 was used in the lysis buffer. Elongation reactions were done essentially as described by Schibler *et al.* (25), and the transcripts were extracted according to Vannice *et al.* (26), followed by precipitation with 10% trichloroacetic acid in the presence of 60 mM sodium pyrophosphate/2 mM GTP and a second precipitation with ethanol. Dot blot hybridizations to filter-bound plasmid DNAs were performed as described (24, 25, 27).

RESULTS

Regulated Expression of Endogenous H4 Genes in 21-Tb Cells. We analyzed the expression of endogenous H4 genes in a heat-sensitive cell-cycle mutant (21-Tb) that had originally been isolated from an undifferentiated P-815 murine mastocytoma line (11). These cells multiply at 33°C, but they become arrested at 39.5°C with a DNA content typical of G_1 phase (11). Within 24 hr of incubation at the nonpermissive temperature, the number of 21-Tb cells in S phase is virtually zero (10, 11). When G_1-arrested cells are returned to 33°C, DNA synthesis is resumed after a lag period of 8 hr and reaches maximal levels after 24–32 hr (28). The heat-sensitive

Abbreviations: gpt, xanthine guanine phosphoribosyltransferase; bp, base pair(s); SV40, simian virus 40; galK, galactokinase.

phenotype of 21-Tb cells has previously been exploited to study cell-cycle-dependent variations in thymidine kinase activity (28).

Steady-state levels of H4 mRNAs were assayed by S1 nuclease mapping of total RNA from 21-Tb cells that had been incubated at 33°C, or for 48 hr at 39.5°C followed by 0–24 hr at 33°C (Fig. 1). The hybridization probe was 5′-end-labeled at a *Tth*111I site located 238 base pairs (bp) downstream from the H4 transcription start site. Due to the presence of multiple expressed H4 genes in the mouse genome (29) and to the high degree of conservation of H4 coding sequences, the probe protects nonallelic H4 mRNAs to various positions near the start site of H4 translation. Thus, the protected bands shown in Fig. 1 reflect the levels of mRNA encoded by the specific H4 gene used as well as by these nonallelic H4 genes, respectively. The levels of all H4 mRNAs are similarly reduced in arrested 21-Tb cells as compared to exponentially growing cells (180-fold for the band protected by the specific transcripts and 120-fold for a group of other protected bands). Within 24 hr after return of G_1-arrested cells to the permissive temperature, H4 mRNAs return to almost the same levels as in exponentially growing cells. In fact, the kinetics of reappearance of H4 mRNAs are very similar to those determined for the relative numbers of DNA-synthesizing cells or for thymidine kinase enzyme activities (28).

We estimated the relative transcription rates of H4 genes in 21-Tb cells during a similar temperature-shift experiment by an *in vitro* transcription elongation reaction performed with isolated nuclei. Labeled *in vitro* transcripts (average size ≈200 nucleotides) were hybridized to dot blots of a plasmid recombinant, pMmH4Alu (29) containing essentially only H4 coding sequences. Control experiments showed that this recombinant, when used as a probe in RNA blot analyses, hybridizes exclusively to poly(A)$^-$ RNA of ≈400 nucleotides—i.e., to H4 mRNA (data not shown). Dots of pAcpr2 (a chicken actin cDNA clone; T. Gerster, D. Picard, and W. Schaffner, personal communication), pCIpr66 (a mouse H-2 histocompatibility cDNA clone; T. Gerster, D. Picard, and W. Schaffner, personal communication), and pBR322 were included as controls. Although actin and H-2 gene transcription varied somewhat after return of arrested cells to the permissive temperature, it becomes evident from the data presented in Fig. 2 that H4 gene transcription in arrested 21-Tb cells is about 1/3rd that in exponentially growing cells. Under the assumption that the *in vitro* elongation of nascent RNA chains is directly proportional to the transcriptional rate *in vivo*, these results indicate that the contribution of transcriptional control to the regulation of histone mRNA levels in 21-Tb cells is relatively unimportant.

Regulated Expression of a Recombinant H4 Gene in Transformed 21-Tb Cells. A 1796-bp *Eco*RI/*Bam*HI fragment containing the cloned mouse H4 gene (29) with 228 bp of 5′ flanking sequences and 1166 bp of 3′ flanking sequences was subcloned into a plasmid vector (pSVE) containing the *E. coli gpt* gene (18), to yield plasmid pSVEH4 (Fig. 3*A*). Separating the *gpt* and the H4 gene, there is a 614-bp fragment of bovine papillomavirus type 1 DNA containing a transcriptional enhancer element (31).

The functionality of the mouse H4 gene was tested by S1 nuclease mapping of RNA obtained 2 days after transfection with pSVEH4 of monkey COS-1 and hamster R1610 cells. Correct expression was clearly evident in COS-1 cells, but not in R1610 cells (data not shown). This was not surprising, because pSVEH4 contains the simian virus 40 (SV40) origin of replication (upstream of the *gpt* gene) and can therefore replicate to high copy number in T-antigen-producing COS-1 cells, but not in R1610 cells. H4 gene expression in R1610 cells became detectable only in transformed cell lines isolated by *gpt* selection (data not shown). These experiments proved that the cloned H4 gene was functional but indicated that expression was low in rodent cells and that cell-cycle regulation studies in mouse 21-Tb cells would therefore be best performed by using transformed cell lines.

To be able to distinguish the transcripts of the cloned H4 gene from those of the endogenous gene copy present in

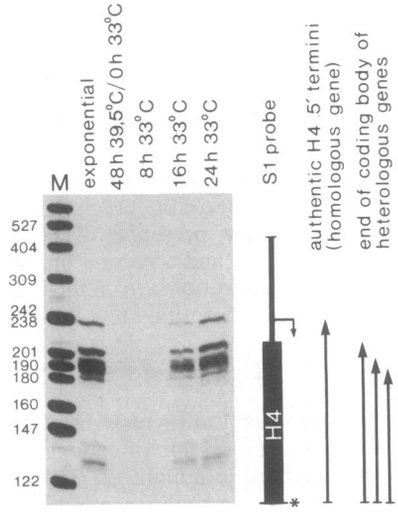
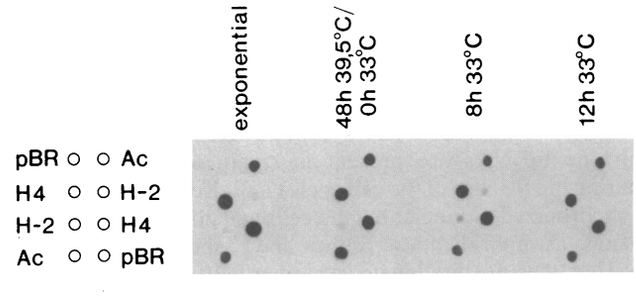


FIG. 1. Regulation of H4 mRNA levels in 21-Tb cells. Cells were incubated at 33°C (exponential) or for 48 hr at 39.5°C followed by 0–24 hr at 33°C; total cell RNA was prepared and hybridized to the ^{32}P-labeled DNA probe. Hybrids were treated with S1 nuclease and the protected DNA fragments were resolved on a sequencing gel. The hybridization probe was 5′-end-labeled at the *Tth*111I site within the H4 gene (see Fig. 3*A*). Transcripts from nonallelic H4 genes protect this probe only within the coding part, whereas mRNAs from this H4 gene protect it up to the cap site (arrow). *, Position of label on S1 hybridization probe; M, DNA size marker (length in nucleotides).



	exponential	48h 39.5°C/ 0h 33°C	8h 33°C	12h 33°C
H4/total cpm	2.8	1.0	1.2	1.5
H4/Actin	4.0	1.0 (1.6)	3.2	1.8
H4/H-2 Ag	3.3	1.0 (43.2)	0.5	1.5
Actin/H-2 Ag	0.9	1.0	0.2	0.8

FIG. 2. Analysis of H4 transcription rates in 21-Tb cells. Cells were incubated at 33°C (exponential) or for 48 hr at 39.5°C followed by 0–12 hr at 33°C, nuclei were prepared, and nascent RNA chains were elongated in the presence of [α-^{32}P]rGTP. The labeled transcripts were isolated and hybridized to denatured plasmid DNAs spotted on nitrocellulose filters. pBR, pBR322; H4, pMmH4Alu, a plasmid containing essentially only H4 coding sequences (29); H-2, pCIpr66, a mouse H-2 histocompatibility cDNA clone (T. Gerster, D. Picard, and W. Schaffner, personal communication); Ac, pAcpr2, a chicken actin cDNA clone (T. Gerster, D. Picard, and W. Schaffner, personal communication). The autoradiographic signals were quantitated by densitometric scanning. The indicated ratios have been normalized, setting the values for G_1-arrested cells (absolute values in parentheses) to 1.0.

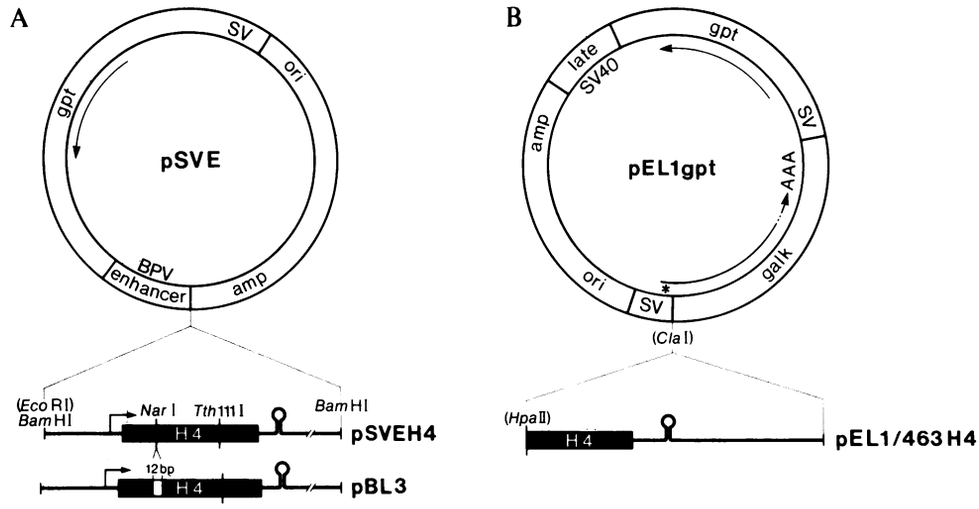


FIG. 3. Structure of plasmid recombinants. (*A*) Structure of pSVEH4 and pBL3. The extended 1796-bp histone DNA insert is drawn below the circle, representing the rest of the plasmid. The transcription start site (arrow) and the 3′-terminal palindrome representing part of the 3′ RNA processing signal are indicated. (*B*) Structure of pEL1/463H4 (30). The 463-bp histone DNA insert contains the last 166 bp of H4 coding sequence, the 3′ untranslated trailer sequence, and 232 bp of spacer sequence. Thick bar, H4 coding sequence; open bar, 12-bp insert; SV, origin of replication and early promoter of SV40; *, *Ava* I site used for 3′ end labeling of S1 nuclease hybridization probe (Fig. 5); ori, origin of replication of pBR322; amp, β-lactamase gene conferring ampicillin resistance; BPV, bovine papillomavirus.

mouse 21-Tb cells in S1 nuclease protection experiments, a 12-bp DNA sequence was introduced at the single *Nar* I site of pSVEH4, to yield plasmid pBL3 (Fig. 3*A*). The *Nar* I site is located 75 bp downstream from the H4 transcription start site and the resulting mutation preserves the H4 translation reading frame, to minimize possible alterations in RNA metabolism due to incomplete translation of the message.

21-Tb cells were transfected with pBL3 and H4 gene expression in individual, and presumably clonal, as well as in pooled *gpt*-transformed cell lines was measured by S1 nuclease protection analysis. Most if not all transformants expressed the H4 gene efficiently at 33°C (data not shown). The pooled transformants (Fig. 4) as well as two individual

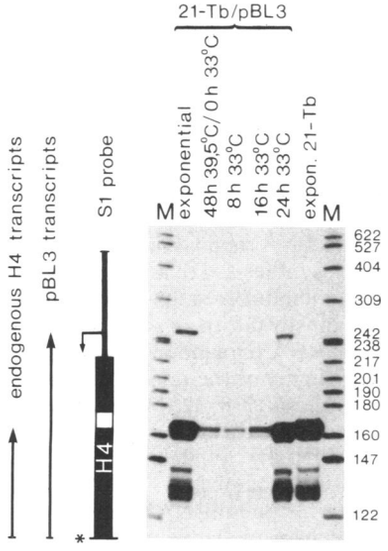


FIG. 4. Regulation of H4 mRNA levels in pBL3-transformed 21-Tb cells. The experimental protocol was the same as in Fig. 1. The hybridization probe was obtained from pBL3, which contains a 12-bp insertion at the *Nar* I site (open bar in Fig. 3*A*) and was 5′ end-labeled at the *Tth*111I site. Endogenous H4 transcripts protect this probe only up to the insertion site, whereas pBL3 transcripts protect it up to the cap site (arrow). *, Position of label on S1 hybridization probe; M, DNA size marker (length in nucleotides).

transformants (data not shown) were subjected to a temperature-shift experiment similar to those described above, and RNA was again isolated and subjected to S1 nuclease mapping. In all three experiments, the recombinant H4 gene was regulated at least as tightly as the endogenous H4 genes (Fig. 4). By densitometric scanning of the autoradiogram, the difference in pBL3-specific transcripts between exponential and G_1-arrested cells was estimated to be 980-fold. Thus, all the genetic information required for correct expression and cell-cycle-specific regulation of the H4 gene resides within the 1796-bp *Eco*RI/*Bam*HI fragment, and this regulation is not affected by the introduction of 12 bp at the *Nar* I site.

3′ Terminal H4 Gene Sequences Are Sufficient for Cell-Cycle Regulation. We have shown previously that histone mRNA-specific 3′ ends are generated from a 463-bp-long 3′-terminal mouse H4 gene segment if it is fused to the early promoter of SV40 and the fusion gene is introduced into hamster R1610 or human HeLa cells (30). To test whether this fusion RNA is subject to cell-cycle regulation, we introduced the recombinant plasmid pEL1/463H4 (Fig. 3*B*) into 21-Tb cells, selected *gpt* transformants, and analyzed RNAs obtained from these transformants during a temperature-shift experiment. An important feature of pEL1/463H4 is that the *E. coli* galactokinase (*galK*) gene followed by SV40 RNA splicing and polyadenylylation signals is positioned immediately downstream of the SV40/H4 fusion gene (Fig. 3*B*; ref. 30). Thus, any transcripts escaping histone-specific 3′ RNA processing and extending through the *galK* gene give rise to stable *galK* mRNA (30), which can serve as an internal control in the present regulation studies. As a hybridization probe that can be used to reveal both these RNAs, we constructed a deletion mutant of pEL1/463H4 in which the 3′ end of the *galK* gene is deleted and replaced by plasmid sequences. The S1 nuclease probe was a 3′-end-labeled DNA fragment extending from the SV40 leader segment to a position beyond the deletion point (Fig. 5). Approximately 10–20% of the hybridizing RNA from transformed 21-Tb cells incubated at 33°C extend beyond the deletion point in the *galK* gene, whereas the remainder are processed to authentic H4 mRNA 3′ ends (Fig. 5). This value is in good agreement with our previous findings in hamster R1610 or human HeLa cells (30). However, the RNA with histone-specific 3′ ends is virtually absent from G_1-arrested 21-Tb cells, whereas the

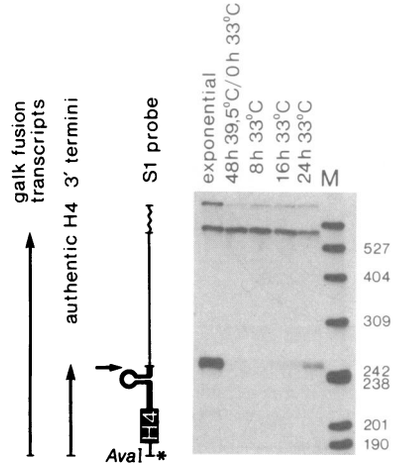


FIG. 5. Regulation of SV40/H4 fusion transcripts in pEL1/463H4-transformed 21-Tb cells. The experimental protocol was the same as in Fig. 1. The DNA probe was 3′ end-labeled at an *Ava* I site 27 bp upstream of the H4 insert to prevent protection by endogenous H4 transcripts. Moreover, the probe is homologous to transcripts extending through the *galK* gene for only 679 nucleotides to allow distinction between the renatured DNA probe and the fragment protected by these transcripts. *, Position of label on S1 hybridization probe; M, DNA size marker (length in nucleotides).

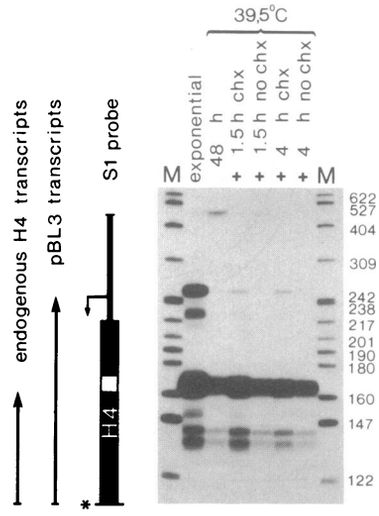


FIG. 6. Effect of cycloheximide on H4-specific transcripts in G_1-arrested 21-Tb cells. pBL3-transformed 21-Tb cells were incubated at 33°C (exponential) or for 48 hr at 39.5°C followed by 1.5 or 4 hr in the presence or absence of cycloheximide at 100 μg/ml. Total cell RNA was prepared and hybridized to the same DNA probe used in Fig. 4. Hybrids were treated with S1 nuclease and the protected DNA fragments were resolved on a sequencing gel. *, Position of label on S1 hybridization probe; M, DNA size marker (length in nucleotides).

concentration of the longer transcripts does not change significantly (Fig. 5). This result indicates that 3′-terminal sequences of the H4 gene are sufficient to create the changes occurring in histone mRNA metabolism during the cell cycle. However, these sequences are ineffective in regulating RNA metabolism when they are contained internally in the much longer *galK* mRNA.

Effect of Inhibition of Protein Synthesis on Histone mRNA Levels. Treatment of cells with inhibitors of protein synthesis is known to stabilize histone mRNA and in particular to reverse a decrease in histone mRNA stability occurring after inhibition of DNA synthesis (refs. 7, 8, 32, and 33; see *Discussion*). We therefore measured the effect of inhibiting protein synthesis in G_1-arrested pBL3-transformed 21-Tb cells on the levels of pBL3-specific and endogenous H4 RNAs. Our prediction was that the levels of both types of RNA should increase in parallel, perhaps up to levels similar to those observed in exponentially multiplying cells. Again, we observed a very dramatic decrease in histone RNA levels when the cells were arrested in G_1 phase by incubation at 39.5°C for 48 hr (Fig. 6). Further incubation of G_1-arrested cells at 39.5°C in the presence of cycloheximide at 100 μg/ml for 1.5 or 4 hr led to an increase of both pBL3-specific and endogenous H4 mRNAs. However, RNA levels were only ≈5-fold higher after 1.5 hr and did not increase further with prolonged inhibition of protein synthesis (Fig. 6). Clearly, the levels of H4 transcripts in cycloheximide-treated G_1-arrested cells were still very much lower than in exponentially growing cells. Essentially identical results were obtained when puromycin at 100 μg/ml was used instead of cycloheximide (data not shown).

DISCUSSION

We have demonstrated that, under all experimental conditions tested, a recombinant mouse H4 gene is faithfully expressed and regulated in 21-Tb cells. In this cell line, H4 transcription rates vary only over a 3-fold range, and histone mRNA levels must therefore be regulated ≈50-fold by some post-transcriptional process(es). Our results indicate that these same post-transcriptional processes must also be regulating our recombinant H4 gene. However, because of the relatively small contribution of transcriptional regulation, it is not yet firmly established whether this gene is also regulated at the transcriptional level, even though its mRNA steady-state levels were always controlled at least as tightly as those of the endogenous H4 genes.

Our results with pBL3- and pEL1/463H4-transformed 21-Tb cells show that insertion of 12 bp at the *Nar* I site, replacement of the 5′ portion of the H4 gene by the SV40 early promoter/leader segment, and deletion of histone spacer sequences >266 bp downstream from the H4 mRNA 3′ end all have little if any effect on cell-cycle regulation of the corresponding transcripts. Thus, the 463-bp H4 gene fragment present in pEL1/463H4 is not only sufficient for the generation of H4-specific 3′ ends (30), but also for producing the correct changes in RNA metabolism during the cell cycle. To determine whether these two processes are in some way related, it will be important to mutagenize the 463-bp fragment systematically. If it is possible to isolate mutants that affect one mechanism but not the other, this could help determine whether the observed regulation occurs before, at, or after the step of RNA 3′ processing.

Much of the current knowledge on the coupling of histone gene expression to DNA replication comes from studies with inhibitors of DNA synthesis. Treatment of S-phase cells with a variety of such inhibitors results in a rapid loss of histone mRNA, which is mostly due to an accelerated turnover of the existing histone mRNA population (6–8). This effect can be prevented by simultaneous treatment of the cells with inhibitors of protein synthesis (7, 8, 32, 33). Based on these results, it has been suggested that histone mRNA concentrations are negatively controlled by a labile or rapidly sequestered protein. It is still not established, however, whether these changes in mature histone mRNA stability reflect the post-transcriptional mechanism(s) involved in histone gene regulation during a normal cell cycle.

We find that inhibition of protein synthesis increases histone mRNA levels in G_1-arrested 21-Tb cells only minimally. Even if we account for the 3-fold difference in histone gene transcription between exponential and arrested cells, cycloheximide treatment could not increase H4 RNA levels back to the levels seen in exponential cultures. We therefore conclude that the regulation we see in G_1-arrested 21-Tb cells

is different from that observed when cells are treated with inhibitors of DNA synthesis. It is possible that histone gene expression in 21-Tb cells is controlled at additional levels other than transcription and mature mRNA stability.

To discuss the possible levels at which this regulation could occur, we must focus our attention on the post-transcriptional mechanisms involved in the maturation of histone gene transcripts to cytoplasmic mRNAs. As is the case for sea urchin and chicken histone genes (34, 35), our mouse H4 gene also generates its mRNA by cleavage of longer precursor transcripts (unpublished observations). These precursor molecules must be extremely short-lived and have not as yet been isolated or directly demonstrated. However, in the case of a sea urchin H2A gene there is indirect evidence indicating that these RNA precursors terminate within 100 nucleotides beyond the 3′ end of mature mRNA (34). Since it is possible that the processing step or the stability of the precursor RNA are the targets of cell-cycle regulation, it becomes important to know more about the precursor of the correctly processed and regulated SV40/H4 RNA of pEL1/463H4. If a very long precursor is used to generate both the short SV40/H4 RNA and the longer *galK* mRNA, then changes in precursor stability or in histone 3′ processing should produce simultaneous changes in the levels of both RNAs. However, this is not what we observe, and therefore the SV40/H4 RNA precursor would have to be different from the *galK* RNA precursor, if regulation were to occur at this level. Clearly, direct experiments will be required to address this question.

If, alternatively, regulation of histone gene expression occurs after 3′ processing, then we must look for structural differences between histone and nonhistone mRNAs. Our results indicate that such a regulatory structure would have to reside within the last 230 nucleotides of H4 mRNA. The most obvious difference between histone and nonhistone mRNAs in this region is the absence of a poly(A) tail and the presence of a conserved palindromic sequence at the 3′ end (36). Perhaps this structure is important for the stability of the processed nuclear histone RNA or for its export into the cytoplasm. The fact that, where analyzed, DNA replication-independent or erythrocyte-specific histone gene variants have been found to lack the conserved terminal palindrome and to produce polyadenylylated transcripts instead (37–39) speaks in favor of a cell-cycle regulatory role for the terminal hairpin structure. However, a completely different situation is encountered during *Xenopus* development. There, the same histone genes containing the terminal palindrome are active throughout development and yet their expression is uncoupled from DNA synthesis during oocyte maturation and the early cleavages but not at later stages (40, 41). Perhaps the control mechanisms occurring in normally dividing cells are not operative during these developmental stages. It may also be relevant that a significant fraction of histone mRNAs present in maturing oocytes and in early cleavage embryos contain short poly(A) tails added to the terminal palindrome (42, 43).

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